CHAPTER ONE
INTRODUCTION

1.1.Background to the study

The biodiversity of nature has provided researchers with arrays of physical, biological and chemical agents, which can perfectly support the existence of humanity in their quest for the discovery and development of drugs for health care purposes.Nature has been and will continue to be a potential source of basic needs such as food, shelter and medicines for mankind(Jamshidi-Kia *et al*., 2018). Natural productsfound in nature have been used since the time immemorial by both man and animals for the treatment of many diseases and thus, played an important role in improving their health and general wellbeing (Mustafa*et al*., 2017). Man's desire for continuous existence in the universe made him develop several technological methods to transform natural resources in the universe into daily essential commodities,which areneeded for his survival (Allan, 2012).

Before 20th century, about 90% of plants' products used as medicines forthe treatment of human and animals ailmentswere obtained directly from different parts of plant such as root, bark and leave of medicinal plants(Sewell and Rafieian-Kopaei, 2014). During this era, plant parts were percolated in readily available fluids such as alcohol and water and the doctors prescribed dosages like tablespoons or teacup of the extract to be taken by the patients for a particular period of time (Sewell and Rafieian-Kopaei, 2014). After 20th century, researchers gained more knowledge and understandingabout the receptor theory of the drug action,that individual chemical compounds present in the medicinal plants extracts were responsible for the healing actions of the plant extracts. Atwenty-one-year-old pharmacist apprentice, Friedrich Serturner in 1806 isolated a pure compound, morphine**,**from the medicinal plant, *Papaversomniferum* L. (Papaveraceae). Since then, many other active components have been isolated from natural sources(Che and Zhang, 2019). The old system of medicine is still inpractice among traditional healers of many cultures till today(Sewell and Rafieian-Kopaei, 2014).

The role of traditional medicine and ethnopharmacology regarding modern discovery and drug development cannot be overemphasized (Andrade-Cetto and Heinrich, 2011; Patil; 2011). Local knowledge about the use of medicinal plants documentationprovides useful database and starting point for drug discovery. Instead of random, chemotaxonomic and bio-rational screening of plants for activities, scientists are able to use local/indigenous knowledge to cost effectively adopts the bioactivity-guided fractionation and isolation of biologically active compounds (Heinrich, 2010).Studies have shown that several drugs which are in use nowadays have their origin from plants used in folk medicine by indigenous people of different cultures, hence, the local use of medicinal plants have played an important role in the health care system(Mustafa*et al*., 2017). For example, the use of*Cinchona officinalis*L. (Rubiaceae) stem bark by the indigenous people of Amazon region for fever treatment has resulted indiscovery of antimalarial drug, quinine by Pelletierand Covetous in 1820 (Willcox, 2004).Also, the use of the herbaceous plant, *Catharanthusroseus*(L.) G.Don(Apocynaceae), by indigenouspeople of the Southeastern Madagascar, is the source of over 75 alkaloids, two of which are used in the treatment of Hodgkinslymphoma and leukaemia in children (Gurib-Fakim, 2006).Many other drugs that are commonly used today including digoxin, aspirin, atropine ephedrine to mention but a few, were discovered by scientific investigation of medicinal plants that are used bythe local peopleworldwide(Moteriya *et al*., 2015;Ram *et al*., 2015).

The use of medicinal plants or their extracts in traditional/herbal medicines by local population fordisease therapy/treatment varies from one culture to the other (Ozioma and Chinwe, 2019). In China, a number of diseases includingcancer disease conditions have been successfully treated through the use of different medicinal plants in Chinesetraditional medicines (CTM) (Kuo*et al*., 2006).In Asian population, Caucasians and African Americans,the use of medicinal herbs and their derivative herbal extracts which are rich in polyphenolic compounds had contributed to reduction in prostate cancerincidence (Syed *et al*., 2007). *Kampo yaku*,a combination of seven herbs traditionally used forcancer diseasetreatment in Japanese traditional medicine had been reported to be therapeutically active(Sakaida*et al*., 1998).In Africa, particularly Nigeria, the use of different plant based remedies by local herbalists has led to the treatment of many disease conditions including cancer-related diseases(Elujoba *et al*., 2005; Omogbadegun,2013; Nwodo *et al*., 2016).

Ethnobotanical surveys aimed at documenting the indigenousplants usedfor the treatmentof cancer diseases have been reported in Nigeria (Ngulde *et al*., 2015: Aliyu and Abubakar, 2016; Segun *et al*., 2018)and in other cultures such as Brazil (de Melo*et al*., 2011), India (Sharma *et al*., 2012; Ramamurthy *et al*., 2015), Iran (Bahmani *et al*., 2017), Ghana (Christian *et al*., 2017), China (Nadin*et al*., 2018) to name a few. Some of these studies have also proceeded to the pharmacological characterisation and identification of active anticancer compounds from the medicinal plants (Pettit *et al*., 1995; Ali and Suaib, 1997;Muriel, 2004: Devi *et al*., 2010; Nwodo *et al*., 2016;Segun *et al*., 2018).

1.2.Cancer disease

The disease,cancer isrecognised by abnormal and uncontrolled cell division which usually results from the prolonged unrepaired DNA damage by electrophilic species(NCI, 2018; WHO, 2018). Cancer cells are capable of forming mass or lump, which can diffusely invade the surrounding tissues. It can arise in any areas of the body, spread and invade almost all the cells available in the human body.Cancer is a non-communicable disease which hardly produces symptoms at the beginning, but it symptomsbecomes pronounced as itprogresses. Sometimes, the manifestation of the symptoms largely depends on the type and location of the cancer in the body, and only few of its symptoms are specific, while many othersymptoms also feature in people with other ailments (WHO, 2018). Environmental factors such asphysical carcinogens, chemical carcinogens, biological carcinogens and dietary carcinogens are the major causes of cancer disease (WHO. 2018).For instance, in 2008, tobacco, an example of chemical carcinogen, contributes about 25-30% of cancerdeaths, while obesity and diet accounted for 30-35%, and infections constituted 15-20% (Islami *et al*., 2018).

1.3.Statement of the problem

Cancer has become a global burden with its epidemiology, which keeps increasing at a rate higher than the people's population; hence,countries all over the world are facing increase in the number of cancer cases. For example, in 2018, Asia accounts for nearly half (48.4%) of the new cases of cancer worldwide, andgreater than half (57.3%) of deaths as a result of cancer were recorded in Asia (IARC, 2018). Worldwide, cancer is the main cause of people's death,with about 9.6 million cancer deaths and 18.1million new cancer cases recorded in 2018 (Bray *et al*., 2018). The cases of new cancer are likely to increasewithin the next twenty years to 22 million (Torre *et al*., 2015).

In 2018, lung cancer (2.094 million, 11.6% of the total), breast cancer (2.089 million, 11.6% of the total), colorectal cancer (1.8 million, 10.2% of the total), prostate cancer (1.3 million, 7.1% of the total), skin cancer (1.04 million, 5.7% of the total) and stomach cancer (1.0 million, 5.7% of the total) were the most commonly diagnosed cancers worldwide, while the commonest mortality due to cancer worldwide were the cancers of trachea and lung (1.8 million deaths, 18.4% of the total), colorectal and colon (881,000 deaths, 9.2% of the total), stomach (783,000 deaths, 8.2% of the total), liver (782,000 deaths, 8.2% of the total) and breast (627, 000 deaths, 6.6% of the total) (WHO, 2018).Thecurrentburden of cancer disease on global healthdemands urgent attention in order to rescue and secure the future generation from this deadly disease.

1.4.Rationale for this study

Despite all medical advancement and efforts of stakeholders towards finding a permanent cure for cancer, the disease continues to be a burden worldwide (Bray *et al*., 2018). However, many drugs have been discovered for the management of various types of cancer, poor selectivity and severe side effects associated with such drugs have limited their use. This has necessitated the need to search for new agents from natural sources with better overall safety profile.

Severalmedicinal plants have been screened for their potential anticancer activity in the past five decades. Africa hosts about 60,000 species of the world's flora and have only utilised about 5,000plants for therapeutic purposes(Mahomoodally, 2013). The ethnopharmacological leads from medicinal plants in Africahas not been fully realised as compared to plants from Indian and Chinese traditional medicine (Bramwell, 2002). For example, the anticancer drug, camptothecin from *Camptothecan acuminate* Decne (Nyssaceae), was discovered from Chinese herbal medicine (Li *et al*., 2010), and the antihypertensive drug, reserpine from *Rauvolfia vomitoria*Afezl (Apocynaceae), was discovered from AyurvedaIndian herbal medicine(Ritu *et al*., 2016). However, the Nigeria flora has been documented as a potential source of pharmaceuticals and therapeutic agents (Gbile and Adesina, 1987), and about a decade ago, the Federal Government of Nigeria set aside one billion USD for the development of traditional medicine and its possible integration into the country's health care sector (Ashidi *et al*., 2010). According to Omogbadegun,(2013), some types of cancers have been successfully managed by traditional medical practitioners in many localities in Nigeria using plant-based preparations.

Unfortunately, theindigenous knowledge about the use of medical plant for the treatment of diseasesincluding cancer is limited to a particular group of people, which are the traditional medicine practioners and the elders. In some cases, the knowledge is believed to be a family secret that should not be revealed but must be protected and passed on to a member of the family, sometimes, orally. However, if this knowledge is not properly passed on, it could be lost when such an individual dies. It is therefore importantto preserve this knowledge by documenting these medicinal plants in order to save them for the future generations andmore so, to validate their potential use as anticancer products. This can be achieved by ethnobotanical survey of a particular locality, hence the reason for this study.

1.5.Research hypothesis

Caesalpinia benthamiana and *Combretum racemosum* used by local people of Gbonyin, Moba and Efon Local Government Areas of Ekiti State, Southwest Nigeria possess anti-tumour and/or anti-cancer compounds that can be exploited for possible clinical development of novel, effective and safe anti-cancer drugs.

1.6.Aim and objectives

This research is aimed at investigating selected Nigerian medicinal plants used by local population of Gbonyin, Moba and Efon Local Government Areas of Ekiti State, Southwestern Nigeria in the management of tumour/cancer for their anticancer potential, isolateand characterise their active compounds which may be useful for development into drugs for use in clinical practice. The specific objectives of this work are as summarised below:

1. Documentation of indigenous knowledge and medicinal plants that are used for different cancer diseasetreatmentin three selected Local Government Areas of Ekiti State, Southwest,Nigeria
2. Identification, authentication,collection and extraction of the selected plants
3. Screening of selected plants against human cancer cell lines for anticancer activity
4. Purificationand isolation of the compound(s) in theactivefractions using different chromatographic methods
5. Characterisation of the isolated compound(s) by various spectroscopic techniques
6. Testing the isolated compounds against human cancer cell lines for their possible anticanceractivity.

CHAPTER TWO
LITERATURE REVIEW

2.1.Natural product and history

The Mesopotamia (2600 B.C.), was the earliest documentation of the use of natural products in health care delivery, where substances derived from plants such as oils of *Glycyrrhiza glabra* L. (Leguminosae)(liquorice), *Papaver somniferum*(poppy juice), *Cupressus sempervirens* L. (Cupressaceae) (cypress),*Commiphora* species(Burseraceae)(myrrh)and *Cedrus*species(Pinaceae)(cedar), were written on clay tablets in cuneiform. All these oils are still being used today for the treatment of many ailments like cold, cough and inflammation. The Egyptian pharmaceutical record (1500 B.C) documented about seven-hundred plant-based drugs. The use of natural products as source of medicines has also documented by several other records, which include: the Wu Shi Er Bing (1100 B.C., 52 drugs), Charaka (1000 B.C., 341 drugs), Shennong Herbal (100 B.C., 365 drugs),Tang Herb (659 A.D., 850 drugs) and the Galen (200 A.D., 30drugs) (Cragg and Newman, 2005).A large number of the drugs used today (about 70%) have their origin in natural product and many secondary metabolites obtained from natural products are the most successful drug leads (Butler, 2004; Rey-Ladino *et al*., 2011). Among the world's 25 best selling pharmaceutical products, 12 were derived from natural sources and one-quarter of all medicationprescriptionsare made with various types of secondary metabolites obtained from natural products (Balogun *et al*., 2019).

Natural product continues to show great relevance in drug discovery industry till today. The joint award of one half of 2015 Nobel prize to Willian Campbell and Satoshi Omura in Physiology and Medicine, for the discovery of avermectin, a bioactive compound, isolated from strains of *Streptomyces* found in the soil samples, which was chemically modified to ivermectin, a more effective antiparasitic drug that is used in the treatment of many parasitic diseases, which affect mostly the people of developing countires, and the award of the other half of 2015 Nobel price to Youyou Tu for

artemisinin discovery from the Chinese plant, *Artemisia annua* L. (Compositae) (Callaway and Cyranoski, 2015) are all indication of the importance of natural product. Figure 2.1 shows the relevance of natural products in drugsdiscovery and Figure 2.2 showsstructures of bioactive natural products.

2.2.Sources of natural products

Products from natural origin are biologically active agents/compounds that are derived from living thing (plants, animals and organisms) and non-living things or minerals. Products derived from living things are classified into four major groups based on their sources and these include, natural products obtained from microorganisms, marine organisms, animals and plants.

2.2.1.Bioactive products from microorganisms

The discovery of popular antibiotic penicillin, produced by *Penicilliumnotatum*, by Alexander Fleming in 1928, marked the beginning of drug discovery from microorganisms. This discovery led to the screening of several other terrestrial and marine microorganisms for drug candidates. In fact, water and soil samples from different parts of the world were collected and investigated in order to discover bacterial and fungal strain that can serve as drug leads. This however, led to the development of many important antibiotics like chloramphenicols, cephalosporins and rifamycins (Chin *et al*., 2006).

Natural products derived from microorganisms are majorly used in antimicrobial therapy. However, microbial metabolites have also found therapeutic applications in the treatment of several health challenges. For example, Asperlicin, isolated from *Aspergillus alliaceus*, is a novel antagonist of the neurotransmitter and cholecystokin used in the control of appetite (Butler, 2004). A metabolide isolated from the fungi, *Aspergillus terreus*, Lovastatin, was found to be the lead compound for the cholesterol lowering group of statins (Goswami *et al*., 2012). Figure 2.3 shows the structures of bioactive products from microorganisms.

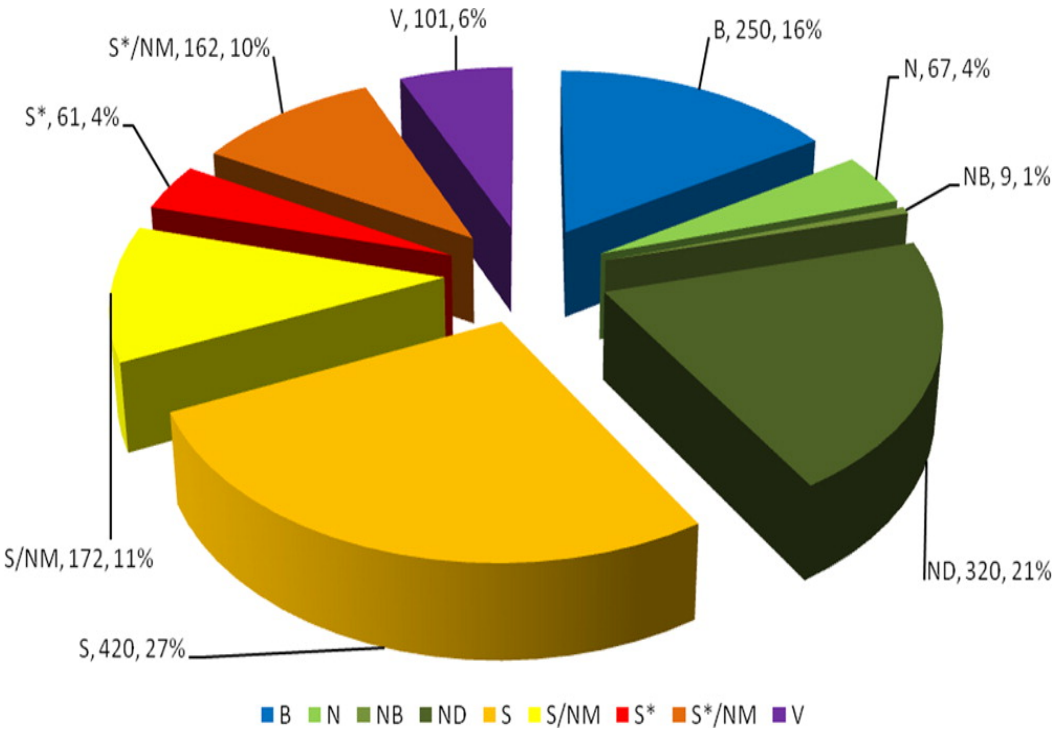


Figure 2.1. Relevance of natural products in the discovery of drugs (Newman and Cragg, 2016).

B = Biological; usually a large (>45 residues) peptide or protein either isolated from a cell line or an organisms

N = Natural product

NB =Natural product botanical drug (these have been recently approved)

ND =Derived from a natural product but, usually a semisynthetic modification

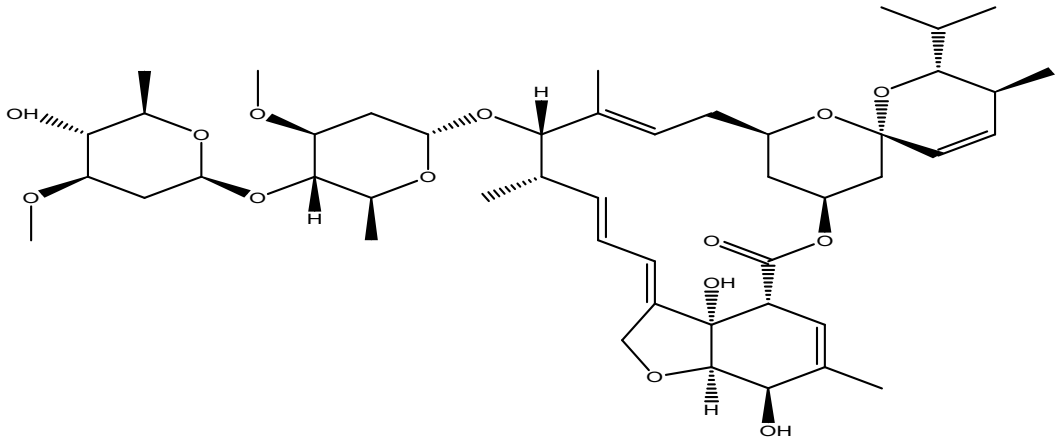
S = Total synthetic drug often found random screening/modification of an existingagent

S/NM = Natural product mimic

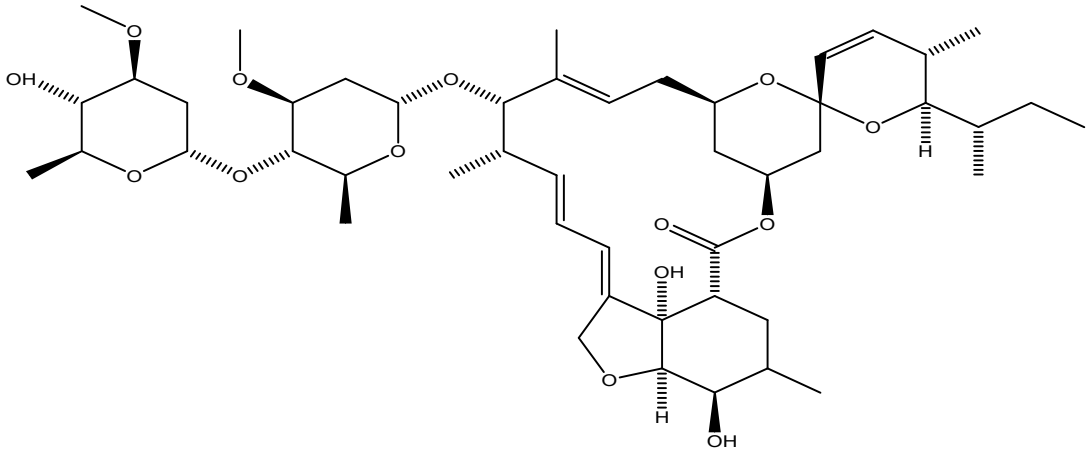
S* = Total synthetic drug

S*/NM = Made by total synthetic, but the pharmacophore was from a natural product

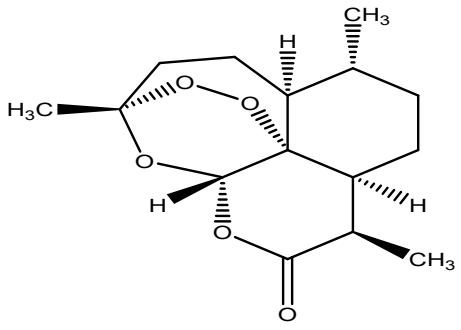
V = Vaccine



Avermectin

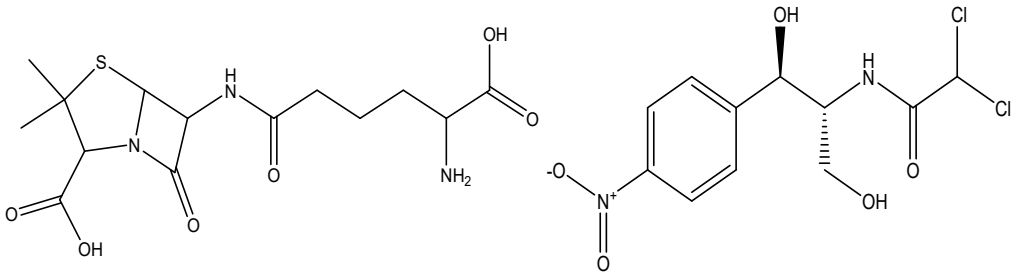


Ivermectin



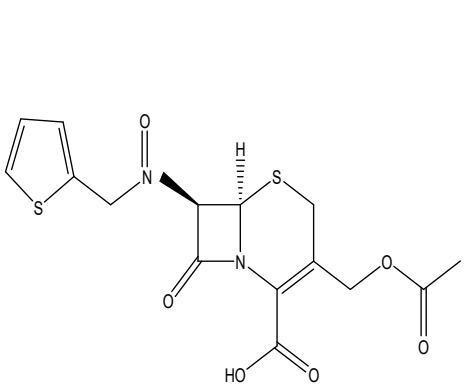
Artemisinin

Figure 2.2.Structures of bioactive natural products(Newman and Cragg, 2016).

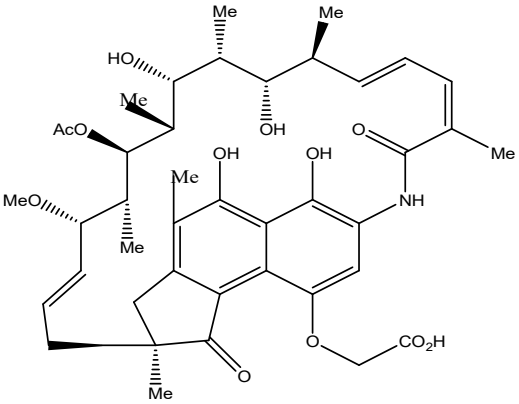


Penicillin

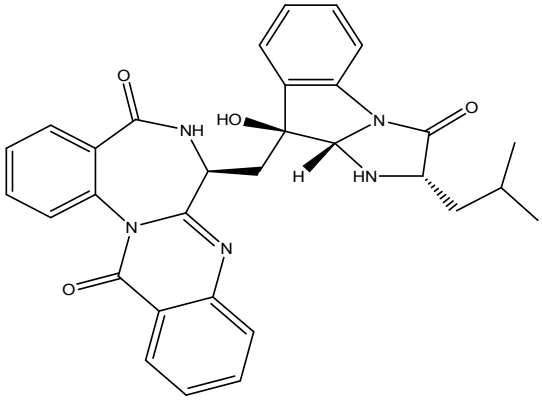
Chloramphenicol



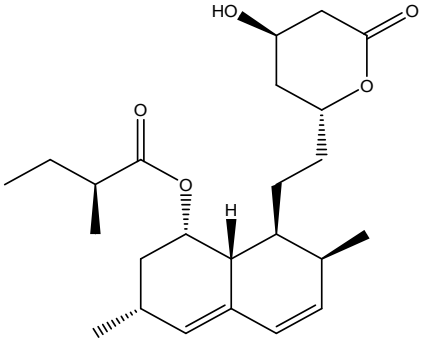
Cephalosporin



Rifamycin B



Asperlicin



Lovastatin

Figure 2.3.Structures of bioactive natural productsderived from microorganism (Dias *et al*., 2012; Craggand Newman, 2013).

2.2.2.Bioactive products from marine organisms

The ocean contains a larger area of the earth surface with about 70% of the total surface area and possessing about half of the total biodiversity. The marine world has a huge potential in the discovery of important drugs for humanity (Cragg and Newman, 2013). According to Newman and Cragg. (2016), the earliest record of the use of marine organism as natural products was the use of flatworm, *Lumbrineris brevicirra* as anti-insecticidal agent. Fishermen used this worm for many centuries as fishing bait. The active constituent in the flatworm, nereistoxin was isolated by Nitta in 1934 and the structure was confirmed by Hagiwara in 1965 (Hagiwara *et al*., 1965). A closely related analogue, Padan, was developed through a structural activity relationship (SAR) studies and marketed by Takeda in 1967 as an insecticidal agent (Haefner, 2003). In addition to this, the isolation of spongouridine and spongothymidine from *Cryptothecacrypta* activated the interest of natural product scientists into the marine world (Mayer*et al*., 2010).

Recent findings have discovered a number of novel metabolites from the marine organisms with significant pharmacological properties. Although very few of these products obtained from marine organisms have made it to the market, many natural products obtained from marine organisms are now in clinical trials. Some of the important commercialised marine natural products arecytarabine (cytostatic agent) and vidarabine (antiviral agent), both from sponge and kanic acid (anthelminthic agent) from red algae (Anjum*et al*., 2016). Other anticancer compounds isolated from marine organisms that are in clinical trial include; Bryostatin-1 from Bryozoan and Yondelis from sea squirt (Rangel and Falkenberg, 2015).The structures of the bioactive products from marine organisms are shown in Figure 2.4.

2.2.3. Bioactive products from animals

Another area of natural product that serves as source of molecule with potent pharmacological effect is animals such as frogs, snakes, spiders, scorpions and insects as well as invertebrate animals like crustaceans and arthropods. Epibatidine, a very potent analgesic agent, isolated from the skin extracts of an Ecuadorian poison frog, is known to be two hundred times more potent than morphine (Daly *et al*., 2000). Another metabolite, Chitosan, is a natural linear polysaccharide obtained from the exoskeletons of crustaceansand arthropods, has been shown to havepotent antifungi andantibacterial activity (Fernandes *et al*., 2008).

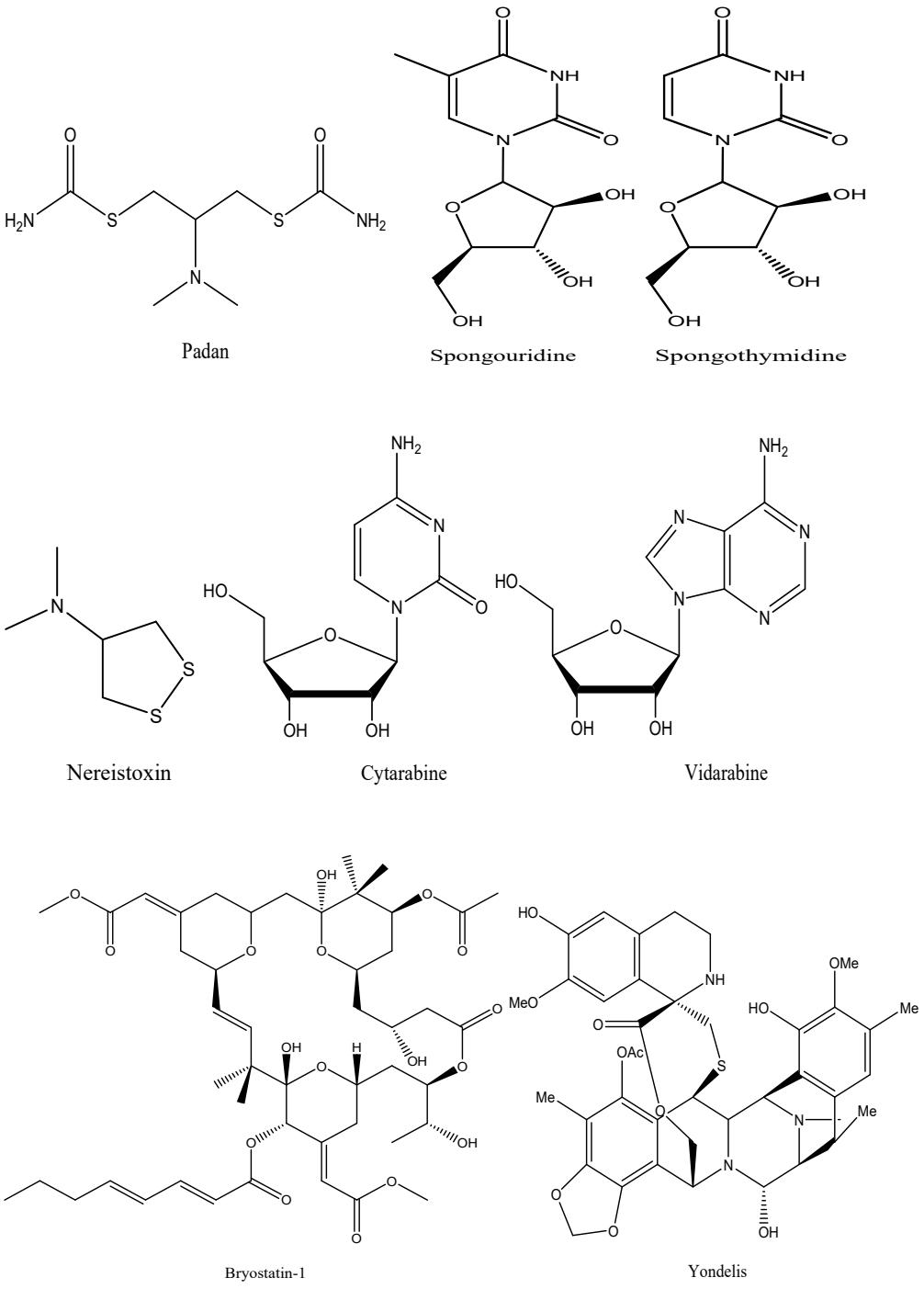


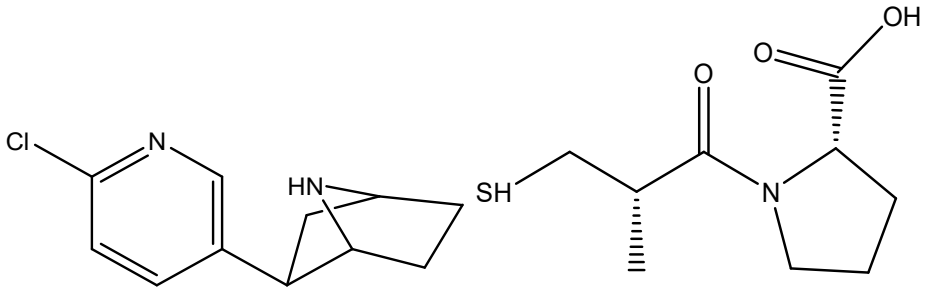
Figure 2.4.Structures of bioactive natural productsderived from marine organisms (Rangel and Falkenberg, 2015)

Cilazapril and captopril, isolated from the Brazilian viper, *Bothrops Jararaca*, are two angiotensin converting inhibitors, which were developed from a polypeptide teprotide, which are effective against hypertension and other cardiovascular diseases (Komajda and Wimart, 2000). The structures of bioactive products from animals are shown in Figure 2.5.

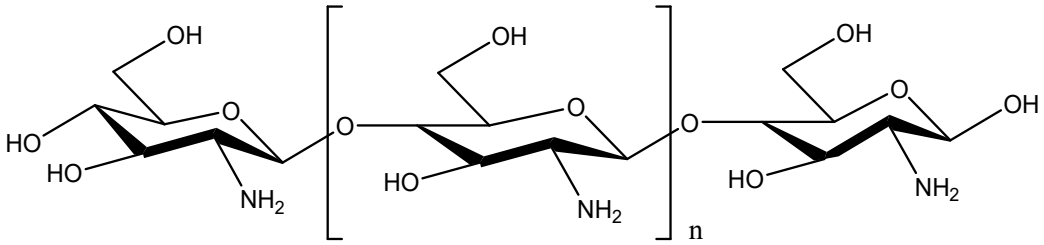
2.2.4.Bioactive products from plants

The use of plants and its constituents for the treatment of various diseases dates back to thousands of years ago and about 50,000 to 70,000 plant species have been screened for their medicinal use (Marwat *et al*., 2009). To this end, a large number of new drugs (about 11% of the 252 drugs known as essential drugs) are of plant origin and many authors have discussed the importance and potential of medicinal plants as sources of new biological and medicinal agents (Che and Zhang, 2019). Many other synthetic drugs obtained from natural precursors also have their origin in plant. Plants have always been a rich source of drug leads, as they contain a structurally diverse secondary metabolites which are not facile to synthesize in the laboratory (Butler, 2004).

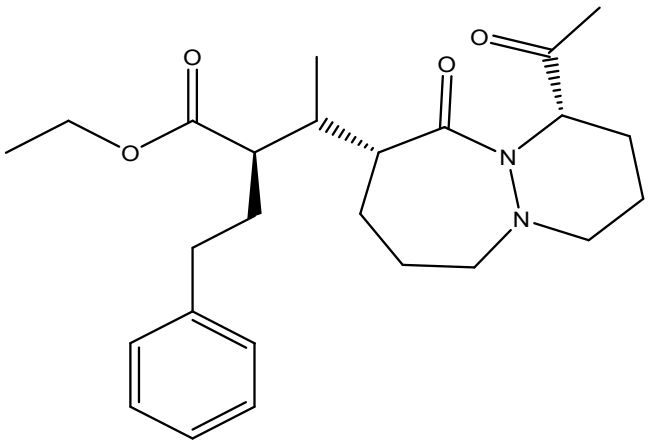
Aspirin, the widely used analgesic and anti-inflamatory agent, was obtained from natural product salicin, which was obtained from the willow tree bark, *Salix alba*L. (Salicaceae) by Johann Buchner (Levesque and Lafont, 2000). Quinine, an alkaloid, which is effective against malaria infection, especially chloroquine resistant *Plasmodium falciparum*, was first derived from *Cinchona succirubra*L. (Rubiaceae) bark. Another plant natural product is digitoxin, a cardiotonic glycoside used in the treatment of congestive heart failure, was isolated from *Digitalis purpurea*L. (Plantaginaceae) (Gheorghiade *et al*., 2004). Paclitaxel (Taxol), one of the most widely used drugs for the treatment of breast cancer, was obtained from *Taxus brevifolia* Nutt.(Taxaceae) bark. A L-histidine-derived alkaloid, pilocarpine, obtained from *Pilocarpus jaborandi* Vahl (Rutaceae), has been used clincally in the management of acute angle-closure glaucoma and chronic open-angle glaucoma for over 100 years (Rosin, 1991). Tubocurarine, isolated from *Chondrodendron tomentosum*Ruiz & Pavon (Menispermaceae), is a muscule relaxant used to reduce the need for deep anaesthesia in surgical operations (Dias *et al*., 2012).Figure 2.6 shows the structures of bioactive products from plant.



Epibatidine Captopril



Chitosan



Cilazapril

Figure 2.5.Structures of bioactive natural productsderived from animals (Newman and Cragg, 2013)

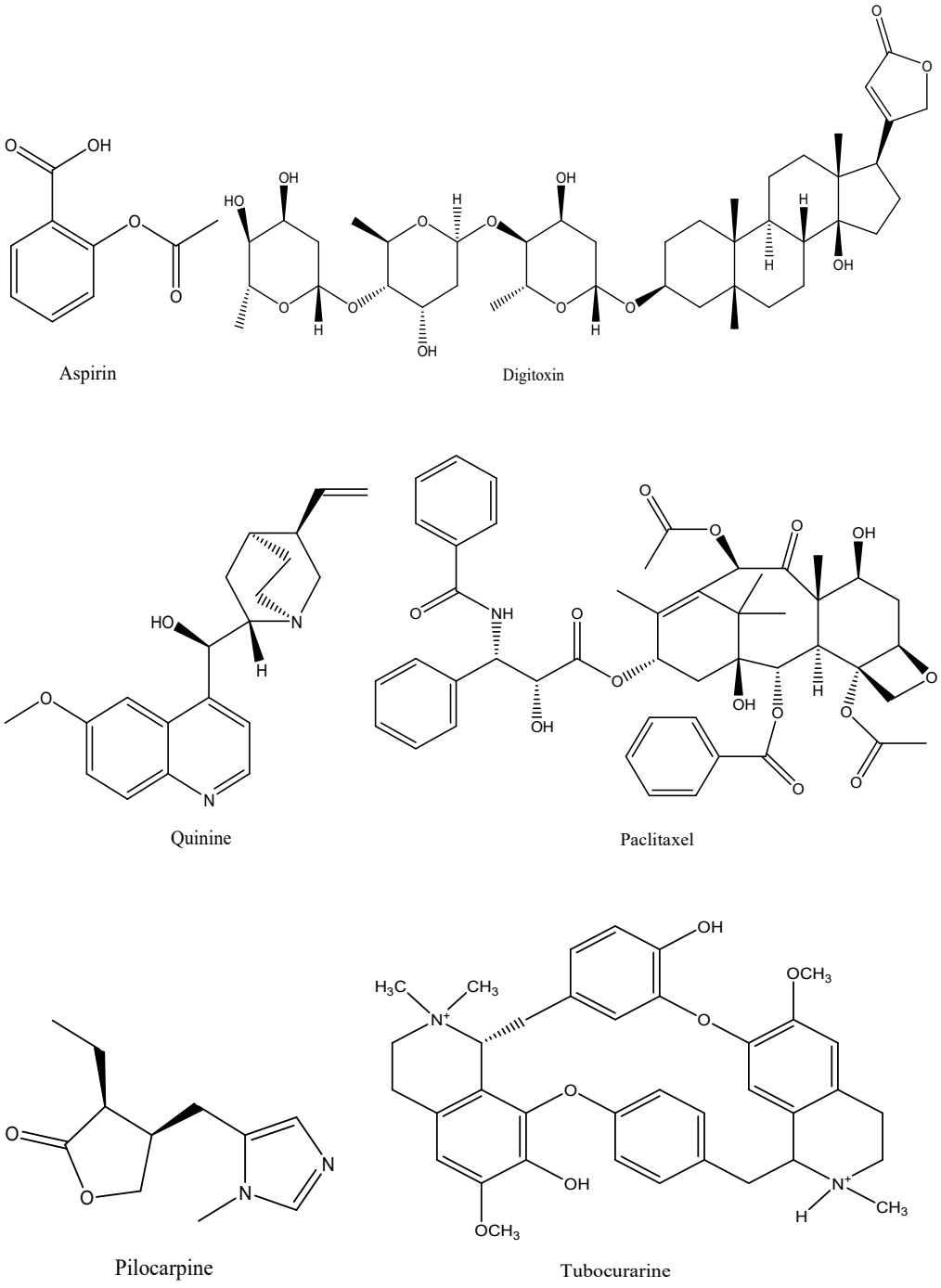


Figure 2.6.Structures of bioactive natural productsderived from plants (Newman and Cragg, 2013)

2.3.Different methods used in selecting plants for drug discovery

There are various approaches used in selection of plants, to be tested for new bioactive compounds and these include; Phylogenic or chemotaxonomic method, Bio-rational method, Random or blind selection and Ethnobotanical/ethnopharmacological method.

Phylogenic or chemotaxonomic method is a plant-collection method in which researchers select similar/related plants. It is based on the assumption that species within the same taxa possess similar chemical properties. For example, bioactive alkaloids have been shown to be present in large amounts in certain plant families like Berberidaceae, Leguminosae, Raunuculaceae, Rubiaceae, Solanaceae and Papaveraceae (Encyclopaedia Botanical, 2018).

Bio-rational method is based on the defensive features of plant against certain predators. It is believed that such plants produce certain chemical that is capable of exerting harmful effect on their predator. For example, it was noticed that the consumption of *Thevetiaperuviana* (L.) Lippoid (Apocynaceae) by herbivores led to the death of such animals.Also, a cardiac glycoside, oleandrin present in oleander was discovered to be responsible for the harmful effects(Barbosa *et al*., 2008). This discovery activated extensive research which led to the use of cardiac glycosides in human heart problems such as heart failure.

Random or blind selection is practiced by researchers to exploit unstudied areas with great diversity. It has resulted in the discovery of some important medicinal compounds. For example, the discovery of some anticancer compounds such as taxol from *Taxus brevifolia*Nutt.(Taxaceae) and vincristine and vinblastine from *Catharanthus roseus*(L) G.Don (Apocynaceae) are through the random screening program (Soejarto, 1996).

Ethnobotanical/ethnopharmacological method is usually employed by researchers to inquire/investigate how people of a particular region/culture make use of indigenous plants found in their localities, for the treatment of different diseases (Cheikhyoussef *et al*., 2011). It is a method that relies on knowledge gained from the use of traditional medicine by the local people of a particular culture.Investigation of indigenous knowledge on medicinal plants used in the treatment of diseases can serve as one of the major ways by which public health can be improved (Cheikhyoussef *et al*., 2011). Often times, due to fear of losing customers as well as the risk of exposure of source of

income or means of livelihood, the people endowed with indigenous knowledge about the use of medical plants for the treatment of disease considered the knowledge as a family secrets that should not be revealed, but must be protected and passed on to a member of the family, sometimes, orally without proper documentation(Ngulde *et al*., 2015). Several drugshave been obtained from ethnobotanical studies, for example, the discovery of antimalarial drug, artemisinin, which was isolated from *A.annua*, a plant used for several centuries in China for malaria (Zhou *et al*., 2012).

2.4.Cancer disease and history

Cancer, a diseaserecognised by abnormal and uncontrolled cell divisionthat usually results from the prolonged unrepaired DNA damage by electrophilic species, was originated from the word '*crab*' in greek languagewhich means carcinos or carcinoma, as used by Hippocrates, the greek physician in 460-370 BC (Nastoupil *et al*., 2012). Hippocrates till today was believed to be the father of medicine. Later after him, in 28-50 BC was a physician from Rome, Celsus who interpreted the Greek word to mean cancer and the disease has been known to have affected human beings as well as other animals as recorded in history to the present world's civilisation. Among fossilised bone tumours, was found one of the earliest cancer evidence, in human mummies, ancient Egypt (1500 BC), where the process of mummification helps to preserve the features of malignancy for centuries before the advent of effective diagnostic instruments. However, the earliest cancer treatment documentation was recorded in the Egyptian Papyrus Ebers (1500 BC) (Borchardt, 2002).

2.4.1.Cancer as a global disease

Worldwide, cancer is the second main cause of people's death,with about 9.6 million cancer deaths and 18.1million new cancer cases in 2018 (Bray *et al*., 2018). The cases of new cancer are likely to increasewithin the next twenty years to 22 million (Torre *et al*., 2015). Globally, cancer is increasing at a rate faster than the population increase, with the developing nations of the world accounting for approximately 56% of the new cases and 70% of cancer mortality worldwide in 2018 (Bray *et al*., 2018). Approximately 8.9 million people were killed by various cancers worldwide in 2016, where tracheal and lung cancers were the largest killer with over 1.7 million (Hannah and Max, 2018). Countries all over the word are facing increase in the number of cancer cases, However, in 2018, Asia accounts for nearly half (48.4%) of the new

cases of cancer worldwide, andgreater than half (57.3%) deaths as a result of cancer were recorded in Asia (IARC, 2018).

The disease,which is the second main cause of people's deathbehind cardiovascular diseases, is responsible for 1 out of 6 deaths recorded for all diseases worldwide (Hannah and Max, 2018), and can arise from any areas of the body, spread and invade almost all the cells available in the human body. In 2018, lung cancer (2.094 million, 11.6% of the total), breast cancer (2.089 million, 11.6% of the total), colorectal cancer (1.8 million, 10.2% of the total), prostate cancer (1.3 million, 7.1% of the total), skin cancer (1.04 million, 5.7% of the total) and stomach cancer (1.0 million, 5.7% of the total) were the most commonly diagnosed cancers worldwide, while the commonest mortality due to cancer worldwide were the cancers of trachea and lung (1.8 million deaths, 18.4% of the total), colorectal and colon (881,000 deaths, 9.2% of the total), stomach (783,000 deaths, 8.2% of the total), liver (782,000 deaths, 8.2% of the total) and breast (627, 000 deaths, 6.6% of the total) (WHO, 2018).Figure 2.7 shows the global cancer incidence and mortality in 2018.

2.4.2. Cancer in Africa

Cancer statistics in Africa have not been fully established over the years, due to lack of adequate case report and sustainable medical information management in most of the African countries. Aliterature update study conducted worldwide in 2010, on cancer registry revealed that only one percent (1%) of the literature information originated from Africa, which was very low compared to 34% obtained from Europe and 42% derived from Asian (Howlader *et al*., 2017). Astudy investigated by Kingham and its co-workers. (2013) showed that 650,000 people of indigenous Africans diagnosed with cancer disease every year out of approximately 965 million people, have a lifetime risk of dying as a result of the disease, which is two times higher than what is obtainable in well developed countries of the world.

The most common cancer disease in Africa region, cervical cancer, which is preventable, accounted for 12% of all newly diagnosed cancer disease in both men and women and 22% of all female cancersannually (WHO, 2017). Twenty-three people died annually out of every 34 cases diagnosed with cervical cancer in every 100, 000 women in Africa (Plummer *et al*., 2016). This is because, cervical cancer usually not recognised or treated early enough due to inadequate access to health facilities and lack

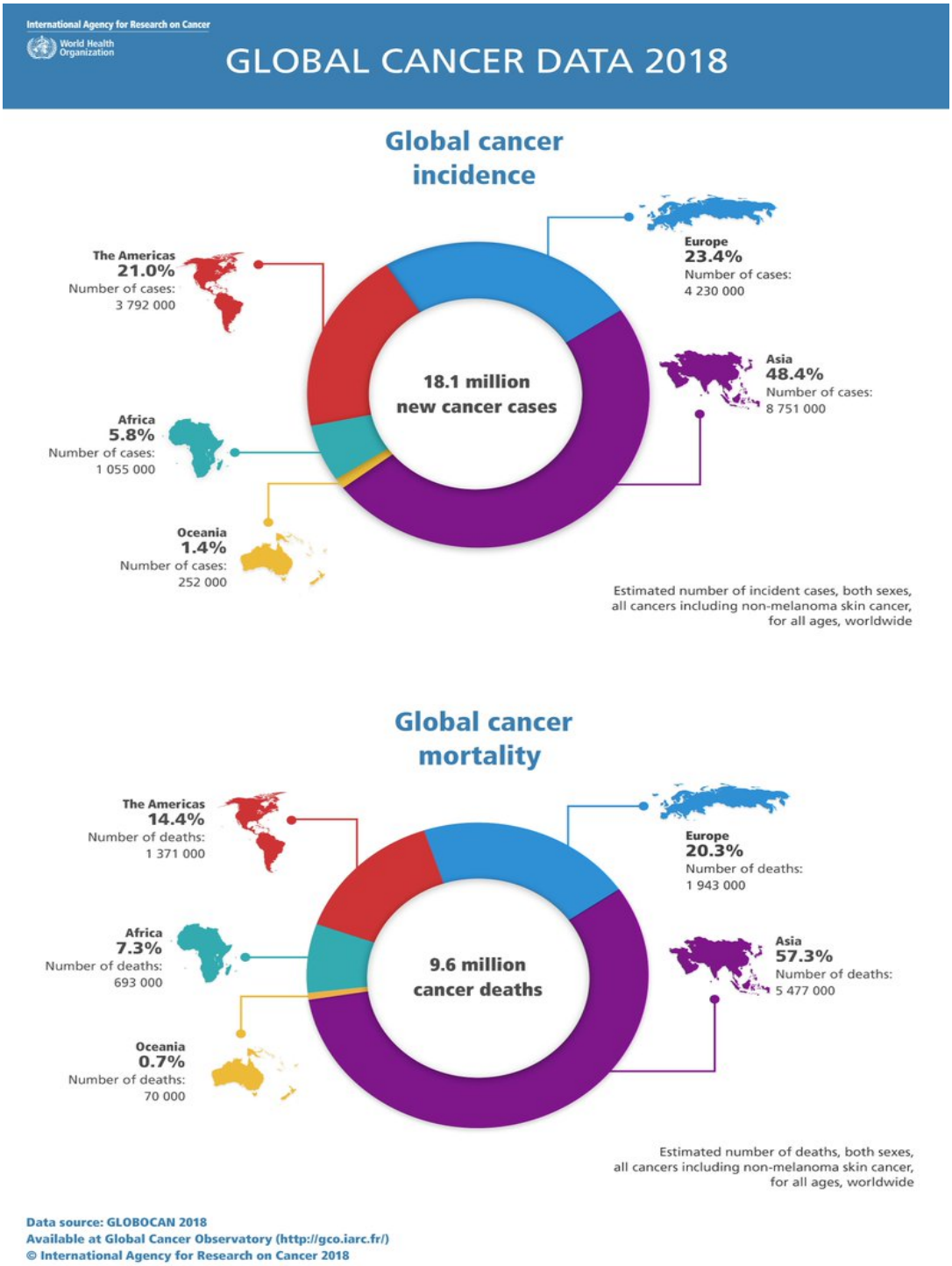


Figure 2.7. Map showing global cancer incidence and death in 2018 (GLOBACON, 2018)

of effective screeninguntil it reaches advanced stages. Meanwhile, routine check to identify precancerous lesions (asymptomatic) in women aged 30-49 years,early treatment and HPV vaccination can help prevent approximately 80% of cervical cancer (WHO, 2017).Also, the World Health Organisation (WHO) reported that 68, 000 of newcases of cancer in Africa are due to infection causing organisms such as human papilloma virus (HPV). However, several health insurance schemes (HIS) has been instituted in many African contries including Ghana, Kenya, South Africa, Eygpt, Rwanda, Kenya, Nigeriaetc. to reduce the burden of diseases including cancer on the populace(Obalum and Fiberesima, 2012). In addition, the use of radioactivity machines in almost every public hospital in Egypt (80 m) and South Africa (45-50 m) has been encouraged.

2.4.3. Cancer in Nigeria

Nigeria, the most populous nation in Africa, is also faced withfactors such as limited number of cancer registries, lack of adequate case report, poverty and sustainable medical information management, regarding cancer statistic data. Cancer registries are where routinely collection and publication of data regarding cancer disease supposed to be obtained. Unfortunately, this information is not readily available in most of the cancer registries of the developing countries (Binu *et al*., 2007; Boyle and Levin, 2008).However, the World Health Organisation (WHO), (2010) reported that Nigeria have the highest cancer incidence as well as death rate, when compared to other Africa countries, with reported 250,000 new cases and 80,000 cancer death each year (WHO, 2017).Breast, cervical, prostate, colorectal, liver and non Hodgkin's lymphoma cancers are the six most common in Nigeria, in descending order (Popoola *et al*., 2013).

The incidence and severity of cancer have been reported to vary across the six geopolitical zones of Nigeria. A report from the north-central part of the country mentioned breast cancer as the leading cancer type among the female inhabitants (Afolayan *et al*., 2012), while another study from the north-western part stated that cancer of the breast was second behind cancer of the cervix (Mohammed *et al*., 2008). A recent study from two cancer registries in Nigeria however, stated that cancer of prostate was far most present among males (Jedy-Agba *et al*., 2012). A cancer registry data for five years review study conducted in the north-central region of the Nigeria revealed that 22.4% of new cancer cases for breast cancer accounted for 35.41% of all cancers registeredfor women (Afolayan *et al*., 2012). Like many other African

countries, various programs such as National Health Insurance Scheme (NHIS) has been put in place by the Government of Nigeria to reduce the burden of diseases especially cancer, which is the most complicated with psychological, social, economic and emotional consequences, thereby reducing untimely death resulting from this disease. The Federal Government of Nigeria through the Federal Ministry of Health (FMoH)in 2018developed a cancer controlplan(2018-2022)*via*theNational Cancer Control and Planning, which stands forthe Ministry envisions cancer control efforts for the country to be achieved within the next five years and beyond. This will subsequently reduce the incidence and prevalence of cancer in Nigeria (Irabor*et al*., 2016).

2.4.4. Economic impact of cancer

Globally, cancer incidence and mortality are rising rapidly, particularly in the developing nations of the world, therefore, there is overwhelming need for countries to adopt and implement control measures. Till now, according to International Agency for Research on Cancer (IARC) and Global Initiative for Cancer Registries (GICR), only 1 out of 5 developing countries has the required data to drive policy and reduce the suffering and burden of cancer (IARC/GICR, 2016). Cancer economic impact is quite noteworthy and keeps growing globally when compared with all other worldwide death causing diseases. For instance, in 2008, cancer economic impact (U$895 billion) is 20% higher than heart disease, second leading cause of economic loss, with (U$753 billion), accounting for 1.5% of the gross domestic product (GDP) of the world (WHO, 2010). Lung, bronchus, and trachea cancers were reported to be the largest drain on global economy with U$180 billion. This may be attributed to an average of 15 years earlier death of smoker compared to nonsmokers. A report by Economist Intelligence Unit of the World Health Organisation, 2009 estimated approximately U$217 billion as the total global economic cost of new cancer cases, including research cost, productivity losses, medical and non-medical costs (EIU, 2009).Similarly, in 2010, the total annual economic cost of cancer was estimated at approximately US$ 1.16 trillion (Stewart and Wild, 2014). In addition, loss of years of life regarding productivity caused by cancer represents a single largest drain on the global economy when compared to other death causing diseasessuch as HIV/AIDS as well as other diseases that are caused by infections (WHO, 2010).

2.4.5. Normal cell cycle

The cell cycle is an ordered set of events, which results in the growth and division of cell into two daughter cells. The cell cycle is segmented into four phases; G1, S, G2 and M phase as shown in Figure 2.8. Each, with its intricacies, and must be fully completed, before the cell can move to the next phase. The cycle begins with the G1 (Gap1) phase, in which mitogenic stimulation causes the activation of the cycle dependent kinases. The kinases phosphorylate various proteins that are needed by the cell for its progression through the cycle. The kinases have dual function of either activating proteins involved in DNA replication or inhibiting proteins that retain cell in a non-dividing state (Schubbert *et al*., 2007).

Cells that fail to be activated for cell division are arrested and enter into the G0 (resting) phase, where they remain quiescent for a long period. However, activated cells progress to the S (synthesis) phase, where DNA replication occurs, with a resultant duplication of the chromosomes. In the G2 phase, the cells ensure that replication of the DNA is complete with the M phase which stands for "mitosis" whereby the chromosomes separate in the nucleus and cytoplasmic division occurs (Sherr, 1996). There are two important checkpoints in the cell cycle. The checkpoint at the end G1,which decides whether conditions are right for chromosome replication to proceed, thereby preventing abnormal cells from entering the S phase. The second checkpoint at the end of G2, which checked for anydamaged and mutated DNA beforeprogressing to mitoticdivision stage (Orford and Scadden, 2008).

2.4.6.Pathology of cancer

Cell growth is a normally carefully controlled process that responds to the needs of a particular organ of the body. Occasionally, the exquisite control systems fail and particular cell type may multiply, even though, a larger population is not required. The resulting cell mass is called a tumour or cancer. Initially, a tumour is established by local invasion of cancerous cells, leading to tissue degeneration. Usually, many cancers arise from just one cell (or from a small number of cells) and needs to acquire several changes to become cancerous (Baselga, 2006). The key important phases in cancer development are shown in Figure 2.9.

Two critical molecular events that are necessary for cancer development include the activation of oncogenes (cancer causing genes) and the inhibition of the tumour

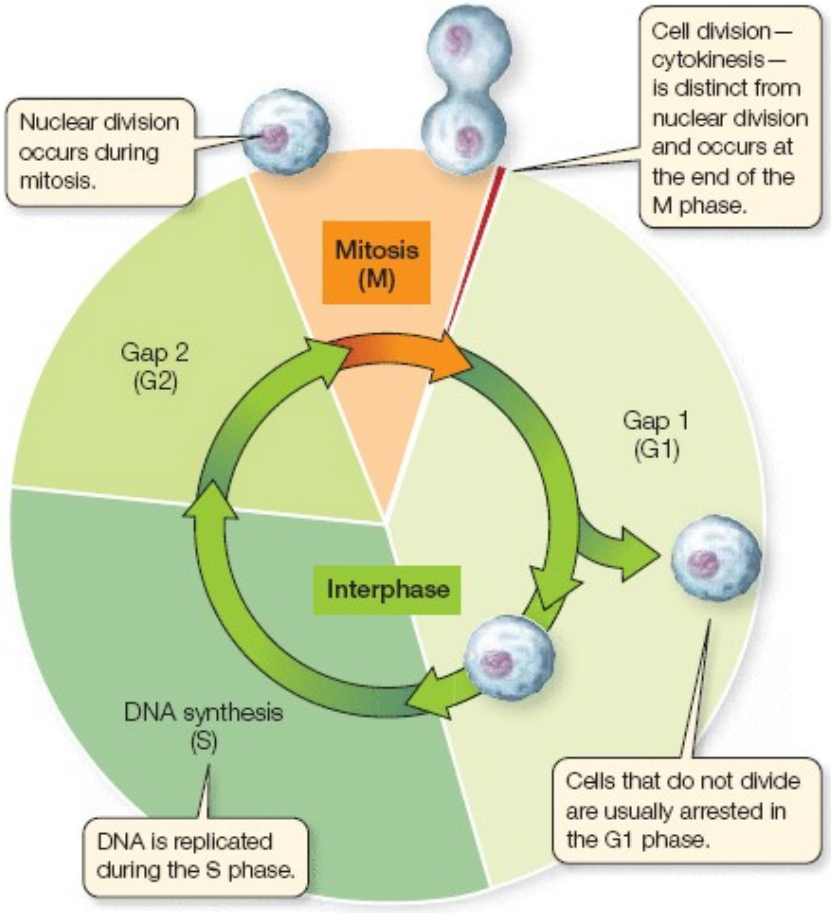


Figure 2.8. The normal cell cycle (Hunt*et al*., 2011)

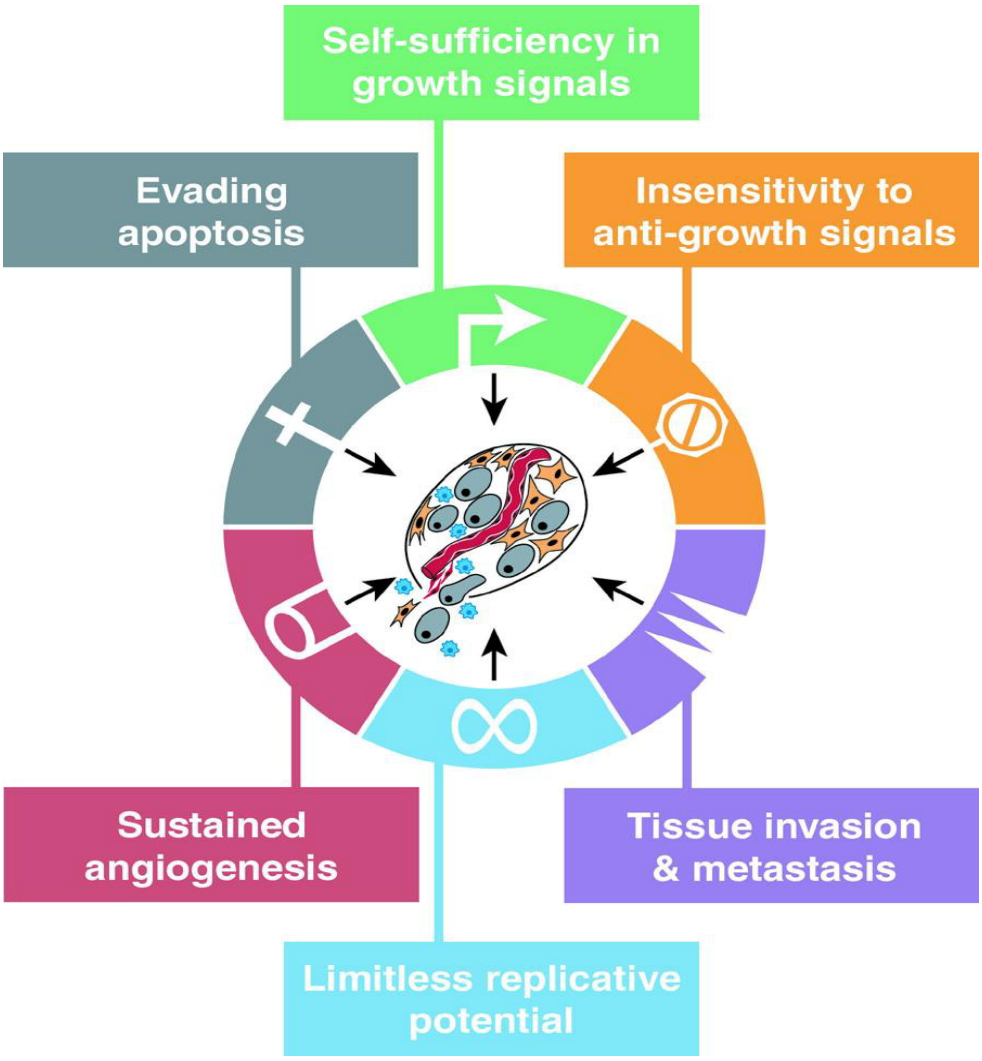


Figure 2.9. The key important phases in cancer development (Hanahan and Weinberg, 2000)

suppressor genes. The proteins that stimulate cell division are coded for by oncogenes also known as mutated genes (anti-oncogenes). Examples include the *erbB* and *k-ras*, which code for the receptor of a growth factor and a protein implicated in the cell signaling pathways respectively,while proteins that inhibit cell division are coded for by anti-oncogenes also referred to as tumour suppressing genes. An example is the p53 gene that codes for the p53 protein. The p53 protein is often referred to as 'the cell policeman', as it stops division of cell at G1 and G2 checkpoints, when there is damaged/mutated DNA. By inhibiting tumour suppressing gene, cancer cells with damaged/mutated DNA escape the cell cycle checkpoints; proliferate with the resultant formation of cancerous daughter cells. It has been observed that many cancers exhibit defective p53 gene (Rudland *et al*., 2002).

The normal living tissue requires a constant supply of oxygen and nutrients from the capillary vessels to survive. In cancer cells, the enlargement of cells means the cells in the centre no longer receive nutrients from normal blood vessels. Without sufficient supply of nutrient and oxygen, tumour cells cannot metastasize or form new tissues. Through a process known as angiogenesis, cancer cell makes growth factors that induce the formation of new blood vessels. The cells to make new blood vessel when divided are inactive in the normal living tissues but are activated by angiogenic factors in tumour/cancer cells. These angiogenic cells metastasize to new location, growing and producing new blood vessels, resulting in speedy growth of the tumour (Veggeberg,2002).

Normal cells grow and die in a controlled and regulated manner through a process known as apoptosis.Before they eventually die, they may divide up to fifty times.During each division, the enzyme, telomerase, replaces the shortened ends (telomeres) of the chromosomes.Adult cells lack the telomerase enzyme and this limits number of times division can take place. However, the telomerase enzyme is fully activated in cancer cells, thereby allowing an uncontrollable division of cell. This makes the cancer cell to evade apoptosis thereby leading to proliferation of abnormal cells (Carmetiet and Jain, 2000).Cancer cells are self-sufficient in growth factors. For the division of a normal cell, external growth factors are needed. Inhibition of the synthesis of these external growth factors by the normal cell regulation will cause them to stop dividing.Whereas, cancer cells have already lost the need for positive growth factors, so they divide with or without these factors. Similarly, when new cells are not

needed by the tissues, anti-growth signals are synthesized to stop the production of new cells. Cancer cells have become independent cells with limitless replicative potentials. Cancer cells travel to other parts of the body by a process called metastasis, through the bloodstream and deposit in other organs leading to secondary tumours (Hanahan and Weinberg, 2000).

2.4.7.Classification of cancer

Cancer can be classified into different types based on some criteria;

(a) Cells from which a cancer arises– The body consists of hundreds of cells and cancer can be grouped into hundreds based on this criterion. For instance, breast cancer starts from the cells of the breast, prostate cancer begins from the cells of the prostate, lung cancer arises from the cells of the lung, and likewise brain cancer starts from the cells of the brain (Bertram, 2001).

(b) Tissue – Cancer can be linked with specific tissue in the body; these include carcinoma; which are cancers that begin in the skin, examples are cancers of the kidney, stomach, bladder, intestines, breast, lungs colon etc. Sarcoma; which are cancers that are formed in the bone and soft tissues, muscle, fat and other connective tissues like tendons and ligaments, examples are malignant fibrous histiocytoma and rhabdomyosarcoma. Leukemia; these are cancers that start in the tissue that forms blood, like the bone marrow, example are myelocytic and lymphatic cancer. Lymphomas; which are cancers that originate from immune system cells, examples are lymphoma and Hodgkin lymphoma. Cancers of the central nervous system are cancers that begin in the brain and spinal cord tissues, examples are pineal parachymal tumours, astrocytic tumours, meningeal tumours, oligodendroglial tumours and germ cell tumours(Ananya, 2012).

(c) System – The system helps to predict the rapidity, development and spread of cancer cell. The abnormality of the cells with respect to the surrounding normal tissues determines the grade of the cancer, increasing abnormality increases the grade, from 1-4. Cells that are well differentiated and closely resemble normal specialised cells belong to low grade tumours (grade 1), cells that are typically differentiated with slight abnormality are classified as grade 2. Very abnormal and poorly differentiated cells are classified as grade 3, while grade 4 is immature, highly abnormal and undifferentiated cells (NCI, 2014).

(d) Cancer stage –The magnitude/severity of individual's cancer based on the origin (primary) tumour as well as the extent, to which cancer has spread in the bodyis described by the word staging. The tumour, node, and metastases (TNM) system is the staging method commonly used, which classifies cancer based on the primary tumour (T), regional lymph nodes involvement (N), and distant metastases (M) (Edge and Compton, 2010). TNM staging system is also used to classify the extent of the disease. This includes stage 0 where the disease is restricted to the surface (*in situ*), stage I where the disease is restricted to the original tissue and there is indication of growth of tumour, stage II where there is partial local spread of the cancerous cells, stage III where there is broad local and regional spread of the cancer and stage IV where the cancer has spread beyond the lymph nodes into other parts of the body (NCI, 2014).

2.4.8.Causes of cancer (Carcinogens)

Generally, there are four categories of external agents, which are referred to as carcinogens, which have been directly linked to cancer. These external agents include physical carcinogens, chemical carcinogens, biological carcinogens and dietary carcinogens. Apart from these external factors, internal agents such as heredity and hormone are also responsible for cancer disease.

Physical carcinogens, such as ionising radiation and ultraviolentray usually increase the risk of skin cancer development, as a result of the prolonged exposure of the skin to unprotected sunlight. The sun emits three types of harmful ultraviolet rays, which are A, B and C. Ultraviolet A possesses long wavelength that penetrates deep into the skin, to damage the skin's collagen, leading to premature ageing and triggers the skin for cancer. Ultraviolet B possess short wavelength which causes sun burn and cancer on the skin. Ultraviolet C has short rays that are lethal to the plants as well as animals and has also been associated with cancer (Sarasin, 1999).

Chemical carcinogens, which include asbestos, components of tobacco smoke and arsenic (a drinking water contaminant) have been attributed to cancer development (Secretan*et al*., 2009). A division of world health organization (WHO) in charge of cancer epidemiology monitoring, international agency for research on cancer (IARC), listed over 400 chemical agents such as benzene, herbicides, coal tar, arsenic etc. as carcinogens (Secretan*et al*., 2009). Asbestos is one of the main occupational carcinogens and exposure to it kills over 90,000 workers, through lung cancer and

mesothelioma, annually (WHO, 2007). Tobacco smoke contains more than 60 mutagens that binds and chemically modified DNA. According to Global Burden of Disease (GBD) study in 2015, tobacco is the most common risk factor of cancers, causing around 22% of global cancer deaths and around 70% of global lung cancer deaths (GBD, 2015). Yearly, approximately 7 million of totalestimated cancer deaths in the world were caused by tobacco smoking (WHO, 2018).

Biological carcinogens include infections from certain bacteria, parasites and viruses. Chronic infections are one of the major risk factors of cancer especially in the developing nations of the world. Approximately 15% of cancers diagnosed in developing countries of the world were attributed to carcinogenic infectionsin 2012 (Plummer *et al*., 2016). *Helicobacter pylori*are known to increase the risk of developing stomach cancer. Human papilloma virus (HPV) is known to be responsible for cervical cancer, the Epstein-Barr virus increases the risk of developing nasopharyngeal cancer and Burkitt lymphoma liver cancers are linked to a hepatitis B virus infection, while HIV infection has been connected to higher risk of developing Kaposi sarcoma (Plummer *et al*., 2016).

Dietary carcinogens include mutagens and carcinogens that are contained in the food, plant, mushroom substances and fungal products. Polycyclic aromatic amines, especially heterocyclic amines (HCAs), remain a great source of carcinogens, as humans withordinary lifestyle are exposed to it. HCAs are imidazolequinolines and imidazoquinoxalines produced from sugar, creatinine and amino acids obtained by heating protein food like fish and meat. HCAs have been demonstrated to increase cancer in various organs such as breast, prostate and colon. Similarly, polycyclic aromatic hydrocarbons (PAHs), a group of contaminants produced by burning of carbon-based materialsgets into our food during food processing.Some PAHs are known to damage DNA, thus cause cancer(Plaza-Bolaños *et al*., 2010).Foods may also be contaminated with toxins, especially aflatoxin B1, (AFB1) that occurs naturally in *Aspergillus flavus*. Experimental animals like rat, monkey and fish have shown that presence of hepatic carcinomas in them is caused by AFB1. Other dietary carcinogens are pyrrolizine alkaloids from various edible plants, hydrazines from mushrooms and cycasin from cycad nuts (Sugimura, 2000).

Heredity is also one of the factors that is responsible for cancer disease. Cancers such as breast and ovary are caused by inheritance of mutated genes. A very few numbers, less than 0.3% of the population are carriers of the mutated gene that causes about 3-10% of cancers (Roukos, 2009). For example, inheritance of BRCA1 and BRCA2 mutated genes can increase cancer of the breast risk with about 75% (Roukos, 2009). The relative risk for developing cancers when the first degree relative (parent) have been diagnosed varies with different cancers, For example, the risk for cancer of the breast is 1.8% when parent have it more than 50 years,and 3.3% ifparent have it less than 50 years (Singletary, 2003), for prostate cancer 1.9% (Bruner *et al*., 2003), lung cancer 1.5% (Cote *et al*., 2012), and colorectal cancer 2.0% (Kampman, 2007).

Hormones such as insulin (the growth factor hormone) as well as their binding proteins have been suggested to be involved in carcinogenesis, by the role they play in proliferation, differentiation and apoptosis of cancer cells (Rowlands *et al*., 2009). Sex-related hormones are important especially in cancers of the testis, prostate, endometrium, ovary, bone, thyroid, and breast (Henderson *et al*., 2000). For instance, a woman with cancer of the breast will have higher level of hormone causing cancer than another woman without cancer of the breast. This may partly explain why the daughters of such women have high risk of having cancer of the breast.

2.4.9.Prevention of cancer

As previously highlighted above, several cancers are originated from environmental exposure and lifestyle of individual, many of which can be controlled. In fact, majority of the cancers can be prevented to a significant proportion and about 50% of cancer deathsare preventable. Even though, scientific evidence has indicated that some cancers may be caused by human genetic make-up which in any ways cannot be prevented (WHO, 2010).

Avoidance of environmental/external factors related to lifestyle, which include tobacco smoking, alcohol intake, canned food intake, occupational hazards, excessive sunlight and body mass index maintenance, proper infection treatment and refuse dump site abstainess could prevent the risk of cancer development. Succinctly, heavy alcohol consumption and the use of tobacco associated cancers can be completely prevented (WHO, 2010).

Physical inactivity/poor nutrition, obesity/overweight related cancers, which accounted for one-fourth to one-fifth of global cancers, as indicated by the world cancer research fund, can be prevented (Bhaskaran*et al*., 2014). A study revealed that about 19% of breast cancer deaths and26% of colorectal cancer mortality are attributable to increased weight and physical inactivity; adhering to a balanced diet lifestyle will help prevent this mortality (WHO, 2007).

Proper vaccination, treatment of infection and behavioral changes could prevent many of the cancers related to infectious agents, such as human immunodeficiency virus (HIV), human papilloma virus (HPV), hepatitis B virus (HBV), *Helicobacter pylori*and hepatitis C virus (HCV). For instance, nearly half of the world's liver cancer has been linked to chronic hepatitis B virus infection and the vaccination of children has been shown to protect them against the virus and prevent liver cancer.Similarly, cancer that has been linked to occupational hazards such as exposure to avoidable carcinogens, like asbestos and unhealthy practices in work place, factories and building sites can be prevented by adhering to health and safety rules (WHO, 2007).

World health organisation stated that approximately 60,000 people die annually from exposure to sun's ultraviolet radiation (WHO, 2007), meanwhile, the ultraviolent radiation from the sun served as a good source of vitamin D.Penetration of the radiation into the skin has been linked to skin cancer. Avoiding direct exposure of the skin to excessive sun can preventskin cancers to a great extent (Siegel *et al*., 2015).Generation of HCAs, a dietary carcinogen, can be reduced by employing useful methods such as preventing direct contact of meat surface to the fire flames, utilisation of foils paper to cover meat before roasting in the oven and avoid microwave catering (Sugimura, 2000).

2.4.10.Cancer therapies (Treatments)

Several options are available for cancer treatment, and the choice of treatment to use depends on the type of cancer, its location and the stage of the cancer disease. The three main options of treatment usually employed are radiotherapy, surgery and chemotherapy.

Radiotherapy involves high energy rays that are targeted directly on cancer cells. This damages the DNA and prevents replication, thereby killing rapidly dividing cancer cells. Radiotherapy may also kill rapidly dividing normal cells, like the hair follicle

cells, leading to side effects like alopecia. Modern instruments that uses radiotherapy include X-ray, ultrasound (sonography), magnetic resonance imaging (MRI scan), computed tomography (CT scans) and position emission tomography (PET scans). Radiotherapy is usually used together with surgery (Armstrong *et al*., 2006).

Surgery is used to remove cancerous growth especially in the tissues and organs and it is usually applied for early stage cancers and benign tumours. Removal of surface tumours through surgery was one of the oldest forms of cancer treatment. The metastasis of cancer from its original point of development to other healthy tissues of the body has delimit the use of surgery incancer treatment, as there is a high tendency for cancer to reappear at another part of the body after being removed by surgery (Sudhakar, 2009).

Chemotherapy involves the administration of cytotoxic compounds, which target rapidly dividing cancerous cells, to destroy them or to stop their metastasis. Chemotherapy is the most widely used for cancer treatment, and research to its usage started during the World War II, when it was discovered that there was a significant reduction in white blood countsof people exposed to nitrogen mustard. This stimulated the interest of scientists in the potential of nitrogen mustard in inhibiting the growth of rapidly dividing cells (DeVita and Chu, 2008). Chemotherapeutic drugs are classified into several groups based on their mode of action as indicated below:

(i) Alkylating agents -Thesekill cells by interacting with DNA during cell synthesis. They perform their pharmacological action by direct interaction with exposed DNA and adding alkyl groups under physiological conditions. This results in permanent damage of the DNA, leading to the death of the cell. Examples include chlorambucil, cyclophosphamide, cisplatin, bendamustine andoxaliptalin (Hurley, 2002).

(ii) Antimetabolites - They are structural analogues of naturally occurring substances (e.g. purine, pyrimidine) that are required for specific biochemical reactions. By mimicking these substances, they fraudulently substitute themselves for these substances and they function by interrupting the cell cycle by preventing DNA synthesis during the S phase, thereby inhibiting nucleic acid synthesis. Examples of such agents include purine analogues (6-mercaptopurine), pyrimidine analogues (5-fluorouracil), amino acid antagonists (alanosine) and folic acid analogues (methotrexate) (Peters *et al*., 2000).

(iii) Topoisomerase inhibitors- Theyarethe class of drugs that inhibit topoisomerase, an enzyme that catalyzes the introduction of transient DNA breaks and also facilitate the re-joining of the DNA strands backbone, phosphodiesterduring normal cell cycle. During DNA transcription and replication of cells, the double-strand nature of the DNA confers a necessity for strand unwinding and this topological problem must be compensated by over-winding elsewhere in the DNA molecule and this balance is made possible by the topoisomerase enzyme. Topoisomerase I binds to the double-stranded DNA and form covalent complexes at 3′-end of the DNA breaks, while at the other 5′-end, covalent complexes are formed by topoisomerase II. Camptotecin, irrinotecan and topotecan are topoisomerase I inhibitors examples while, etoposide, teniposide and mitoxanthrone are examples of topoisomerase II inhibitors (Nitiss, 2009).

(iv)Mitotic spindle poisons - They act during the mitotic phase of the cell cycle by interfering with the microtubules within the nucleus. The microtubules are polymers of α and β tubulin dimers that are involved in the maintenance of cell structure, cell movement, intracellular trafficking and mitosis. They also form a major constituent of the mitotic spindles, and during mitosis, the microtubules enable chromosomal segregation (Jordan and Wilson, 2004). By interfering with microtubule polymerisation and subsequent mitotic spindle formation, spindle poisons suppress spindle work force, leading to mitotic arrest and cell death in speedily dividing cells. Examples of this agent include the vinca alkaloids - vinblastine and vincristine, taxols - paclitaxel and docetaxel, epothilones and eribulin (van Vuuren *et al*., 2015).

(v) Endocrine drugs -They act by interfering with specific hormonal pathway in the human body. Tamoxifenused for breast cancer treatment,was discovered in 1950s during the AstraZeneca's (formerly called ICI Pharmaceutical division) oral contraceptive program (Quirke, 2017). Tamoxifen stick competitively to the steroid binding site on the estrogen receptor (ER) of mammalian cells, thereby stopping the cellular proliferation at the GI/S transition stage (Howell *et al*., 2005). Antiandrogens act on androgen receptor in a similar way to tamoxifen and they are majorly used for the treatment of prostate cancer as alternatives to orchiectomy. Testosterone and dihydrotestosterones are the major hormones produced by the testis and are needed for the proliferation of prostate cancer cells. Antiandrogens lowers the androgen level, thereby starving the prostate cancer cells the needed hormone, leading to slow cellular

proliferation and ultimately cell death. Examplesof clinically used antiandrogens include bicalutamide, flutamide, nilutamide and enzalutamide (Cookson *et al*., 2013).

(vi)Cytotoxic antibiotics - These are produced from bacterial and fungi cultures, they are used for the treatment of cancer and they have varying modes of action. The anthracyclines antibiotics intercalate with the DNA and inhibit the DNA gyrase, which is needed to split and reconnect DNA helix during the process of replication. Anthracyclines stabilise the DNA topoisomerase II thereby preventing the reconnection of the DNA strands. Examples of such agents include doxorubin, daunorubin and epirubicin (Booser and Hortobagyi, 1994). The antibiotic, actinomycin D, intercalates between guanine and cytosine base pairs thereby interfering with DNA transcription. Other antibiotics that are used in the treatment of cancer are bleomycin and mitomycin C (Payne and Miles, 2008). Figure 2.10 shows mode/mechanism of action of these anticancer agents/drugs on cell cycle.

2.5.Plants as a source of anticancer drugs

Since time immemorial, man has depended on plants for food and healthcare (Belay *et al*., 2016). For this reason the early humans developed the art of growing useful plants around where they lived to increase accessibility. Plant is a typical biosynthetic laboratory, the daily physiological and defence roles of plants result in the expressionand synthesis of primary and secondary metabolites (Toshiya and Kazuki, 2010).Many of these secondary compounds can be very active in small amounts and are thususeful as source of drugs to humans for the treatment of their diseases including cancer. Although, many synthetic chemotherapeutic agents are available for the treatment of cancer, the problem of poor selectivity and associated side effects is a limitation to their effectiveness.This has necessitatedthe continuous search for less toxic and more potent anticancer drugs which will be selective against cancer cells, thereby reducing side effects.

The discovery of anticancer compounds from medicinal plants has attracted scientific research, with the discovery of the vinca alkaloids, vincristine and vinblastine, in the 1950s. The success stirred the United States National Cancer Institute (NCI) to screenseveral plants, mainly from the temperate regions, between 1960 and 1982. This resulted in the discovery of many novel agents with potent anticancer activities, including the taxanes and camptothecin (Cassady and Douros, 1980).

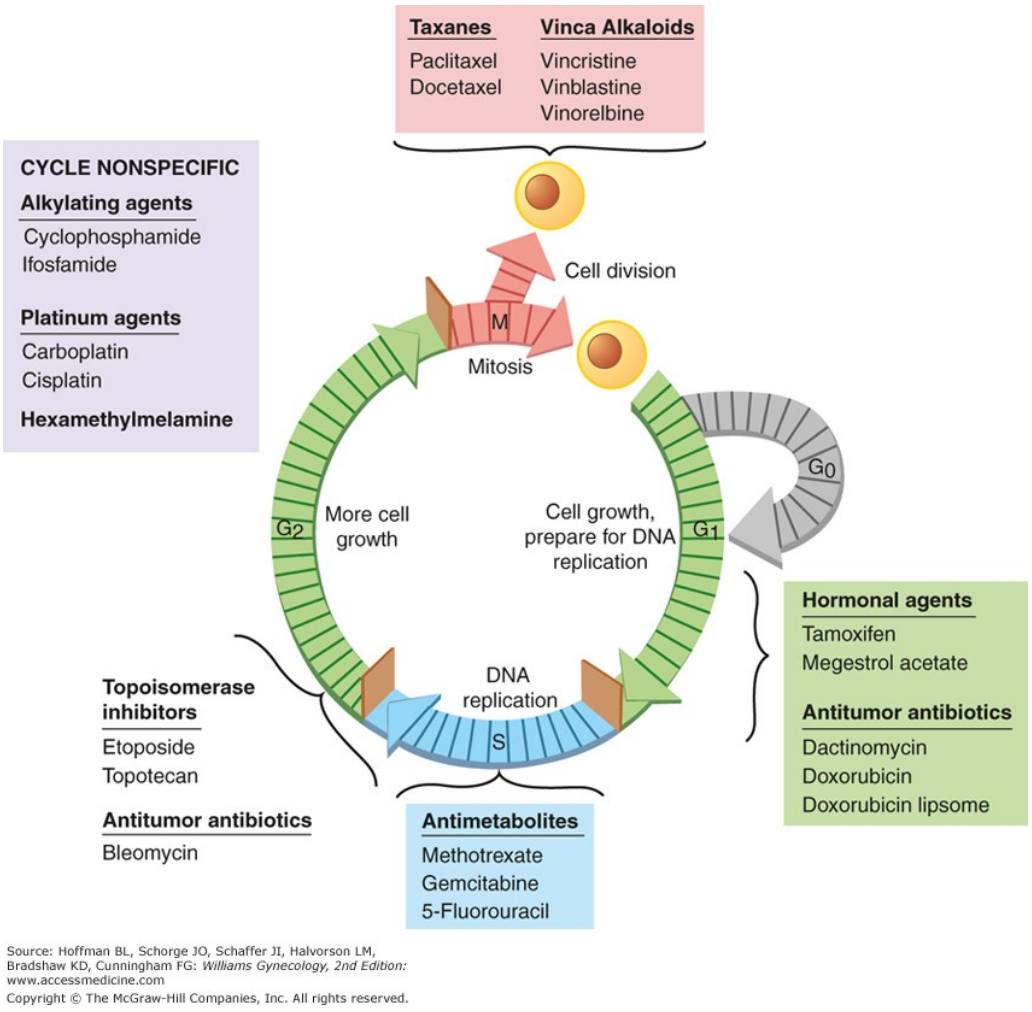


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Figure 2.10.Mode/Mechanism of action of anticancer agents/drugs on cell cycle (Newman and Cragg, 2016).

In 1986, the NCI began another extensive screening of plants with anticancer activity mainly focusing on the tropics and sub-tropical regions of the world (Cragg and Newman, 2013). It was reported that none of the drug made it to clinical trial.Since 1950s, medicinal plants have contributed immensely to the discovery of chemotherapeutic agents. A recent study showed that natural products and its derivatives, contributed nearly half of the anticancer drugs discovered between 1940 and 2014 (Newman and Cragg, 2016).

2.5.1.Anticancer agents of plant origin in clinical use

Vinca alkaloids- Vincristine and vinblastine – isolated from the Madagascar periwinkle *C. roseus*, were the first constituents derived from plant to receive approval for cancer treatment. *Catharanthus roseus*used in folklore for diabetes treatment, while under investigation to determine its active antidiabetic constituents,it was discovered to causebone marrow depression andreductionin white blood cell in ratsexposed to the plant extracts. Afterwards, the extracts were found active in mice with lyphocytic leukaemia. Hence, the discovery of vincristine and vinblastine (Noble, 1990). The vinca alkaloids stick to tubulin in an irreversible manner, thereby hindering proliferation, leading to the death of the cell (Mukhtar *et al*., 2014).

Semisynthetic derivatives of Epipodophyllotoxin, teniposide and etoposide, are use clinically for lymphomas, bronchial and testicular cancers treatment (Lee and Xiao, 2005).Podophyllotoxin was first isolated from*Podophyllum peltatum* L. (Berberidaceae) in 1880, and its structure was described in 1950s. This drug inhibits topoisomerase II, thus stopping thecleavage of the enzyme-DNA complex and arresting cell growth, and eventually cell death (Srivastava *et al*., 2005).

During the extensive screening of plants with anticancer properties from the tropics in the 1960s by NCI, an extract of the bark ofplant *T. brevifolia***,**was discovered to possess excellent anticancer properties (Cragg, 1998). In 1967, Monroe Wall and Mansukh Wani isolated paclitaxel, (Taxol) as the active principle responsible for the anticancer activity of the plant and also determined its structure (Cragg, 1998). The most widely used breast cancer drug, paclitaxel, stick to β-tubulin in an irreversible way, thereby encouraging stabilisation of microtubule which eventually causes apoptosis (Wilson and Jordan, 1995). Paclitaxel is also used for lung and ovarian cancers treatment. Taxol is present in small amounts from natural sources. For

example, the bark of about six mature 100-year-old *Taxus brevifolia*tree is needed to isolate two grams of paclitaxel, which is the approximate quantity needed for a course of treatment (Cragg and Newman, 2005).

A more recent agent derived from plant is camptothecin and its more effective derivatives, topotecan and irrinotecan. Camptothecin was isolated from *C. acuminate*,the Chinese ornamental tree(Cragg and Newman, 2013). Although camptothecin was advanced by NCI in 1970s to clinical trials, its poor solubility profile and severe bladder toxicity effect has led to its more effective derivative, irrinotecan and topotecan development. Camptothecin and its derivatives inhibit topoisomerase-I thereby damaging the DNA, resulting to death of the cell. Topotecan is used for ovarian and lung cancers treatment, while irrinotecan is used for colorectal cancers treatment (Cragg and Newman, 2005). The structures of plant-derived anticancer agents in clinical use are shown in Figure 2.11.

2.5.2.Anticancer agents of plant origin in clinical development

A collaborative work between the United State National Cancer Institute (NCI) and South African's Botanical Research Institute in 1970s, led to the isolation of combrestatins, from *Combretum caffrum* (Eckl. & Zeyh.) Kuntze (Combretaceae),a plant found in South Africa. An anti-angiogenic agent, combrestatins, shutdown vessel in tumours, thereby resulting into tumour necrosis. Combrestatin A4 phosphate is a water-soluble analogue of the combrestatins and has shown promising result in early clinical trials (Pinney *et al*., 2005).

Another promising plant-derived cytotoxic product is thapsigargin. This product was isolated from*Thapsia garganica* L. (Apiaceae), harvested on the Meditarranean island of Ibiza, it induces programmed cell death in proliferating cells of prostate cancer, and is a potential agent in human prostate cancer treatment (Denmeade *et al*., 2003).

Flavopiridol is another agent of future interest, although is a totally synthetic product, which has its basis for design from a natural product, rohitukine, isolated from *Dysoxylum binectariferum* Hook. f. (Meliaceae), as the constituent that is in charge of the immunomodulatory activity observed in the plant. It is in phase II clinical trials against a range of cancers, including lymphomas and leukaemias (Sauville *et al*., 1999).The structures of plant-derived anticancer agents in clinical development are shown in Figure 2.12.

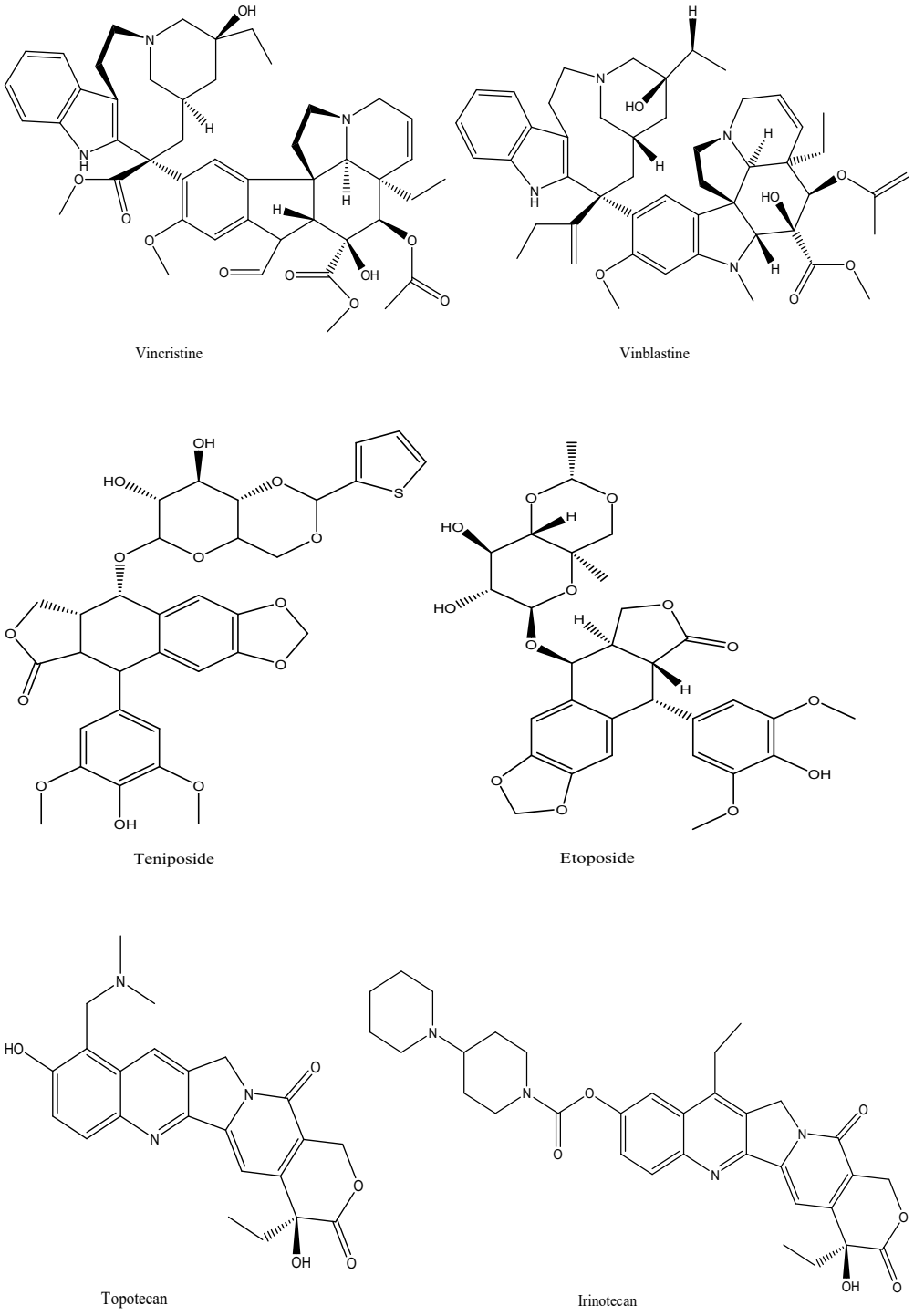


Figure 2.11.Structures of plant-derived anticancer agents in clinical use (Nwodo *et al*., 2016)

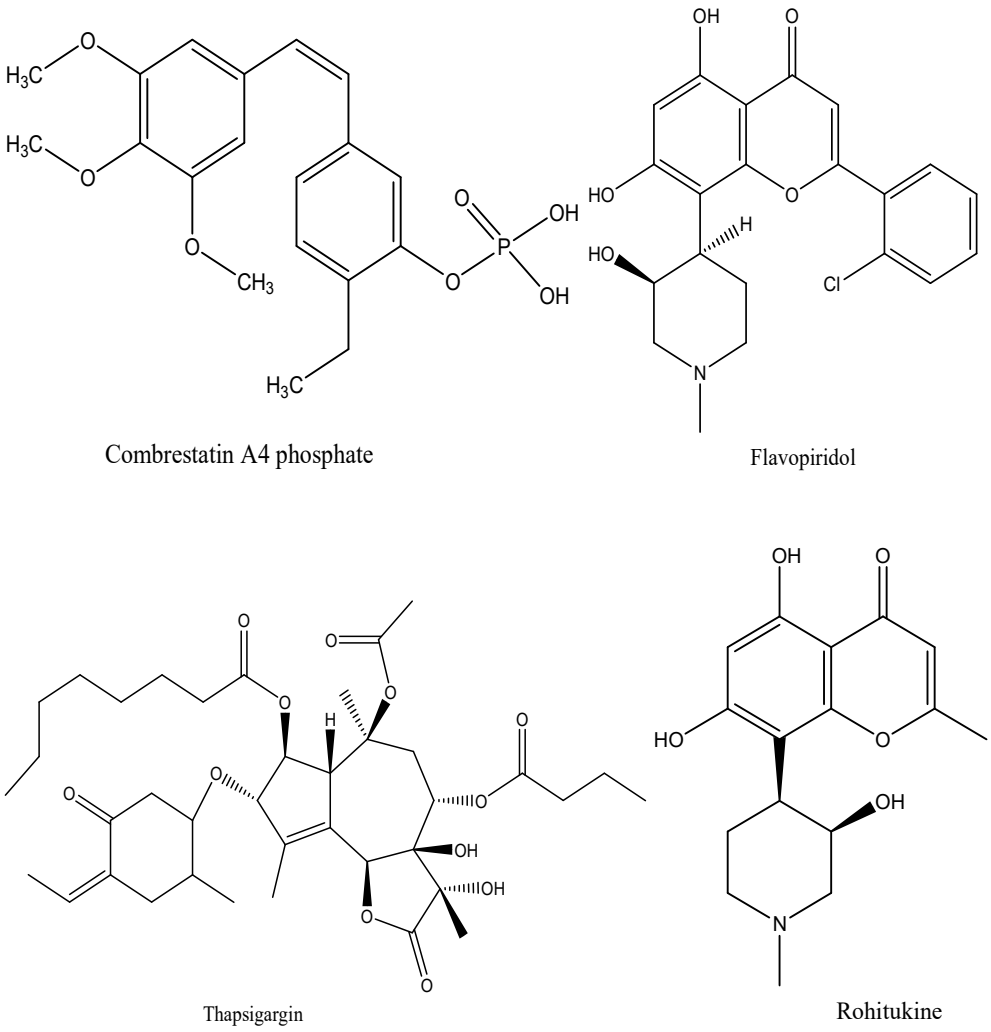


Figure 2.12.Structures of plant-derived anticancer agents in clinical development (Nwodo *et al*., 2016)

2.6. Major groups of compounds with anticancer activityfrom ethnomedicinal plants

2.6.1. Alkaloids and their derivatives

Alkaloids are plant secondary metabolites that mostly possess a basic nitrogen atom, and due to their bitterness, protect plants from the aggression of animals (Nwodo *et al*., 2016) Alkaloids possess wide range of pharmaceutical activities and occur in various families of plant including Apocynaceae, Amaryllidaceae, Meliaceae, Rubiaceae, Rutaceae and Solanaceae (Li *et al*., 2006). Alkaloids are pharmacologically active and their actions are felt in different parts of the body (Trease and Evans, 1989).Currently, about 50% of clinical drugs, especially anticancer drugs, contain the alkaloid moiety, with the chief being the vinca alkaloids, camptothecin analogues and paclitaxel (Wall, 1998; Stanton *et al*., 2011).An example from this group is the pregnane alkaloids, sarcovagine D and sarcorucinine A1, isolated from the leaves of *Sarcocca saligna* (D.Don) Muell.-Arg. (Buxaceae).They displayed potent cytotoxic activity against human breast cancer SK-BR-3,pancreatic cancer PANC-1 and leukemia K562, with the IC_{50} values of 2.25μM, 2.70 μM and 2.87μM, respectively (Yan *et al*., 2011).

2.6.2 Flavonoids and their derivatives

Flavonoids are groups of secondary plant metabolites that have structure of a phenyl benzopyrone, which is C6-C3-C6. Based on their level of saturation, C-ring substitution pattern and central pyran ring opening, they are sub-grouped into flavonoids, flavones, chalcones flavanols,isoflavones and flavanones (Middleton *et al*., 2000). Flavonoids exist naturally in various fruits such as the citrus fruits, apple, berries, grapefruits, onions, grape etc. They are found in every plant parts especially the coloured parts such as flowers. Isoxanthohumol andxanthohumol isolated from hops (flowers or seed cone)of the plant, *Humulus lupulus* L. (Cannabaceae) are example of this group. They have been shown to exhibit antiproliferative activities on human colon cancer (HT29), breast cancer (MCF-7), and ovarian cancer (A2780) cells in *in vitro*study with IC_{50} values of 6.87μM, 3.47μM, and 4.69 μM,respectively (Miranda *et al*., 1999).

2.6.3. Terpenoids and their derivatives

Terpenoids are natural products that constitute one of the largest classesand serve as a repository of important constituents forthe discovery of drugs. Based on structures, terpenoids are grouped into several classes, including monoterpenoids,

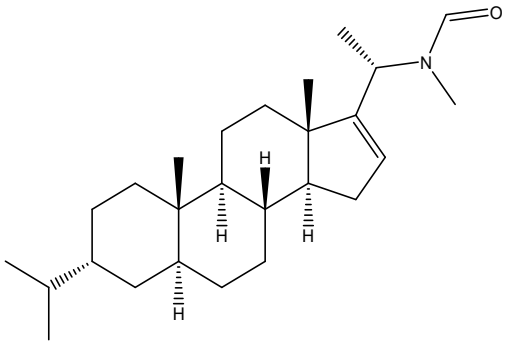
sesquiterpenoids, diterpenoids, triterpenoids and tetraterpenoids. An example from this group is D-limonene, a monocyclic monoterpene found in the essential oils of citrus fruit(Rutaceae), recognised to possess chemopreventive properties for more than two decades. In an orthotopic mouse model for human gastric cancer, D-limonene inhibits tumour growth and metastasis, probably via its antiangiogenic, proapoptotic and anti-oxidant effects (Rabi and Bishayee, 2009).Terpenoids are found in families such as Actinidiaceae, Gentianaceae,Bignoniaceae and Loganiaceae.

2.6.4. Quinones and their derivatives

Quinones represent a group of secondary metabolites with the quinone skeleton. Based on their benzene ring number in the structural skeleton, they are divided into four maingroups; anthraquinone, benzoquinone, phelanthrenequinone and naphthoquinone. Quinones are widely distributed in many plant families including Boraginaceae, Lamiaceae, Leguminosae, Rhamnaceae, Polygonaceae and Rubiaceae. Various quinone derivatives have been shown to possess anticancer activities.An example from this group is Aloe-emodin, an anthraquinone isolated from *Rhamnus fragula*Mill (Rhamnaceae) and *Aloe vera*(L.) Burm.f. (Xanthorrhoeaceae), reported to displayed anticancer activities on manyhuman cancer cells like leukemia, colon,lung, neuroectodermal tumour,hepatocellular and nasopharyngeal in *in vitro* and *in vivo* studies (Pecere *et al*., 2000). Aloe-emodin inhibits cancer cell proliferative by prolonging G1, S, or G2/Mcell cycle phase, cell arrest or apoptosis, depending on treatmentprocedure and type of cancer cell.

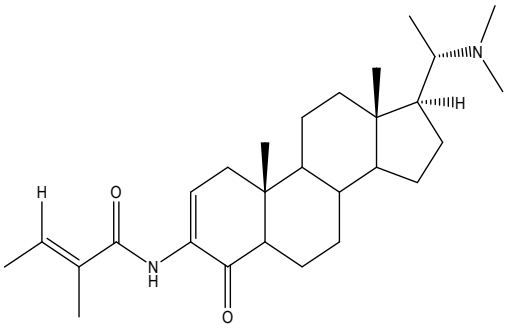
2.6.5. Xanthone and its derivatives

Xanthones are simple three-membered ring compounds with diverse biological profile, which include antihypertensive, antioxidant and anticancer activities (Simoben *et al*., 2015). The xanthone, α-mangosin, isolated from the pericarps of mangosteen, *Garcinia mangostana* L. (Clusiaceae) is an example from this group, it effectively subduedproliferation of the human leukaemia HL60 and colon DLD-1 cancer cells by inducingapoptosis in the mitochondria of the cell (Nakagawa *et al*., 2007). Also, Garcinone E, a xanthone isolated from the hull of *G. mangostana*, exhibited potent cytotoxic activity against five carcinoma cells including Hep3B, HCC36,HA22T, lung and gastric with IC_{50} less than 10μM (Ho *et al*., 2002).Figure 2.13 shows the structures of some ethnomedicinal plant-derived compounds with anticancer activity.



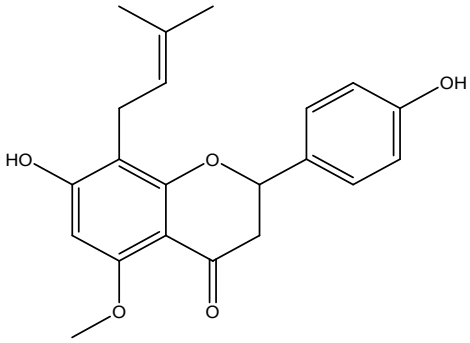
Sarcorucinine A1

(Alkaloid)



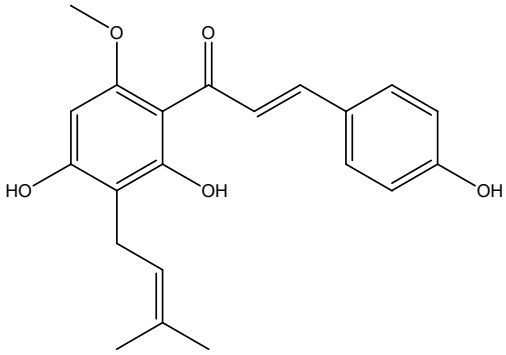
Sarcovagine D

(Alkaloid)



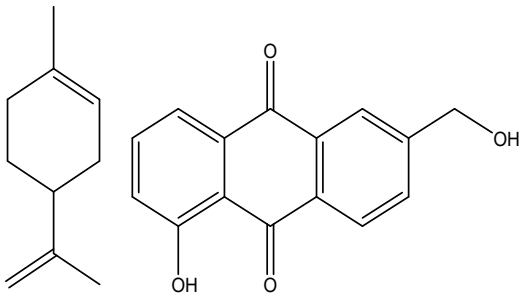
Isoxanthohumol

(Flavonoid)



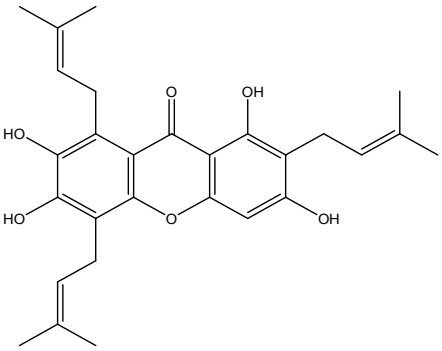
Xanthohumol

(Flavonoid)



D-limonene

Aloe-emodin



Garcinone E

(Terpenoid) (Quinone) (Xanthone)

Figure 2.13.Structures of plant-derived agents with cytotoxicity activities (Simoben *et al*., 2015; Nwodo *et al*., 2016)

2.7. Strategies used in the discovery of plant-derivednatural products

In general, the plant parts used as medicine in traditional medicines were usually treated with locally available material such as mineral substances, water and local gin, and obtained as impure crude extracts in the form of liquid, semisolid or powder prior to oral or topical administration (Evans, 2009). Nowadays, the exploration of active compounds contained in the plants used in traditional medicine has become of interest in drug development research. Over the last few decades, the strategies for research in the area of natural products have been gradually established. The strategy of natural product isolation has shifted from the old approach to the modern day method. Old approach focuses on the chemistry (isolation and identification) of compounds from natural sources followed by biological activity testing (mainly *in vivo*) of the isolated compounds. While, the modern strategies focus on the bioassay-guided (mainly *in vitro*) isolation and identification of active 'lead' compounds from natural sources. Meanwhile, both strategies may begin with the selection of the plants to be investigated based on ethnobotanical information, folkloricreputations, or traditional use (Seidel, 2012).

2.8.Method of assessing biological activity of plant-natural products

In isolation of bioactive compounds from medicinal plants, the bioassay method for successful process should be carefully selected. An ideal bioassay must be veryresponsive to active materials in small amounts; this would help to facilitate the screening of the natural product. In addition, the bioassay should be selective for a particular bioactivity, easy to carry out, not expensive and easy to maintain (Rahman *et al*., 2001). Generally, bioassays are grouped into two: Mechanism-based and cell-based assays.

2.8.1. Mechanism-based assays

This assay involves drugs/plant extracts activity measurement in relation to enzymes, proteins, receptors, DNA etc. One of the effective ways in discovery of drug is by targeting the isolated systems in the metabolic pathways. When properly designed, mechanism-based assay provides robust, sensitive and accurate information about the activity of a compound or extract, even at a very low concentration; however, there are some limitations to the use of mechanism–based assays in drug discovery. One disadvantage of the assay is that some pathway may be missing out since its deal with enclosing environment. Also, it could be difficult to ascertain the potency of some

compounds as they could inhibit an enzyme by a different pathway. More so, mechanism-based assay active agents may not be active *in vitro* due to inability to penetrate cell membrane (Seethala and Fernandes, 2001).

2.8.2. Cell-based assays

Unlike the mechanism based assay where isolated systems are used, cell-based assays depend on interactions of the drug with intact cell. Mode of action of extracts/agents is usually not possible with these types of assaysbut it allows a wide range of potent agents to be discovered. It also provides opportunity to screen out agents that cannot pass through the cell membrane. These assays have a lot of advantages such as broad screening ability and high hit rate than mechanism-based assay despite its high maintenance cost and difficulty to perform (Rahaman *et al*., 2001).

2.8.2.1. MTT assay (3-(4,5-Dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide)

MTT assay is a quantitative,reliable and sensitive colorimetric assay, it measures cell viability due to an enzyme ability, mitochondrial dehydrogenase in living cells to reduce 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide, a water-soluble yellow substrate, to a product, formazan, that is dark blue/purple in colour, which is water insoluble. The amount produced is directly proportional to cell number in a range of cell lines (Mosmann, 1983; Gerlier and Thomasset, 1986). In the investigation of chemicals for the treatment of cancer,this assay is widely used as an initial step (Skehan *et al*., 1990). Compounds/chemicals found potent in inhibiting growth of cancer cellsor killing cancer cells are screened further in animal modelsfor theirtumour growth suppressor ability.

2.8.2.2. XTT assay (2,3-Bis(2-methyoxy-4-nitro-5-sulforphenyl)-2H-tetrazolium-5 carboxanilide)

The need to stabilise formazan crystals formed upon the reduction of MTT prior to spectrophotometric measurement and the nature of the assay's endpoint, brought about development of tetrazolium analogues. XTT salt is one of those analogues in which a negatively charged sulfonate group is attached to the phenyl moieties. These adjustments brought about generation of water soluble formazan crystals, while in the culture medium, eliminating need for the solubilisation procedure. Intermediate electron acceptors are frequently used with the MTT dye to facilitate the XTT dye

reduction. The reduced ability to penetrate cell membrane is attributed to increased negative charge of the molecules (Micheal *et al*., 2005). In the same vein with MTT, XTT assay is applied in the measurement of cellular proliferation where reduction of dye is proportional to number of viable cells in exponential growth phase (Marshall *et al*., 1995).

2.8.2.3. Alamar blue assay

Thisis a simple, sensitive, reliable, rapid, safe, and cost effective assay thatmeasures viability of the cell. It is a sensitive redox indicator assay that fluoresces and changes colour upon reduction by living cells. The reduction of oxidised, non-fluorescent alamar blue to a pink fluorescent dye by the activity of the cells in the medium is believed to be mediated by mitochondrial enzymes; however, it may be due to cellular metabolism impairment (de Fries and Mistuhashi, 1995). Alamar blue does not obtain measurement by killing of cells, as it is non-toxic to cell, as with MTT. Therefore, it allows reuse of cells for further investigations, especially in primary tissue cultures. In addition, alamar blue is more valuable as an endpoint measurement for cytotoxicity rather than a kinetic measure for monitoring cell growth (O'Brien *et al*., 2000). The absorbancecan be taken at 570nmand 600nm.

2.8.2.4. Flow cytometry assay (Live/dead assay)

Sometimes dead cells often give a false positive result, as they tend to stick non-specifically to many reagents, thus making it difficult to get accurate result and analysis. The flow cytometry, a rapid and reliable assay, was designed to help researchers distinguish between live and dead cells in a cell suspension more accurately. Determination of viable cell is crucial when evaluating the response of cytotoxic drugs. The dye exclusion is an example that can be used in this assay. It works based on the fact that live cells have intact membranes that are impermeable to a variety of dyes, which easily penetrate the damaged, compromised, permeable membrane of non-viable cells. The excitation and emission absorbance are obtained at 528nm and 617nm respectively (Ashcroft and Lopez, 2000; Perfetto *et al*., 2004).

2.8.2.5. Cyquant direct cellproliferation and Caspase 3/7 detection reagent assays

Other examples of live/dead assays are the cyquant direct cell proliferation assay and caspase 3/7 detection reagent assay. Cquant direct cell proliferation assay works based

on the ability of the binding dye to permeate the DNA of the cell, in blend with a background suppression reagent. The dye is irresponsive to dead cells as well as cells with compromised cell membranes, thereby causing only healthy cells to be stained (Abraham *et al*., 2008).The advantages of this assay includes; no-wash, homogenous format and fast add-mix-read protocol. This assay can be used to assess cell growth, cell viability, or compound toxicity in a range of application, from high-throughput screening to biproduction (Abraham *et al*., 2008). On the other hand,caspase 3/7 detection reagent assayhas a unique nucleic acid binding dye, which after activation in apoptotic cells, bind to DNA, and produces a bright, fluorogenic response (Cobanoglu *et al*., 2016). One main advantage of this assay over others is that, it does not require wash steps, hence, this helps preserve the fragile apoptotic cells typically lost during wash steps. Caspase 3/7 detection reagent assay can be used an endpoint assay to visualise apoptotic cellsor kinetic assay to monitor the induction of apoptosis of the cells under treatment(Cobanoglu *et al*., 2016).

2.8.2.6. Sulforhodamine B assay

Sulforhodamine B (SRB) is an assay used for determination of cell density, by cell protein content measurement. This assaywas established for National Cancer Research Institute, initiated in 1985for drug discovery program for large-scale anticancer screening.The ability of the dye to bind electronically to protein based amino acid residues of trichloroacetic acid-fixed cells is the principle. The dye behind this method has two sulfonic groups, andis a bright-pink aminoxanthene that binds to basic amino-acid residues under mild acidic conditions. In the presence of weak base such as Tris base, it can be easily dissociated and solubilised for optical density (OD) measurement. The optical wavelength for measuring OD of SRB is 564 nm and as SRB binds, the amount of dye obtained is directly proportional to cell mass (Skehan *et al*., 1990). SRB assay does not differentiate between death and viable cells. Though, it has some benefits over MTT assay. Interference in the MTT reduction without a significant effect on the cell viability has been observed for some compounds, while the SRB assay is rarely affected by interference like this (Plumb *et al*., 1989). Also, SRB assay is not dependent on cell metabolic activity, therefore, it requires fewer steps than the MTT assay (Keepers *et al*., 1991).

2.9.Extraction, isolation and characterisation ofcompounds from plant natural products

2.9.1. Extraction

In an attempt to identify, separate, isolate and characterise a large number of chemical constituents present in medicinal plants, extraction which is usually the first step must be properly carried out to ensure that, potentially active components of the plant are not lost or destroyed during the extraction process. Before extraction, care must also be taken in performing basic steps such as drying and grinding of the plant materials in order to keep the plant constituents intact (Sasidharan *et al*., 2011). Solvent extraction is widely used over the other methods such as distillation, sublimation and pressing and the bioactive constituents being targeted determinethe choice of solvent to use. For instance, methanol or ethanol, which are polar, are usually used for hydrophilic compounds extraction, while a mixture of methanol/dichloromethane (1:1) or dichloromethane only are used for the moderately polar compounds extraction. However, hexane can be used to extract lipophilic compounds(oily compound) as well as to remove chlorophyll from the plant materials (Cos *et al*., 2006). Various techniques which include solid-phase microextraction,microwave assisted extraction, Supercritica fluid extraction, pressurized liquid extraction are modern methods that have emerged and possessed many advantages such as organic solvent consumption reduction, elimination of sample degradation, improvement in extraction efficiency and selectivity as well as elimination of concentration steps before chromatographic analysis (Huie, 2002).

2.9.2. Chromatographic purification and isolation methods

Chromatography is simply the migration of a multi-component mixture through a packed bed, usually silica and they take advantage of a mobile phase and a stationary phase for their separation.

2.9.2.1.Thin layer chromatography (TLC)

Thin layer chromatography represents a method to analyse mixtures via separation of compounds inthe mixtures. It is often used to determine number of components in a multi-components mixture, identify chemical constituents in the mixture, and purify the constituents. The development of the TLC plate commences after the application of samples to the plate. The development process is associated with the distribution of the analyte mixture between two unique phases (mobile and stationary). The stationary

phase of an adsorption chromatography consists of silica gel, aluminum oxide, kieselguhur or magnesium silicate. Thin sheet of glass, plastic and aluminum are solid supports usually used. Solvent is used to dissolve the plant material/extract, whose component is to be separated and the resulting mixture is spotted very close to the bottom (solvent origin) of thealuminum thin layer plate. The eluent, solvent or solvent mixture is allowed to move up the plate by capillary action. TLC can thus be used for the identification of substances in addition to the traditional separation.

2.9.2.2.Preparative thin layer chromatography (Prep TLC)

Preparative thin layer chromatography is a technique useful for small sample purification, and isolation of the compounds, in amount ranges between 10-1000 mg. It enhanced rapid separation of a number of components in a mixture,especially natural extracts (Sherma and Fried, 1995). The operational procedure is almost the same with thin layer chromatograph (TLC), in that it purifieschemical constituents in the mixture making use of the two unique phases (mobile and stationary). Unlike TLC, where the samples are spotted, samples areapplied in long streaksin Prep TLC. After development, specific components of interest in a region of the plate are recovered by scraping the sorbent layerfrom the plate in that region and eluting the separated material from the sorbent using a strong/desire solvent (Sherma and Fried, 1987). Some of the instrumentations used include TLC plates (usually 20 cm x 20 cm, 25 µm thickness), TLC tank and cover, chemical fume hood, organic solvents, ruler, pencil, scissor and aluminium foil paper.

2.9.2.3.Column chromatography (CC)

Column chromatography is another chromatographic technique employed in purification and isolation of plant constituents into its individual compounds. The CC utilise two major phases, the mobile phase (which is the suitable solvent or solvent mixture that elutes the compound mixture in their order of polarity) and the stationary phase (that contains silica gel, usually 60-120 or 60-200 mesh size). Glass tube with a wide circle inlet and a small outlet with a tap or plug as well as retort stand are used as supports in CC. In order to stop the stationary phase from escaping the column, a piece of cotton wool is made to enter the glass column and placed over the outlet. CC principle is based on the movement of compound mixture along with mobile phase via stationary phase, leading to separation of each component in the sample or compound mixture depending on their degree of adhesion to the mobile phase. CC can be used on

scales from micrograms up to kilograms and the main advantage of CC is the relatively low cost as well as easy disposal of the stationary phase which prevents cross contamination due to recycling of stationary phase. However, one of the disadvantages is that it is laborious and takes a longer duration of time to perform (Ranjith*et al*., 2014).

2.9.2.4.Vacuum liquid chromatography (VLC)

Vacuum liquid chromatography is a fast and time saving technique for fractionation, purification and separation of plant natural product constituents based on the interactions of the plant extract that moves along with the mobile and stationary phase.The VLC apparatus consists of a sintered glass funnel with fritted disk of varying grades; G1 (90-150µ) orG2 (40-90µ) orG3 (15- 40µ) and a laboratory water pumpwhich provides 20-70 mmHg vacuum is required for suctioning. VLC is considered superior to column chromatography (CC) in the bioactivity guided purification of natural products, mainly because it allows the fractionation process to be performed in a short period of time. Unlike CC, stationary phase in VLC can dry and sample can be loaded direct without preparing slurry. Also, VLC column can be reused after washing with methanol (100%). VLC is economical and very useful in separation of both large and small quantities of mixture/crude extracts constituents. In contrast to the gradient flow in CC techniques, pressure is applied in VLC to increase flow rate and hence speed up the fractionation (Anupam *et al*., 2018).

2.9.2.5.High performance liquid chromatography (HPLC)

High performance liquid chromatography is a robust and versatile method extensively used in natural products purification and isolation (Cannell, 1998). HPLC instrument consists of a sample introduction device (an auto-sampler or a manual injection valve), a guard column, an analytical column, a detector, a solvent delivery pump, as well as a printer. Separation of plant chemical constituent with the HPLC is usually accomplished due to, different compounds that have variable rates of migration, with a specific column and mobile phase. Degree or extent to which separation will occur is dependent of the mobile and stationary phases(Cannell, 1998). HPLC purifies compounds of interest or targeted compounds through the chromatographer, which selects the ideal circumstances like suitable mobile phase, appropriate rate of flow, right detectors as well as column under which optimum separation will take place. A detector is selected optimally, such that clean peak of the identified sample observed

from the chromatograph, helps in identification of compounds in HPLC technique. The identifying peakmust be well separated from extraneous peaks at the detection levels and must have a retention time. UV detectors are the most popular among UV because they are highly sensitive (Li *et al*., 2004) and most of the natural compounds have absorbance at a very low wavelength (190-210 nm) (Cannell, 1998).

2.9.3.Characterisation (Structural elucidation) methods

2.9.3.1. Infrared spectroscopy (IRS)

Infrared spectroscopy is a simple and reliable analytical technique, which is widely used in natural product researchfor measurement and identification of functional groups present in molecules (Smith, 2011).The IRS instrument consists of a radiation source, measuring and reference cells, wavelength selector, and a detector (transducer).It is a techniquethat is based on the interaction between a molecule and the electromagnetic spectrumradiation.The radiation causes the excitation of the vibrations of covalent bonds within the molecules, thus, a molecule signal is proportional to the square of the change of dipole moment that occurs during vibrational motion of the molecule (Smith, 2011). The entire IR region is divided into two groups;the frequency region (4000-1500 cm-1), where the peaks correspond to different functional groupsand fingerprint region (1500-400 cm-1), where each atom of the molecule is connected by bond, that requires region of different IR for their characteristic peaks to be observed. Most of the information interprets asIR spectrum is obtained from the region known as functional group,while the region of the fingerprint, however, can be useful for confirmation of unknown structure by direct comparison with a known spectra. Fourier transform infrared (FTIR) spectrometer is the most common one usually used in the laboratory (Smith, 2011).

2.9.3.2. Mass spectrometry (MS)

Mass spectrometryis an analytical method in science useful for identification of type and how much of a chemical compound is present within a given sample via measurement of mass-to-charge ratio and abundance of gas-phase ions (Yates, 1996). In other words, mass spectrometry is a continually evolving analytical chemical technique which measures the mass-to-charge ratios of chemical compounds in a sample in order to demystify the amount present (abundance) as well as the type of chemical compound. Mass spectrometers take advantage of the variation in the mass-to-charge (m/z) ratio of ionised molecules to effect their separation. An idea of the

chemical and structural details of a molecule is possible from its m/z ratio. This possibility arises from the highly specific fragments obtainable from the mass spectrometer which provide unique structural information about the molecule (Peters*et al*., 2001). Basically, mass spectrometer is designed to evaporate and ionise molecules in a vacuum, producing gas phase ions, separate the ions in space and/or time based on their m/z ratios, andthen measure the amount of ions with specific m/z ratios.

2.9.3.3. Nuclear magnetic resonance (NMR)

Nuclear magnetic resonance is an analytical method in natural product research employed to determine the molecular structure of a compound by analysing the spectra. It is used to determine the purity of a compound as well as quantified certain product in a reaction mixture (Shah *et al*., 2006). It consists of a sample-holder surrounded with a very strong magnet, a radio-frequency emitter, a radio-frequency receiver and a display, which shows the spectrum. NMR woks based on the spinning charges generated by a magnetic field around it, with a magnetic moment (μ) proportional to the spin. Two spin states, which are +1/2 and -1/2 were generated in the presence of an external magnetic field (B_0), The nucleus of low energy +1/2 spin state generates magnetic field, which is the same as the direction of external magnetic field and the nucleus of higher energy -1/2, spin state produces magnetic field opposite to the direction of external magnetic field. The difference in energy between these two levels dependent on the external magnetic field applied and is always small. Thus an energy transfer is possible between these two energy levels (Paudler, 1974). The signal that matches with this energy transfer is then measured and processed to show the NMR spectra of the nuclei. Apart from ^{1}H and ^{13}C, which are the most common, there is two dimensional NMR such as, Correlation spectroscopy (COSY) and nuclear overhauser effect spectroscopy (NOESY) etc.

2.9.3.4. Ultraviolet spectroscopy (UV)

Ultraviolent spectroscopy is one of the methods in natural product research used for structural elucidation of compounds. A portion of its radiation is absorbed as it passes through the sample, and the energy (wavelength) absorption is dependent on the electronic energy differences possessed by the sample molecules (Pavia *et al*., 1996). Depending on the chromospheresof a sample; its molecules exhibit different energy transitions. However, they absorb energy when transisting from the ground state to excited statesandusually emit light when returning to their ground states (Wang *et al*.,

2005). UV spectroscopy is useful in determination of conjugated double bounds and characterisationof carbonyls, aromatics, and alcohols as well as alkenes (Pavia *et al*., 1996).UV is carried out with a spectrophotometer, made up of a deuterium lampthat provides wavelengths within the visible region (190 to 800 nm), a photomultiplier, a detector and a recorder (Skoog and Leary, 1992). UV light is split and converged toward the sample molecules and a referencemolecule, this portion of the light, between 200 to 400nm exiting the sample or reference molecules, is absorbed by photomultiplier, where their intensities/frequency are detected and recorded.

Following the elucidation of the chemical structure of the active compound responsible for bioactivity in a plant extract, it is needful to determine the actual mechanism responsible for the biological activity. This can be determined by conducting some laboratory experiments including genomics and proteomics studies that are focused on observing the changes of gene and protein expressions respectively. Subsequent to this, *in vivo* assays and toxicological studies will be conducted before proceeding to the clinical trial phase, for further pharmaceutical production process (Sarker and Nahar, 2012).

2.10.Review of research plants

2.10.1.*Caesalpinia benthamiana*

Synonym(s): *Mezoneuron benthamiana*

Eglish/Common name(s): Tiger's claw

Local name(s) by West Africa:**Nigeria**-Yoruba-Oyo(Amuranju), Ekiti (Egun ọ̀ganiboloda); ***Senegal***-Balanta(Némin)and**Gambia**- Hallam (tiger's claw)

2.10.1.1. Taxonomic description of the plant

Kingdom: Plantae

Phylum: Tracheophyta

Class: Magnoliopsida

Order: Legumes

Family: Leguminosae

Genus: *Caesalpinia*

Species**:** *benthamiana*

2.10.1.2. Origin and geographical distribution

*Caesalpinia benthamiana*originated from Africa and is widely distributed in West and Central Africa, from Gabon to Senegal. It is usually found in humid and waste ground localities, deciduous woodland and savanna. It is also very common, especially in rain forest and on the road side (Bosch, 2007).

2.10.1.3. Family: Leguminosae

The Leguminosae is a large and economically important family of flowering plants, and it is sometimes being regarded to as, the legume family(Tucker, 2003). The name 'Legumes' was obtained from the look of typical fruits of the majority of the plants in this family. Unlike the other name 'Fabaceae' which was derived from the defunctgenus 'Faba', a Latin word, which means 'bean'. After the families, Orchidaceae and Asteraceae, Leguminosae is the third largest familyof flowering plant, with about 751 genera and over 19,000 species and second in economic importancebehind cereal (Christenhusz and Byng, 2016).*Astragalus* is the largest genera of this family, with more than 2,000 species, followed by *Acacia* (more than 900 species) and *Indigofera*(about 700 species). Other genera include *Crotalaria* (600 species), *Mimosa* (500 species) and *Caesalpinia* (200 species) (Magallon *et al*., 2001). Leguminosae are cosmopolitan family of flowering plant, which only absent in Antartica. Many genera of this family are extremely widespread, while some are endemic to a single country. Leguminosae are widely distributed in most major land biomes, spreading from arid to wet tropical, grassland as well as coastal (Doyle and Luckow, 2003).

2.10.1.4. Genus: *Caessalpinia*

Caesalpinia is the name of a genus of enormous size and of ancient origin. It is named after the Italian naturalist, Andreas Caesalpino, of Arezzo (1519-1603) (Wagner *et al*., 1999). *Caesalpinia* consists of about 200 species, made up of shrubs, climbers, tall and small trees, fully equipped with curves, spines, sharp thorns and hooks. *Caesalpinia* members are widely distributed in the tropics, subtropics, in America and Asia, Australia, and Africa (Thulin, 1983; Ulibarri, 1996). About 25 species are found in the Caribbean, 10 species in Cuba and the Bahamas, 2-3 species in Mexico and a few extending to Central America (Polhill and Vidal, 1981). *Caesalpiniapulcherrima*(L.) Sw. (Leguminosae) is found in the Philippines and the Caribbean where it is used as ornamental plant. *Caesalpinia* is also found in Colombia, Ecuador, Peru, Paraguay and Argentina and is very popular in Brazil (Allen and Allen, 1981). Nine species are

widespread in Asia with two confined to China (Lewis, 1998). The genus *Caesalpinia* is also popular in Thailand and Indonesia (Roengsumran *et al*., 2000). In Africa, especially Nigeria, *Caesalpinia* is widespread in the western and southern areas, with *C. benthamiana* being the most common. Plants in this genus have been used globally in folkloric healthcare for numerous diseases treatment and various investigations have been carried out to validate their folklore uses (Zamble *et al*., 2008).

2.10.1.5.Botanical description

Caesalpinia benthamiana is a climber or shrub, with dimensions of up to 20 m height by 8 cm width, fully equipped with hard spines and thorns (Bosch, 2007). The species, *benthamiana* was formerly in the genus, *Mezoneuron*, distinguished by thin and winged pods. The leaves of *C. benthamiana* are alternate in arrangement and compound bi-pinnate in form, with 5-6 pinnae on a leaf. The stipules are very small and not clearly visible (Bosch, 2007). The petiole is about 5-10 cm long with a swollen base. Rachis is long of about 15-20cm with several recurved spines, especially at the base of the pinnae. Leave base and apex are rounded in shape and free from hair. Inflorescence is about 20cm long,branched or not branched terminal raceme, with hairs and numerous flowers. Flowers are bisexual with 5-merous and 5-10 cm long pedicel. Sepals are not equal, with the lower oncehaving hood-shape embracing each other. Petals of about 2-2.5 cm wide are free, yellow in colour and unequal (the upper once are larger). Stamens are about 11mm long, free and hairy. It has a superior ovary located on a slender style. Fruits, usually 4-6 seeded are linear to oblong in shape with bright red to pink flat pod (Bosch, 2007).Figure2.14 showsphotograph of (a) leaves, (b)flower and (c) stem of *C.benthamiana*plant at a forest in Ode-Ekiti, Ekiti State.

2.10.1.6. Cultivation

The plant is usually harvested from the wild for local use, mainly medicinal, but also as a source of water. However, it is sometimes planted in the hedges of houses and lands to make them impenetrable (Burkill, 1995).

2.10.1.7.Economic uses

The plant has vicious, recurved thorns and so, it is grown in hedges and wall sides for protection. The leaves are used as a masticatory and the stem, when cut, secrete water which is believed to scare witchcraft (Burkill, 1995).

(a)

(b) (c)

Figure 2.14. Photograph of(a) leaves, (b)flower and (c) stem of *C.benthamiana*plant at a forest in Ode-Ekiti, Ekiti State, picture taken by Famojuro T.I, in 2015

2.10.1.8. Ethnomedicinal uses

In Africa, the aqueous decoction from the roots of *C. benthamiana* is used locallyfor the management of many diseases especially, erectile dysfunction (Zamble *et al*., 2008). In Nigeria, Senegal and Guinea, the decoction of the leaf, root and stem bark is used for the treatment of infectious diseases such as urethral discharge (Bosch, 2007). Infusion of the powdered root is taken orally and topically to clear any form of discomfortin Senegal. The young leaf is consumed fresh in Guinea, to purify and detoxify the body system. In Cote d'Ivoire, the juice obtained from stem of *C. benthamiana* is dropped into eye to get rid of cataract and other eye problems (Dickson *et al*., 2006) and the mash-up of the leaf is used as paste to treat snakebites (Bosch, 2007). In Nigeria and Cote d'Ivoire, the alcohol extract obtained from the root and stem is used as dental hygiene to cure toothache and increase sexual urge (Zamble *et al*., 2008). Decoction of the root of *C. benthamiana* is considered effective remedy for dysentery in Ghana (Irvine, 1961). The powdered roots are mixed with Shea butter or palm kennel oil to treat skin diseases and wounds (Dickson *et al*., 2012). The tincture of leaf twig of *C. benthamiana* is used in Sierra Leone for patients suffering from venereal diseases and impotence. The root of *C. benthamiana* is extracted with palm wine and used to increase strength and sexual urge (Zamble *et al*., 2008).

2.10.1.9.Previous related biologicalstudies

Moronkola *et al*, (2009) evaluated the topical anti-inflammatory activity of the essential oils isolated from*C. benthamiana*leaves at 5 mg and 2.5 mg dose, using the 12-O-tetradecanoyIphorobol-13 acetate (TPA) induced ear edema in mice. Results indicated that all mice treated with 5 mg and 2.5 mg dose of the oil had reduced ear edema with 92.3% and 76.9% respectively. It was shown that the oil performed significantly better against Indomethacin, a standard anti- inflammatory drug used in the experiment with 75.2%.

Mbagwu and Adeyemi, (2008) investigated anti-diarrhoea activity of *Castor* oil (0.2mL/animal) induced diarrhoea in Winstar (170 to 200g) and Swiss 119 (25 to 30g) mice of the aqueous extract of *C. benthamiana*. The mice were fed orally with 400-1600 mg/kg of the plant aqueous extract 30 minutes prior to the administration of *Castor* oil. A dose dependent and significant ($p<0.05$) anti-diarrhoea activity was observed for the extract of *C. banthamiana* in the experiment, indicating strong anti-diarrhoea effect of the plant extract. It was reported that the frequency of stooling in

the mice was drastically reduced and a general decrease in weight was observed in wet, hard, mild and copious stools.

Modulation property of the ethanol, petroleum spirit and chloroform extracts of the root of *C. benthamiana* using the standard antibiotics; norfloxacin, erythromycin and tetracycline in a 96-well plates on multi-drug resistant strains of *Staphylococcus aureus* was investigated by Dickson *et al*. (2006). Ethanolic and chloroform extracts of the plant was observed to have 4-fold potentiation activity against norfloxacin – resistant strain of *S. aureus*, while 2-fold potentiation activity was recorded for the petroleum spirit for the same antibiotic. The observed property was compared with the extracts at 10 µg/mL and 20 µg/mL for reserpine which served as the standard MDR (Multidrug resistance) inhibitor and the results was found to be promising.

Dickson *et al*. (2006) conducted a comparative study on antioxidant and scavenging properties of the chloroform and petroleum spirit extracts of three medicinal plants; *C.benthamiana*, *Microglossa pyrifolia*(Lam.) Kuntze (Compositae) and *Securinega virosa*(Roxb. ex Willd.) Baill (Phyllanthaceae). The chloroform and petroleum spirit extracts of *C. benthamiana* was reported to have the highest antioxidant properties among the three plants with IC_{50} values of 15.55 µg/mL and 19.72 µg/mL for petroleum and chloroform extracts respectively, 23.15 µg/mL for petroleum spiritand 30.36 µg/mL for chloroform extracts, as the inhibition concentration of lipid peroxidation of bovine liposomes in free radical scavenging activity.

Investigation of the aphrodisiac property of the root aqueous extract of *C. benthamiana*, by Zamble *et al*. (2008), was carried out in an experiment where five sexually matured rats divided into 2 groups were used. The first (control group) was given 1mL of tap water and the second (test group) were given 50 mg/kg body weight aqueous extract of *C. benthamiana.* The results indicated that the mounting frequency (MF) ($p<0.001$) increased significantly while the latency (ML- how long it takes a rat to mount or insert his penis into female) decreased after 30 min, 1.15hr and 3.15hrs of observations as compared to the untreated rats with lower (MF) and higher (ML). He suggested that enhancement of libido observed in the rate and the latency reduction with the male showed a remarkable aphrodisiac property of *C. benthamiana.*

Zamble *et al*. (2008) reported the vasorelaxing property of aqueous extract of the root bark of *C. benthamiana* in an experiment involving the use of rats, whose aorta ring

were contracted with induced phenylephrine using rat strip, the application of the aqueous extract (1-20 mg/L) on the aorta ring, resulted in an immediate relaxation of the aorta which continued to reach a plateau after 15 minutes. Furthermore, the quantitative polymerase chain reaction (QPCR) analysis revealed that the root bark extract triggered eNOS mRNA expression ($p< 0.001$) with 2.4 ± 0.5, 4.3 ± 0.7 and 5.7 ± 0.7 at 0.1, 1 and 10 mg/L concentrations, respectively, indicated that the results are of great interest.

Dickson *et al*. (2006) investigated the antimicrobial activity of petroleum spirit, chloroform and ethanol extracts of the root bark of *C. benthamiana* against Gram-positive and Gram-negative bacteria and some dermatophytes such as *Micrococcus flavus* (NCTC 7743)**,** *Bacillus subtillis* (NCTC 10073), *Staphylococcus aureus* (NCTC 4163), multidrug-resistant *S. aureus* SA- 1199B etc. He reported that the MIC values against these organisms ranged from 31.2 to 1000 µg/mL after treatment with three extracts of the plant root bark.In addition, Dickson *et al*. (2007) investigated the antimicrobial activity of three compounds (deoxycaesaldekarin C, benthaminin 1 and benthaminin 2) isolated from the petroleum ether fraction of *C. benthamiana* root bark and reported that the highest antibacterial activity was observed with benthaminin 2 against *Staphylococcus aureus* and *Micrococcus flavus* with MIC of 47µM and less effective against *Bacillus subtillis* (MIC 95 µM), *Pseudomonas aeruginosa* (MIC 398 µM) and *Staphylococcus aureus* (MIC 98 µM). Similarly, Fayemi and Osho (2012) investigated the extracts obtained from the leaves of *C. benthamiana* against *Candida* species and reported that the leave extract was active at 5 mg/mL against *C. glabrata*, 6 mg/mL against *C. torulopsis*, 8 mg/mL against *C. albicans* and *C. stellatoidea* and 15 mg/mL against *C. krusei*.

2.7.1.10. Phytochemical constituents

The leaf of *C. benthamiana* is rich in saponins and tannins (Fayemi and Osho, 2012). Mbagwu and Adeyemi. (2008) revealed the plant contains flavonoids, tanins, reducing sugar, saponins, phenols and anthraquinones in the phytochemical analysis conducted. The root bark has also be shown to contain phenolic compounds such as resveratrol, tannins and gallic acid (Zamble *et al*., 2008). Binutu and Cordell (2000) conducted a study on the chloroform (CHCL3) and butanol (BuOH) extracts of *C. benthamiana* and indicated that it contains methyl gallate, gallic acid, shikimic acid-3-0-gallate, epicatechin, 1-0-methyl-D-chiro-insotol, epicatechin-3-0-gallate and Kaepferol-3-(6-

galloyl). *C. benthamiana* root back contains Benthaminin A-B, Deoxycaesaldekarin C, β-sitosterol and stigmastenone (Dickson *et al*., 2007). The leaves of *C. benthamiana* contain monoterpenes, 36%; sesquiterpenoids, 19.6%; sesquiterpenes, 20.4% and a non-ubiquitous apocarotenoid-$C_{20}H_{30}O$, 16.7% (Moronkola *et al*., 2009).

2.10.1.11. Phytochemical components isolated from *C.benthamiana*

(Dickson*et al*.(2012) isolated deoxycaesaldekarin C, benthamianin 1, benthamianin 2 and a novel cassane-type furaditerpenoid, benthaminin 3from *C. benthamiana*. Binutu and Cordell (2000) also isolated methyl gallate, gallic acid, shikimic acid-3-0-gallate, epicatechin, 1-0-methyl-D-chiro-insotol, epicatechin-3-0-gallate and Kaepferol-3-(6-galloyl) from the chloroform and butanol extracts.Structures of some the isolated compounds from *C. benthamiana*are shown in Figure 2.15.

2.10.2.*Combretum racemosum*

Synonym(s): *Combretum corymbosum* Schumach.**.** *Combretum leucophyllum* G.Don**,** *Combretum trigonoides* Perr. ex DC.**,** *Combretummacrocarpum*Beauv.,*Combretumflammeum* (Welw. ex M.A.Lawson) Hiern.

English/Common name(s): Bushwillows, Christmas rose (Port Harcourt, Nigeria) False bougainvillea (Kenya).

Local name(s) by West Africa**: Nigeria**-Edo(Ọkọ́sọ́), Yoruba-Oyo(Ọgàn pupa), Ekiti (Ọgàn), **Ghana**-Asante(O-hwirɛmo, Wotã),**Ivorycoast**-Akye(Bétzo), **Mali**-Manding-bambara(Kotri)**, Senegal**-Balanta(Kindé kindé), **Sierraleone**-Limba (Bede), **Gambia**-Manding-mandinka kotura (Fox)(Burkill, 1985).

2.10.2.1. Taxonomic description

Kingdom: Plantae

Phylum: Tracheophyta

Class: Magnoliopsida

Order: Myrtales

Family: Combretaceae

Genus: *Combretum*

Species: *racemosum*

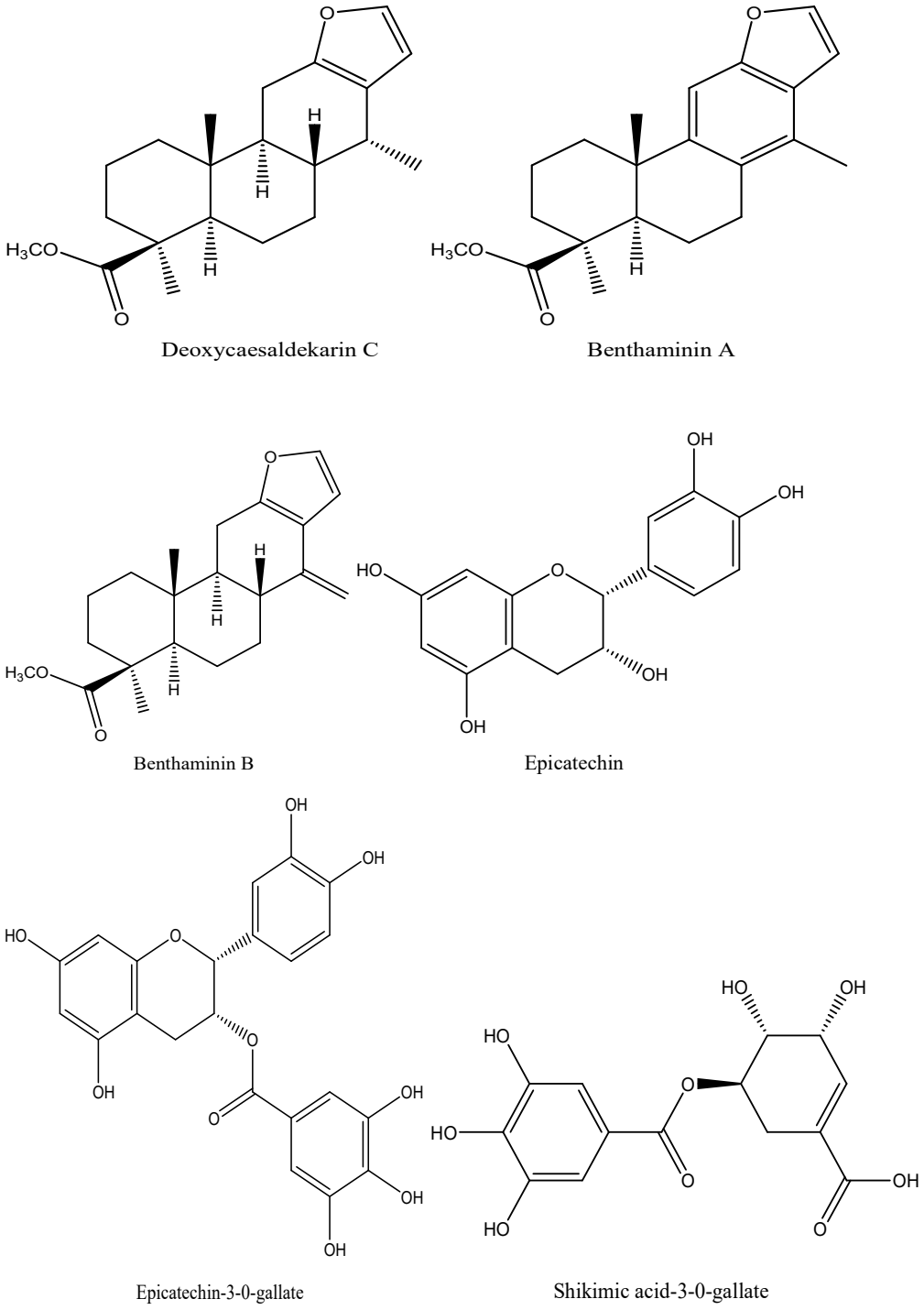


Figure 2.15.Structures of some isolated compounds from *C. benthamiana*

2.10.2 2. Origin and geographical distribution

Combretumracemosum is confined to tropical Africa and represented in Nigeria as a climbing shrub (Okwuosa *et al*., 2012). It is found in riverine forest, margins of rain-forest and secondary deciduous forest. It is widely distributed across Africa;from Sudan to Cameroon, Congo Democratic Republic, Gabon, Kenya and Uganda (Burkill, 1985).

2.10.2.3. Family: Combretaceae

The Combretaceae from the order Myrtales, is one of the largest family of flowering plant, which is made up of climbers, shrubs, herbs and trees, comprises of about 20 genera and 600 species, which are distributed tropically around the world and particularly centers of diversity in Africa and Asia (Exell and Stace, 1966; Heywood *et al*., 2007; Stace, 2007; de Morais*et al*., 2012; Christenhusz and Byng, 2016). The combretaceae family includes the leadwood trees and the *Combretum* imberbe species, which are large to medium size tree with sparse canopy of grey-green leaves, found in the southern Afrotropics. Three other genera - *Laguncula*, *Conocarpus* and *Lumnitzera* are growing in the mangrove habitat (Stace, 2007; Puntiwa and Pimwadee, 2015).*Combretum* and *Terminalia*, with about 380 and 200 species respectively were the largest genera of this family (Pietrovski *et al*., 2006). Many members such as *Terminaliaivorensis*A.Chev. (Combretaceae), use in the construction of timber produced useful dye such as indigo. Their flower is pentamerous, sepals are 4-5, valvate, petals are small sometimes absent, stamens are 10 in two series; Carpel is one, inferior, unilocular; fruit is 2-5 angled. Disc is located at the tip of the ovary on the receptacular tube and is represented by hairy outgrowths sometimes. Tannin containing cells occur throughout in the plant body, particularly in the pericarp of the fruit (Puntiwa and Pimwadee, 2015).

2.10.2.4. Genus: *Combretum*

The genus, *Combretum* sometimes called the *combretums* or bushwillows, comprises of about 380 species made up of climber, shrub and tree. About 300 of these species are native to tropical and southern Africa, tropical Asia (25 species), tropical America (40 species) and Madagascar (5 species) (Hogan, 2012). Bush willow, important tree plants which are majorly found in the savannah habitats of Africa, particularly, in granitic soils. The genus is dominated by *Combretum* and *Terminalia*, its close relative. *Combretum apiculatum*Sond. (Combretaceae) found in the Angolan

woodlands eco-region is a notable example of tree growing in Kunene river basin in Southern Africa (Hogan, 2012). Many other species of this genus are majorly found in the Southwestern forests of Amazon. Some of the species in this genus are usually pollinated by mammals such as bats, while insects and birds conveniently pollinate many other species (Fleming *et al*., 2009).

2.10.2.5.Botanical description

Combretumracemosum is a liane (a woody climbing plant that usually hangs on other trees, especially in the tropical rainforest)or sometimes a scandent (having a climbing habit) shrub, of about 15m height, with hard and long thorns/spine round the stem (Burkill, 1985). The Leaf is about 16 cm long and 6 cm wide, with oblong-ovate to ovate lamina, usually arranged in opposite or sub-opposite, or sometimes ternate manner. Petiole is about 5 mm long, and leaf base is rounded or subcordate, with a prominent midrib that have 6-10 pairs of lateral nerves and apex is acuminate in shape (Wickens, 1973). Inflorescence have terminal and axillary contracted racemes, with pubescent rachis, and similar to foliage leaves are the flora leaves of about 6 cm long and 2-5 cm wide. Flowers with 4-merous, usually located in the lower receptacle, are about 3-5 mm long, sometimes located above or below the ovary (Wickens, 1973). Sepals are triangular and acuminate of about 0.5mm long. Petals are oblong-elliptic, 2-3 mm long and 0.1-2 mm wide, heavily pubescent externally. Stamen and filaments are 12 mm long, anthers are 0.5 mm long. Fruits are triangular to obovate in shape of about 1.8-2.5 cm long and 1.7-2.5 cm wide (Wickens, 1973). Figure2.16 show the photograph of (a) leaves, (b)flower and (c) stem of *C.racemosum*plant at a forest in Ode-Ekiti, Ekiti State.

2.10.2.6.Cultivation

Combretum racemosum like other species in the genus is usually grown in the wild but due to its medicinal value, it is harvested from the wild for variety of local uses. However, a species, *Combretumindicum*(L.) DeFilipps (Combretaceae), commonly called Chinese honey suckle or rangoon creeper, found in Asian is the only one found in many other parts of the world being cultivated as ornamental plant (Dalziel, 1973).

(a)

(a) (c)

Figure 2.16. Photograph of(a) leaves, (b)flower and (c) stem of *C.racemosum*plant at a forest in Ode-Ekiti, Ekiti State, picture taken by Famojuro T.I, in 2015

2.10.2.7. Economic uses

The flowers of *C. racemosum* attracts bees, therefore, it may prove useful in the production of honey. And the leaves are used in Sierra Leone to season soup (Dalziel, 1973). In Gabon, where it grows at entrance of villages, a branch of the plant hang over a house-door is believed to keep away spells (Walker and Sillans, 1961).

2.10.2.8. Ethnomedicinal uses

In Africa ethnomedicine, *C. racemosum* have been used for the treatment of several diseases such as tuberculosis, hemorrhoids, toothache, convulsive coughing, male sterility, genital-urinary and gastrointestinal infections (Oliver-Bever, 1986; Jossang *et al*., 1996; Burkill, 2000). The young leaves are used traditionally in Gambia for the treatment of helminthic and to kill roundworm in children (Dalziel, 1973). In Casamance region in Senegal, the leaf twigs serve as the basis for several preparations made for the treatment of internal parasites (Kerharo and Adam, 1974).The plant is used in folklore medicine in South Eastern part of Nigeria for the treatment of urinary tract infection (UTI) (Esimone *etal*., 2010). In Nigeria, the leaves are used for diarrhoea and several skin diseases treatment (Ajibesin and Ekpo, 2002).In Akye area of Ivory Coast, a drought is made with pimento and salt from squeesing young leaves of the plant or cooking with the leaves wrapped with *Thaumatococcusdaneilli*(Benn.) Benth. (Marantaceae) to be taken in the morning as a vermifuge (Adjanohoun and Ake Assi, 1972).

In Congo, the plant is used for the management of all genito-urinary and gastro-intestinal infections accompany with bleeding and a draught of the root maceration or decoction is taken for dysentery(Burkill, 2000). The leaf sap is used for haemorrhoid, while the root bark pulp is used for the treatment of bleeding during pregnancy(Burkill, 2000). Powdered leaves or roots are used for the treatment of hematuria, convulsive coughing and tuberculosis, while the sap is used as haemostatic and cicitrisant(Burkill, 2000). The powdered bark or leaf is applied to circumcision wounds based on theory of signature, because of the red colour of flower (Bouquet, 1969). In Ivory Coast, the gel obtained from the mixture of the leaf sap with water is used for the treatment of male sterility (Bouquet and Debray, 1974). The gum produced by the bark of the plant is used in Gambia for the treatment of toothache (Williams, 1907) and in Nigeria; the leaf extract ofthe plant is used for ulcer treatment (Okwuosa *et al*., 2006).

2.10.2.9.Previous related biological studies

Schepekin *et al*. (2013) investigated immunomodulatory activity and biochemical properties of two isolated polysaccharides (polysaccharides-acid unbound (CP-AU) and polysaccharides-acid bound (CP-AB)) from the leaves of *C. racemosum* and reported that, CP-AU had potent immuunomodulatory activity, induced interleukin production and tumour necrosis factor-α (TNF-α), while CP-AB was found inactive.

The methanolic extract of *C. racemosum* leaf (CrLv) and root bark (CrRB) was investigated*in vitro* by Nsuadi *et al*. (2012) for antioxidant activity and vasorelaxant property in rats whose aorta rings were pre-contracted with 1 µM of phenylephrine in the presence or absence of endothelium,He reported that both fractions, CrRB and CrLv have antioxidant activities and vasorelaxant effect on the rats in an endothelium dependent manner.

Okwuosa *etal.* (2012) investigated the methanol leaf extract of*C. racemosum* for protective effect in male rats against cyclophosphamide induced pancytopaenia and liver injury and reported that *C. racemosum*leaf extract possess liver and bone marrow protective properties in cyclophosphamide induced cytotoxicity.

Okwuosa and co-workers conducted a comparative study on the protective property of aqueous and hexane extracts of *C. racemosum*leaf in three induced ulcer model albino rats. Comparing the protective valves of rats administered with 400 mg/kg body weight and rats administered with 100 mg/kg body weight of cimetidine, they deduced that results shows high protective valve ranging from 47-64% (using standardised ulcer index) for both extracts and reported that extract protection compared favorably with cimetidine protection on the three ulcer models, They however, suggest that water and hexane extract of *C. racemosum* may yield a double action anti-ulcer effect (Okwuosa *et al*., 2006).

Five fractions (hexane, chloroform, ethylacetate, butanol and aqueous) obtained from the ethanol extract of *C. racemosum*leaf were evaluated for antimicrobial activity against five microorganisms (*Staphylococuss aureus, Candida albican, Pseudomonas aeruginosa, Trichophyton rubum and Klebsiella pneumoniae*). The ethylacetate fraction showed mild activity while the chloroform, butanol and aqueous fractions showed significant activity against all the microorganisms. The hexane fraction yield least activity but ethanol extracts showed no activity against the test organisms

(Ajibesin and Ekpo, 2002).Onocha*et al*. (2005) also evaluated the antimicrobial activity of ethylacetate and methanol extract of *C. racemosum* against *Salmonella typhi*, *Esherichia coli*,*Pseudomonas aeruginosa, Staphylococuss aureus*, *Bacillus subtilis*,*Candida albican*, *Aspergillus niger*, *Dermatophyte* species and reported that both extracts showed antibacterial activity against test organisms but only methanol extract displayed antifungi properties against *Candida albican*, *Aspergillus niger* and *Dermatophte* species.

Eze *et al.* (2012) investigated *in vitro* and *in vivo* anti-trypanosomal activity of methanol extracts of *C. racemosum* leaf against *Trypanosoma brucei* using mice and reported that leaf extract inhibited the movement of trypanosome and render them powerless to the mice *in vitro* at 125 to 0.2559 mg/mL, They indicated that, there was a reduction in the parasitaemia and the packed cell volume of the affected mice at 50, 100 and 200 mg/kg body weight was increased by the extract, which was given to them interperitoneally. The result obtained shows that the plant extract is save for the treatment of *Trypanosoma brucei* when compared with the group that received diaminazene aceturate, which resulted in total death of the mice.

Gossan *et al*. (2016) investigated antibacterial and cytotoxicity activity of a new pentacyclic triterpenoid glucoside identified as 28-0-β-D-glucopyranosyl-2α,3β,21β,23-tetrahydroxyolean and fourteen other known triperpenoid compounds which includes terminolic acid, betulinic acid, quadranoside II, arjungenin, 3-0-β-acetyl-ursolic acid, chebuloside, bellericagenin B, bellericaside B, combreglucoside, kaji-ichigoside, combregenin, arjunolic acid, arjunglucoside I and 24-hydroxy-tormentic acid, isolated from the root of *C. racemosum* against promyelocytic leukemia HL-60 and human erythromyeloblastoid leukemia K562 cell lines. They reported that, 3-0-β-acetyl-ursolic acid, betulinic acid and quadranoside II had significant cytotoxicity with IC_{50} values ranges from 13 to 50 μM and arjungenin, terminolic acid and 3-0-β-acetyl-ursolic acid exhibited moderate antibacterial activity against the test organisms.

Oluyemi *et al*. (2019) investigated the phytochemicals in the crude methanol extract, chloroform and n-butanol fractions of *C. racemosum* leaf for antiplasmodial activity against chloroquine-sensitive (D10) and chloroquine-resistant (W2) strains of Plasmodium falciparum. They reported eleven isolated compounds including four

ursane-type [(19α-hydroxyasiatic acid (1), 6β,23dihydroxytormentic acid (4), madecassic acid (8), nigaichigoside F1 (10)], four oleanane-type [(arjungenin (2), combregenin (5), terminolic acid (7), arjunglucoside I (11)] and a isomeric mixtures of triterpenes and abscisic acid (9) that exhibited moderate to low antiplasmodial activity, with madecassic acid being the most active against D10 ($IC_{50} = 28 \pm 12$ µg/mL) and W2 ($IC_{50} = 17.2 \pm 4.3$ µg/mL).

2.10.2.10. Phytochemical constituents

Phytochemical screening of ethyl acetate and methanol extract from *C. racemosum* indicated the presence of flavonoids, alkaloids, saponins, steroids, tanins, cardiac glycoside and terpenes (Onocha *et al*., 2005).

2.10.2.11. Phytochemical components isolated from *C.racemosum*

Gossan *et al*., 2016 isolated a new pentacyclic triterpenoid glucoside identified as 28-0-β-D-glucopyranosyl-2α,3β,21β,23-tetrahydroxyolean and fourteen other known compounds, which are terminolic acid, betulinic acid, quadranoside II, arjungenin, 3-0-β-acetyl-ursolic acid, chebuloside, bellericagenin B, bellericaside B, combreglucoside, kaji-ichigoside, combregenin, arjunolic acid, arjunglucoside I and 24-hydroxy-tormentic acid from the root of *C. racemosum*. Structures of someof the isolated compounds from *C. racemosum*are shown in Figure 2.17.

2.10.3. Other plants used in the study

Summary of the review of other plants including *Chasmanthera dependens*Hochst. (Menispermaceae), *Enantia chlorantha*Oliv. (Annonaceae), *Euphorbia opuntioides*Welw. ex Hiern (Euphorbiaceae), *Lagenaria breviflora*(Benth.) Roberty (Cucurbitaceae), *Nymphaea lotus* L. (Nymphaeaceae), *Olax subscorpioides* Oliv. (Olacaceae)and *Ritchiea longipedicellata*Gilg (Capparaceae)used in this study are reported in Table 2.1.

In summary, the literature review has shown that natural productsobtained from nature have been used since the time immemorial to improvethe health and general wellbeing of the people. It has shown that cancer disease has been a major global heath burden and has responsible for the death of millions of people wordwide resulting in huge economic loss. The review indicated that several agents derived from ethnomedicinal plants have been used and shown to be effective for the treatment of cancer, while others are still in clinical development. It was noted that many drugs currently used in

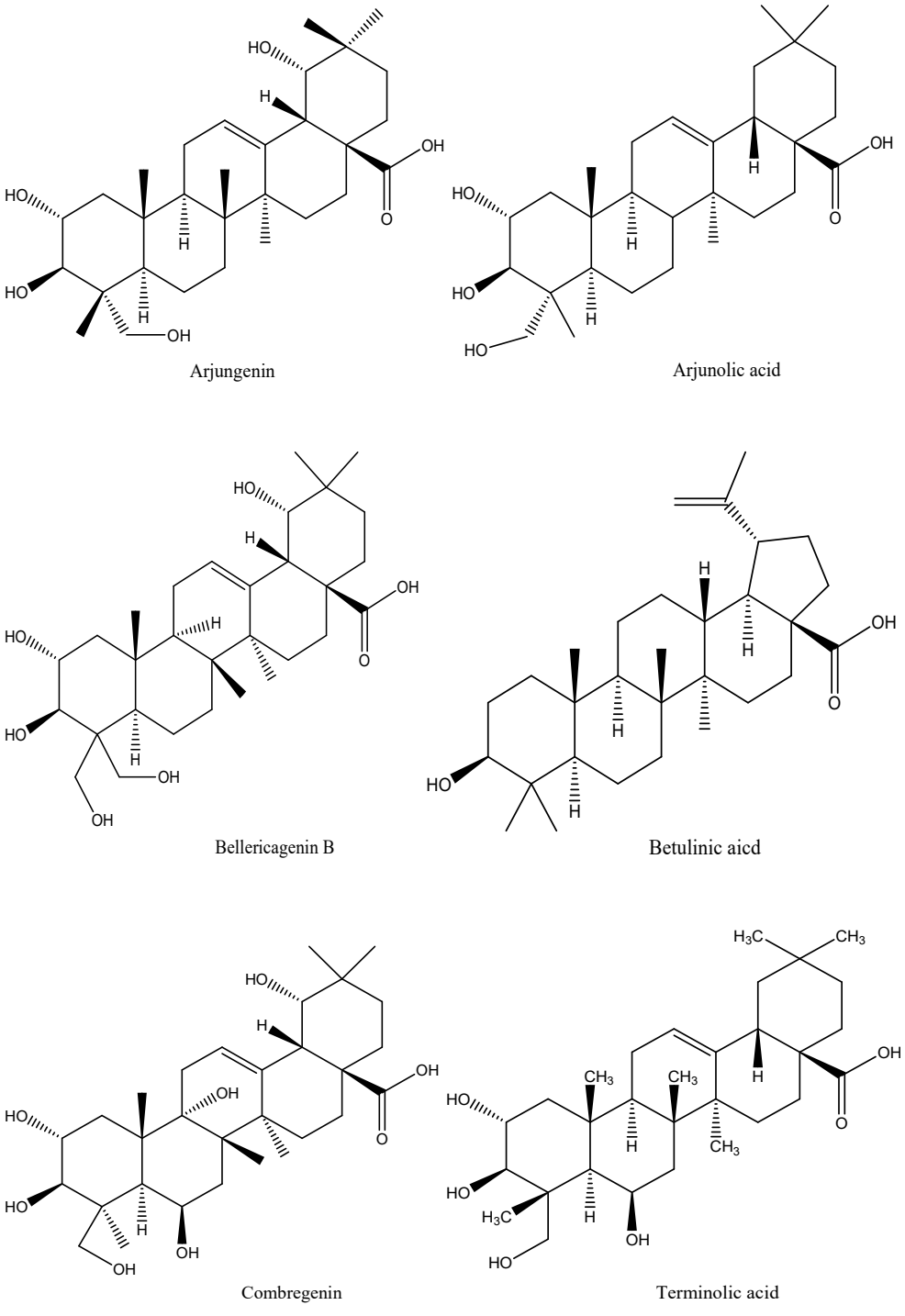


Figure 2.17.Structures of some isolated compounds from *C.racemosum*

Table 2.1. Summary of literature survey on other plants used in this study

S/N	Plant name	Family name	Local name	Ethnomedical uses	Chemical constituents
1	*C. dependens*	Menispermaceae	***Nigeria (Yoruba):*** àtó, **Kenya:**kugiei	Pain, snakebites, stiffness,malaria, inflammation,convulsions,dementia, epilepsy, cough andwounds (Iwu, 1993; Mosango, 2008).	Jateorrhizine, pseudicolumbamine, palmatine (berbericinine), anonaine, columbamine,tetrahydropalmatine, liriodenine, O-dimethylcorytuberine, lysicamine (oxonuceferine), glaucine, magnoflorine, norglaucine, govanine, oxoglaucine, 8-hydroxycolumbine, nornuceferine, coreximine, furanoid diterpene (Ohiri *et al*., 1982)
2	*E.chlorantha*	Annonaceae	***Nigeria*** (Yoruba): Awopa or Dokita (Igbo): Oso- molu	Ulcers, malaria, worms, wounds, chills, boils, urinary tract infections, vomiting, fever, cough, sore, sexual asthenia, hepatitis, intestinal spasms, malaria, aches, spleen in children,jaundice, yellow bitter, typhoid fever and tuberculosis (Tsabang *et al*., 2012; Siminialayi and Agbeje, 2004).	Alkaloids, saponins and reducing sugar (Olamide and Mathew, 2013).
3	*E.opuntioides*	Euphorbiaceae	**Nigeria** (Yoruba): Oro alagogo	Asthma, dysentery, respiratory tract infections, diarrhoea, gonorrhea, tumours, cancer, coughs, and skin ailments (Olounladé *et al*., 2017).	Triterpenoids, tannin, diterpenoids, flavonoids, and polyphenol (Olounladé *et al*., 2017).
4	*L. breviflora*	Cucurbitaceae	**Nigeria** (Yoruba): Tagiri	Fertility, smallpox, schistosomiaisis, headache, vermifuge, convulsion wound antiseptic, digestive disorders, measles, coccidiosis diseases, cold (Ajayi *et al*., 2002; Tomori *et al*.,	Saponin, oxalate, tannin, terpenoid, triterpenoids, alkaloid, phenol, carotenoid, phytate and oxalate content (Elujoba *et al*. 1990; Adeyemi *et al*., 2017)

2007)

Table 2.1. Summary of literature survey on other plants used in this study(cont')

S/N	Plant name	Family name	Local name	Ethnomedical uses	Chemical constituents
5	*N.lotus*	Nymphaeaceae	**Nigeria** (Yoruba): Oju oro	Fever, skin diseases, gonorrhea and bronchitis aphrodisiac, astringent, cardiotonic, anti-inflammatory agent, insomnia, cancer, anxiety, diabetes, diarrhoea, dysentery, dyspepsia and general weakness, anti-hemorrhagic indigestion, stomach, heart diseases, aches and cancer (James, 2008; Akinjogunla *et al*., 2009).	Myricetin-3-0-rhamnoside, Nympholide A and B, Myricitrin, 1,2,3,4,6 pentagalloyl glucose, nupharine, nympheine, nelombine, nupharidine, phenyl alanine, threonine, alanine, valine, tyrosine, arginine, butanoic acid, leucine, D, L-isoleucine, 2-amino-7-methyl octanoic acid, aspartic acid, serine-arginine, (Elegami *et al*., 2003; Sowemimo *et al*., 2007).
6	*O. subscorpioidea*	Olacaceae	**Nigeria** (Yoruba): Igi ifon	Rheumatism, arthritis,pain-killers,asthma, mental illness, anxiety,fever, cold, parasitic infection, constipation, venereal diseases and dysentery (Burkill, 1985)	Glycosides, flavonoid, saponins, steroids, alkaloids and aromatic substances (Ayandele and Adebiyi, 2007)
7	*R. longipedicellata*	Capparaceae	**Nigeria** (Yoruba): ológbe-kuyan; **Ivory Coast**: Akye dzotinkin	As tonic, antianaemic, chest-troubles, headache,antiseptic, aphrodisiac; kidney-pains, general pains, nausea, ear-ache, arrow-poisons and antidote (Bouquet and Debray, 1974).	Alkaloids, tannins, anthraquinones, saponins, glycosides, flavonoids, phenols and terpenoids (Trease and Evans, 2002)

cancer chemotherapy also have their base in natural bioactive agents. It also highlighted several methods that are being employed in the purification, isolation, and testing of the plant-derived bioactive agents. This study focuses on purification and isolation of more bioactive compounds (cytotoxic), that can serve as lead for the treatment of cancer disease from two ethnomedicinal plants (*C. bethamiana* and *C. racemosum*) used in Nigerian ethnomecine.Both are important plants with variousethnomedicinal uses, they are relatively abundant in their natural habitat and their collection for research purpose cannot in any way disturb the ecosystem.

CHAPTER THREE
MATERIALS AND METHODS

3.1.General materials

3.1.1. Chemicals

Analytical grade solvents were re-distilled before usein this study. The analytical grade/HPLC grade solvents used are methanol, n-hexane, ethyl acetate, dichloromethane, diethyl ether, ethanol toluene, acetone, acetic acid, formic acid (Sigma-Aldrich, Germany).

3.1.2. Reagents

Phosphate buffer saline solution (PBS), Dimethylsulphoxide(DMSO) (Thermo FisherScientific, UK), Oxytocin, 0.2% trypsin (GSK, UK), Dulbecco's Modified Eagle's Medium (F-12), 10% foetal bovine serum (FBS), 1% penicillin-streptomycin antibiotics suspension(Invitrogen, Carlsbad, CA, USA), nucleic acid stain, Bakground suppressor I, caspase 3/7 reagent and Na pyruvate.

3.1.3. Instrument/Equipment

Aluminum TLC plates, 20 x 20 PTLC glass plates (Merck, Germany), Ultraviolet lamp (Allen 425LCF-750-Q), Rotary evaporator (Buchi, Switzerland), Microbiology Safety cabinet (Nure, UK), Inverted microscope (Evos Life Technology, UK), Texan F50 microplate reader(Thermo FisherScientific, UK),Hemocytometer (Hausser Scientific Company, New York USA), Refrigerator, Freezer (Lec Electronic, UK), Centrifuge (Labnet International, Inc. Global), Centrifuge tubes, Magnetic stirrer(Thermo FisherScientific, UK), Vortex mixer (Stuart Equipment, UK), Multi-channel pipette, Micro-pipette, Sterile pipette tips, Microtiter plate (flat-bottomed)(Corning® Costar® TC-treated,Merck, UK), Cell culture flask (T75, T25), Cell culture tube (50 mL), Serological pipettes, Serological pipette holder, Aspirator, Disposable hand gloves, Desiccator, Falcon tubes(Fischer Scientific, UK), Malex Electric blender (800 series) (India), Incubator (Triple Red Laboratory Technology, UK), Filter paper, Filters, Buckner funnel (Sigma-Aldrich, Germany), Desktop computer (Stone, UK), Weighing

balance (Sartorious analytical balance, UK), Eppendorf tubes, Graduated syringes, Conical/volumetric flasks, Funnels (glass), Capillary tubes,Measuring cylinders, Separatingfunnel (glass)(Thermo Fischer Scientific, UK), Test tubes, Test tube racks, Column tube (glass), Spatulas,Weighingboats, Crucible(plastic),Beakers (glass),Razor blades (Tiger), Cotton wool, Retort stand (SLS, Britain),24-well culture dishes (Millipore, USA), NMR instrument, IF instrument and MS instrument.

3.2.Plant material

3.2.1.Ethnobotanical survey

3.2.1.1. Study area

Study area is made up of three Local Government Areas of Ekiti State, Southwestern Nigeria. Like others States in Nigeria, Ekiti State was selected for this study based on the prevalence of cancer incidence and death statistics as reported by previous workers (Awololaet *al*., 2011; Babatundee*t al*., 2013). In addition, each of the three LGAs selected for this study represented each of the three geo-political zones:Gbonyin (Ekiti South), Moba (Ekiti North) and Efon (Ekiti Central) (Figure 3.1) found in the State. Ekiti state lies in the south of Kwara and Kogi State, east of Osun State and bounded by Ondo State in the east and in the south.The studyarea lies between longitudes 40°51′ and 50°451′ east and 70°151′ and 80°51′ north of the geographical limits.

The study areais mainly an upland zone measuring about 455 m (1,493 ft) above the sea level.It covers land area of 2168 km² with a population of 381,436 in the 2006 national census (NPC, 2010). Yoruba language is the major language of the people of this region. Religions of the people in this region are mainly Christianity and African Traditional Religion (ATR), while a small percentage of the people are Muslim. Two distinct seasonsof tropical climate, rainy season (April - October) and dry season (November-March), were found in the study area. A temperature range of between 21^{o}C and 28^{o}C, with high humidity was found in this region. The vegetation of the study area is in two forms; tropical forest which occurs in the south and central, and guinea savanna which is predominant in the northern periphery area.The main occupation of the people of this region is Agriculture.

3.2.1.2. Study design

The ethnobotanical survey was conducted in three selected LGAs of Ekiti State with each representing each of the three geo-political zones in an attempt to have an

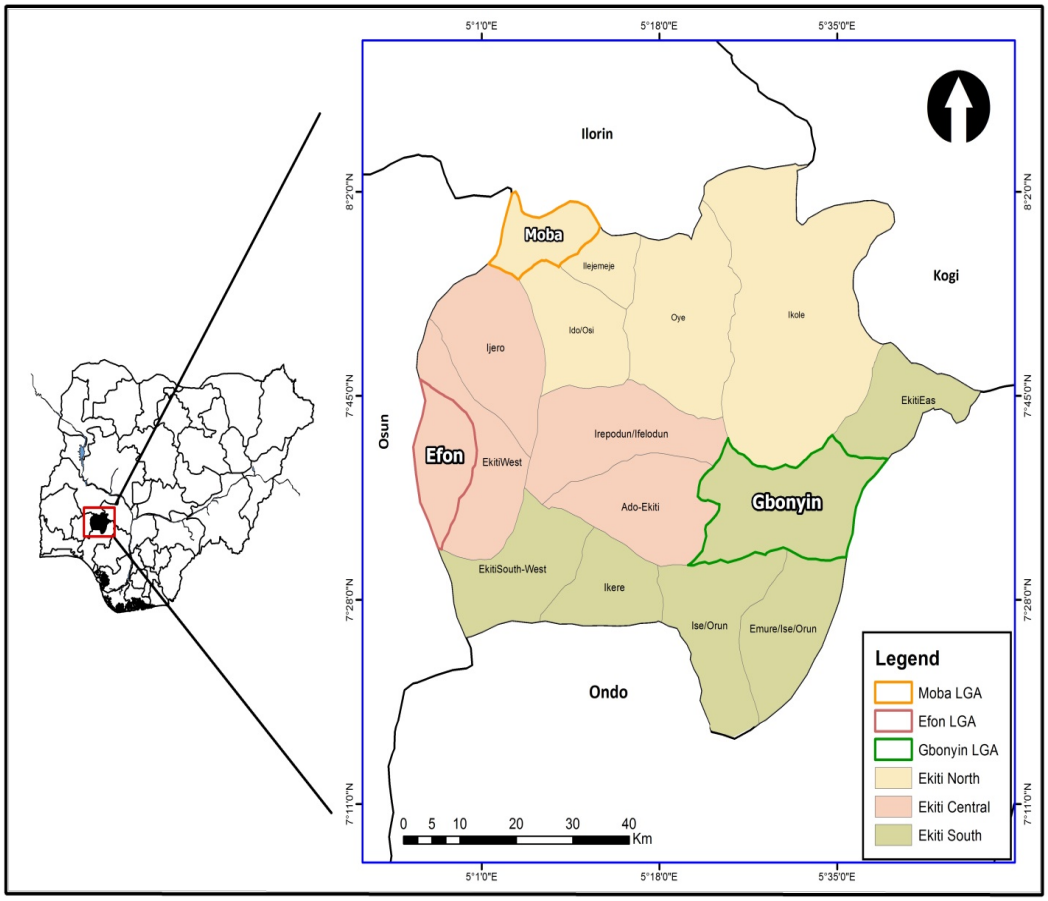


Figure 3.1. Map of Ekiti State showing the study area of the ethnobotanical survey

overview of the indigenous knowledge of treatment of cancer by the traditional medicine practitioners (TMPs) of the state. It was carried out between April 2015 and October2015. Personal interviews and semi-structured questionnaires (Appendix 1) were used to obtain relevant ethnomedicinal information/data fromdifferent categories of respondents which included diviners, herbalist, herb sellers and primary health care giver on indigenous plants used locally fortreatment of cancer disease in the selected Local Government Areas of the State.

3.2.1.3. Respondents permission

Prior tothe interview, a brief consultation was held with the village heads and the head of the traditional medicine practitioners (TMPs) in each of the study communities under the three selected LGAs in order to know the namesof the respondents before contacting them for interview. A total of thirty-eight (38) respondents comprising of thirty-two (32) TMPs including herbalists, diviners, herb sellers and traditional birth attendants and six (6) other relevant people consisting ofpeasant farmer, hunter and nursewere consulted as directed by head of the TMPs in each of the communities. While in contact with the respondents, the purpose of the interview was made known to them and their permission to publish the findings of this research were obtained orally from all the participants.

3.2.1.4. Administration of questionnaire

Although, English language was used to prepare the questionnaire, but discussion was communicated to the respondents in Yoruba language and in case where respondent did not fully understand, it was further communicated to them in Ekiti dialect by myself and an interpreter. Three major sections were captured in the questionnaire; (1) covers demographic data like sex, ethnic group, age, religion, educational background, source of knowledge of herbal practice as well as duration of practiceof the respondents. (2) Includes respondent'sfolk classification of cancer disease and other questions like 'what do you know about the disease called cancer?' Factors responsible for cancer, cancer diagnosis method, symptoms of cancer and cancer types which has been treated before by respondents. (3) Involves information on medicinal plants used for cancer management,method of preparation, plant parts used, mode of administration,treatment duration, likely side effects of the recipes, availability of the plants used in the treatment as well as how they preserve their recipes etc.

3.2.1.5. Ethnobotanical data analysis and plant selection

The data obtained from the field survey were examined according to the Use-Mention index (UMi), which is defined as the number of mentions for one plant for a defined category, divided by the total number of respondents interviewed for the use category (n_u) (Andrade-Cetto and Heinrich, 2011) $UM_i = UM/ n_u$.From the data analysis, a total of 57 medicinal plants were realised from the survey, out of which nine (9) plants (Table 3.1) were selected based on factor such as; most mentioned plant species(*C. racemosum*, *C. benthamiana*, *E. chlorantha*, *E. opuntioides*and *O. subcorpioides*) with previously unreported anticancer activity. This was validated through literature searching.Some plant species (*C. dependens*, *L.breviflorus*, *R. longipedicellata* and*N. lotus*) that are least exploited was also considered.

3.3. Methodology

3.3.1. Identification, authentication and collection ofselected plants

Each of the selected plant specieswere collected fresh in July, 2015 from selected Local Government Areas for this study, in Ekiti State, Southwestern Nigerian.Each plant was identified and authenticated by Mr. Adeyemo, A. and Mr. Shosanya, O. S. at Forest Herbarium of the Forest Research Institute of Nigeria (FRIN). Ibadan, Nigeria where their voucher specimens were deposited.

3.3.2. Drying and extraction

The various plant materials collected were thoroughly cleaned to avoid contaminants and air-dried for about two to three weeks. They were then dried in the oven for 72 hrs at 40°C prior to grinding. They were ground into coarse powder using plant milling machine and about 200g each of the powder were separately macerated with 100% methanol for 48 hours. Each extract was decanted, filtered and concentrated *in vacuo*. The percentage yield was calculated. Percentage yield of extracts was calculated as:

Yield%= (weight of extract / weight of plant)x 100

3.4. Phytochemical analysis of plant powdered sample

Phytochemical screening was carried out on the powdered samples of ninespp for their various secondary metabolites using the standard protocols (Adesanya and Sofowora, 1983; Harborne, 1983).

Table 3.1. **Selected plants for the preliminary study**

S/No	Names and families of plant	No of times mentioned	FHI NO	Parts used
1	*C. benthamiana*	3	110847	Leaf
2	*C. dependens*	2	110619	Root
3	*C. racemosum*	4	109781	Root bark
4	*E.chlorantha*	3	101821	Stem bark
5	*E. opuntioides*	3	111088	Whole plant
6	*L. breviflora*	2	110618	Fruit
7	*N.lotus*	1	110617	Leaf
8	*O. subscorpioides*	3	110739	Root bark
9	*R. longipedicellata*	2	110624	Leaf

3.4.1. Test for Alkaloids

About 5g each of the plant powder was poured into a clean test tube and 10mL of 5% hydrochloric acid (HCL) was added. The mixture was boiled on water bath for 10 minutes, filtered. The pH was adjusted to about 6-7 by adding few drops of concentrated Ammonia. About 0.5mL of each filtrate was taken into separate testtubes, 2-3 drops each of Dragendorff's and Mayer's reagent were added, anorange or cream precipitate indicated presence of alkaloid.

3.4.2. Test for Tannins

Dried powdered samples (1g each) were separately boiled in distilled water (20 mL) for 5 min, while hot, filtered and allowed to cool. The filtrates were adjusted with distilled waterto 20mL.Four (4) mL of distilled water was used to dilute 1mL each of the solution above to make 5mL. Two drops of ferric chloride (0.1%) solution was added to the final solution. A blue – black, green or blue green colour indicates presence of tannins.

3.4.3. Test for Phenol

About 1 g of eachpowdered samples were dissolved in water andallow to stand.The extract was then filtered, and a few drops of dilute ferric chloride ($FeCl_3$) solution was added. The formation of a red, blue, green, or purple coloration indicates the presence of phenols.

3.4.4. Test for Cardiac glycosides

Dried powdered samples (1g each) were dissolved in 80%alcohol (10 mL) for 5 min on a steam water bath. Each extracts filtered separately and diluted with an equal volume of distilled water. Lead acetate solution in few drops was added; shaken together vigorously and allowed tostand for about 10 mins before filtered. Each filtrate was extracted with chlorine(2 vols), the chloroform extracts were combined. The extracted portions were made to dry using steam bath and reserved for the following test.

a. Killer- Kiliani Test

Zero point three mililitre ferric chloride reagents (0.3mL of 10% ferric chloride in 50mL glacial acid) was added to the cooled residue of concentrated chloroform extracts obtained from above in clean test tubes. Then 2mL of concentratedtetraoxosulphate vi acid (H_2SO_4) was added carefully down the side of

each tube to form a layer below(acetic acid). Reddish brown “ring” observed at the interface as well as green colour in the acetic layer were theindicatorof presence of cardiac glycosides.

b. Kedde Test

The cooled residue obtained from above (for each plant) was mixed with 1mL of 2% 3, 5-dinitro benzoic acid in ethanol. Then 5% sodium hydroxide (NaOH)was added to the resulting solution. The presence of brown-purple colour indicated a positive result for cardenolides.

3.4.5. Test for Saponins

Dried powderedsamples (1 g each) were seperately boiled in 10 mL of distilled water for about 10 min andwhile hot, filtered. On cooling, the filtrates were tested for:

a. **Frothing**: Formation of froth indicated a positive result.
b. **Emulsifying properties**: Noted on addition of 2 drops of olive oil to each testtube conntaining the dried sample.

3.4.6. Test for Anthraquinones

Dried powdered samples (1g each) were placed in a dry test tube,5 mL ofchloroform ($CHCl_3$) was added and test tube shaken for 5 min. Extracts were filtered and 10% ammonia solution in equal volumewas added to the filtrate, and then shaken. In the aqueous layer (upper part), appearance of a rose pink colour indicated the presence of anthraquinone.

3.4.7. Test for Flavonoids

Dried powered samples (1 g each) boiled seperately in 10 mL of distilled water for about 10 min andwhile hot, filtered. On cooling, about 1 mL of each filtrate dissolved in methanol and heat on the water bath at 50°C.While on the water bath, metallic magnessium and concentrated tetraoxosulphate vi acid (H_2SO_4)were added, a red colour showed the presence of flavonoid.

3.4.8. Test for Steroids

About 0.5 g of eachpowdered samplewas dissolved in 3 mL of chloroform and allowed to stand. The extract was then filtered, 2-3 drops of concentrated H_2SO_4 was then added to each filtrate to form a lower layer, a reddish-brown ring colouration was taken as positive for steroid.

3.5.Cytotoxicity studies

3. 5.1. Cell culture and Cell lines

Human adenocarcinoma breast cancer cells (MDA-MB-453) and normal human kidney cells (HEK-293) collected from Pune, India, National Centre for Science (NCCS),and human adenocarcinoma breast cancer cells(MCF-7), human adenocarcinoma prostate cancer cells(PC3), and human adenocarcinoma lung cancer cells(A549) collected from Public Health England, UK,European Collection of Authenticated Cell Cultures (ECACC), were cultured in T75 flasks having DMEM, enhanced with 10% fetal bovine serum (FBS), Na pyruvate and 1% penicillin-streptomycin antibiotics suspension. Cells were preserved in a humidified atmosphere, containing 5% CO_2 at 37ºC and changes in cell morphology, corresponding to the effect of the culture medium was checked through an inverted microscope at every 48 hours. Cells were subcultured and split in a 1:3 ratio into another cell culture flask when they reached confluence by washing with 0.02% EDTA in phosphate buffer saline solution (PBS) and dissociated with 0.2% trypsin.

3.5.2.Preliminary cytotoxicity screening of extract ofthe selected plants

Invitro cytotoxic activity of the various concentrations of the selected plant extracts were evaluated using the viability assay (MTT) method as decribed by Deepa *etal*., (2011) with slight modifications. Briefly, each extractwas dissolved in dimethylsulphoxide (DMSO) at 37°C to give a stock solution of 1 mg/mL, three- fold serial dilution were prepared from the stock to provide the working concentrations of 1.37 – 1000 µg/mL(1.37, 4.12, 12.35, 37.04, 111.11, 333.33 and 1000µg/mL). Breast cancer cell (MDA-MB-453) and normal human kidney cell (HEK-293)were seeded into96- well microtiter plates containing 200 µL of medium and placed in incubator for 48 hto reach confluent, after which exactly0.2 µL of various concentrations of test compounds in triplicates as well as negative control were added onto plates containing 4000 cells per well. The plates were incubated at 37°C in humidified CO_2 (5%) incubator for 24 h. The negative control was performed using DMSO to replace test extracts, and the blank contains only growth medium, while methotrexate was used as the positive control. At the end of 24 h, the supernatants were aspirated from each wells and 200µL MTT solution (final conc. of 0.5mg/mL in PBS) was added. Plates incubated again for 4 h at 37°C in humidified CO_2 (5%) incubator after which the MTT solution was carefully removed and insoluble formazan product formed was

dissolved in 150 µL DMSO. The plates were placed in a multi-well spectrophotometer and the plates were shaked thoroughly before absorbance was measured at 570 nm. Percentage cell viability was determined as [(absorbance of treated cells- absorbance of blank / absorbance of control cells – absorbance of blank)]×100).

3.5.3. Selection of plants for bulk extraction

Following the preliminary cytotoxicity screening, the two most active plants, *C. benthamiana* (Figure 3.2) and *C. racemosum*(Figure 3.3) were then selected to be the research plants in focus for large scale extraction, purification, isolation andcharacterisation as well as biological assays.

3.5.4.Bulkextraction of *C. benthamiana*and*C. racemosum*plant materials

The powdered leaf sample of *C. benthamiana* (0.8kg) and root sample of *C. racemosum* (1.7 kg) were macerated separately with 100% methanol for 72 hours at room temperature (20ºC - 25ºC). The sample mixtures wereoccasionally stirred. Eachplant extract was separately decanted, filtered andconcentrated *in vacuo*.

3.5.5. Cytotoxicity activity of partitionedfractions of *C. benthamiana* and *C. racemosum*

3.5.5.1.Viability assay(MTT)

Invitro cytotoxic activity of the n-hexane, dichloromethane and aqueous fractions of *C. benthamiana* and*C. racemosum* were evaluated using the viability assay (MTT) method described by Deepa *etal*., (2011) as mentioned above. Each fraction was dissolved in dimethylsulphoxide (DMSO) at 37°C to provide a stock solution of 1 mg/mL, two- fold serial dilution was prepared from the stock to provide the working concentrations of 50 and 100µg/mL. Breast cancer cell(MCF7), prostate cancer cell(PC3) and lung cancer cell(A549) were seeded into96- well microtiter plates containing 200 µL of medium and placed in incubatorfor 48 hto reach confluent, after which exactly0.2 µL of various concentrations of test fractions in triplicates as well as negative control were added into plates containing 4000 cells per well. The plates were incubated at 37°C in humidified CO_2 (5%) incubator for 24 h. The negative control was performed using DMSO to replace test fractions, and the blank contains only growth medium, while methotrexate was used as the positive control. At the end of 24 h, the supernatants were aspirated from each well and 200µL MTT solution (final conc. of 0.5 mg/mL in PBS) was added. Plates were incubatedagain for 4 h at 37°C in

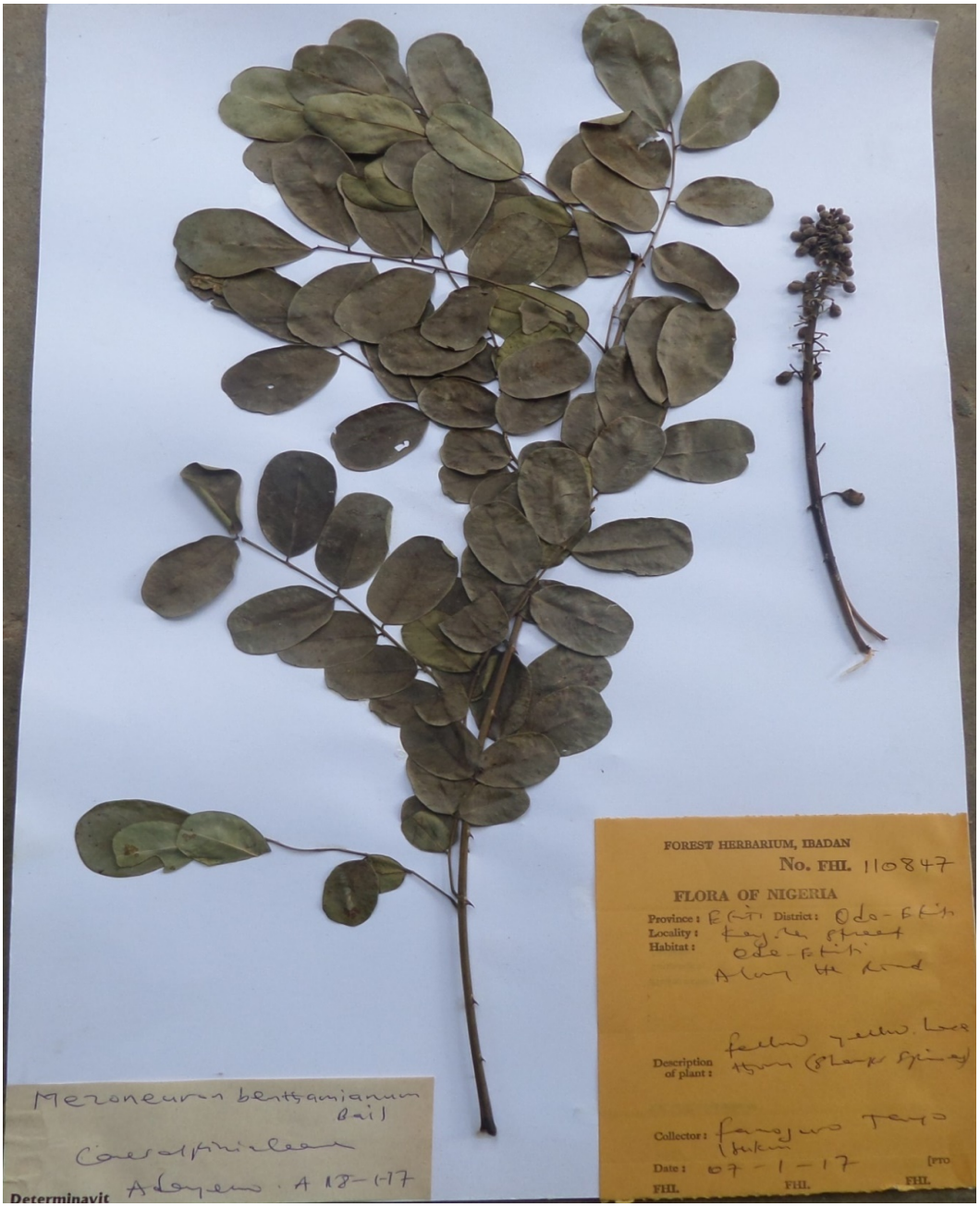


Figure 3.2.Photograph of*C. benthamiana* (110847) herbarium specimen

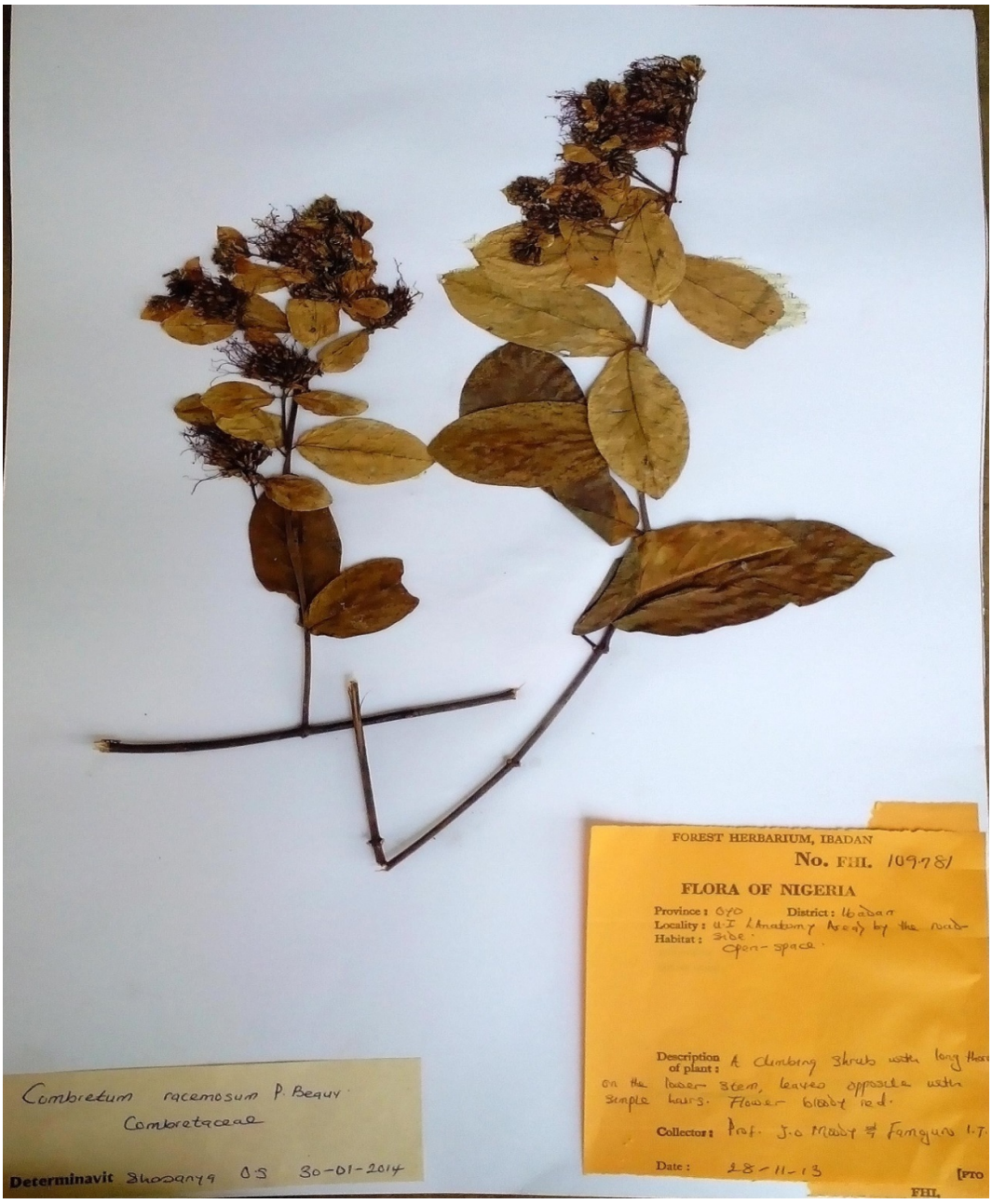


Figure3.3.*C. racemosum* (109781) herbarium specimen photograph

humidified CO_2 (5%) incubator after which the MTT solution was carefully removed and insoluble formazan product formed was dissolved in 150 µLof DMSO. The plates were placed in a multi-well spectrophotometer and the plates were shaked thoroughly before absorbance was measured at 570 nm. Percentage cell viability was determined as [(absorbance of treated cells – absorbance of blank / absorbance of control cells – absorbance of blank)]×100).

3.5.5.2. Cyquant direct cell proliferation assay (microscopy assay format)

The effect of two most active fractions (CBD and CRD) on the cancer cells was also evaluated using cyquant® direct assay as described by (Abraham*et al*., 2008). Briefly, Each fractionand the standard, methotrexate were dissolved in dimethylsulphoxide at 37°C to provide a stock solution of 1 mg/mL, two- fold serial dilution was prepared from the stock to provide the working concentrations of 50 and 100µg/mL. About 2000 of breast cancer cell(MCF7), prostate cancer cell(PC3) and lung cancer cell(A549) were seeded in 48- well microtiter plates containing 400 µL of mediumand placed in incubatorfor 48 hto attach, after which exactly0.2 µL of various concentrations of test fractionsand standard were added.The negative control was performed using DMSO only. Plates were incubated at 37°C in humidified CO_2 (5%) incubator for 24 h. Then, 200 µl of the supernatants were aspirated from each wells and exactly 200 µL of 2X detection reagent was added. Plates were incubated again at 37°C in humidified CO_2 (5%) incubator for 60 minutes.Cells imaging were obtained using a standard green filter microscope, fromat least five fields of view per sample.The dead cells remain colourless while the live cells appeared green in colour.

3.5.5.3. Caspase-3/7 green detection assay (endpoint assay format)

The caspase-3/7 detection assaywas further performed on the two fractions (CBD and CRD) as described by (Cobanoglu *et al*., 2016). Briefly, each fraction and the standard(methotrexate)were dissolved in dimethyl sulphoxideat 37^0c to provide a stock solution of 1 mg/mL, two- fold serial dilution was prepared from the stock to provide the working concentrations of 50 and 100µg/mL. About 2000 of breast cancer cell(MCF7), prostate cancer cell(PC3) and lung cancer cell(A549) were seeded in 48-well microtiter plates containing 200 µL of mediumand placed in incubatorfor 48 hto attach, after which exactly0.2 µL of various concentrations of the test fractions and standardwere added. The negative control was performed using DMSO only. Plates were incubated at 37°C in humidified CO_2 (5%) incubator for 24 h, after which all the

medium wascarefully aspirated from each wells and exactly 200 µLof caspase-3/7 green detection reagent was added. The plates were incubated again at 37°C in humidified CO_2 (5%) incubator for 30 minutes. Cells imaging were obtained using a standard green filter microscope,fromat least five fields of view per sample. The live cells remain colourless while dead cells appeared green in colour.

3.5.6.Cytotoxicity activity of isolated compounds

Invitro cytotoxicactivity of theisolated compounds were assessed using the viabilty assay (MTT) method by Deepa *etal*, (2011) as mentioned earlier. A stock solution of 200µg/mLwas made with dimethylsulphoxide for each isolated compounds, two- fold serial dilution were prepared from the stock to provide the working concentrations of 12.5, 25, 50 and 100µg/mL. Also, for methotrexate (positive control), a stock solution of 200µg/mLwas made with dimethylsulphoxide, thereafter a two- fold serial dilution was prepared from the stock to provide the working concentrations of 12.5, 25, 50 and 100µg/mL. Breast cancer cell(MCF7), prostate cancer cell(PC3) and lung cancer cell(A549) were seeded into96- well microtiter plates containing 200 µL of medium and placed in incubator for 48 hto reach confluent, after which exactly0.2 µL of various concentrations of test compounds in triplicates as well as negative control were added into plates containing 4000 cells per well. The plates were incubated at 37°C in humidified CO_2 (5%) incubator for 24 h. For the negative control, DMSO was used to replace the test compounds, and the blank contains only growth medium, while methotrexate was used as the positive control. At the end of 24 h, the supernatant wascarefully aspirated from each well and 200µL MTT solution (final conc. of 0.5mg/mL in PBS) was added. Plates were incubated again for 4 h at 37°C in humidified CO_2 (5%) incubator after which the MTT solution was carefully removed and insoluble formazan product formed was dissolved with 150 µL DMSO. The plates were placed in a multi-well spectrophotometer and the plates were shaked thoroughly before absorbance was measured at 570 nm. Percentage cell viability were determined as [(absorbance of treated cells- absorbance of blank / absorbance of control cells - absorbance of blank)]×100).

3.6.Statistical analysis

Statistical analysis was accomplished using the Microsoft Office 2010 Excel software (Microsoft Corporation, Redmond, Washington, USA)and Graph Pad Prism software version 7.0.The cytotoxicity assay was performed in triplicate and data were expressed

as mean ± standard error of mean (SEM).Sigmoidaldose-response curve of datafitting obtained from non-linear regression analyses (log of inhibitior versus normalized response) was used to determine the IC50 and analyse differences in cytotoxicity activity. In addition, one-way ANOVAwith Dunnett's multiple comparison tests were conducted at$\alpha_{0.05}$to detect differences in cytotoxicity activities of the isolated compounds compared with methotrexate (standard). Differences inthe activity lower than $p<0.05$ were considered statistically significant.

3.7.Chromatographic purification experiments

3.7.1. Solvent-solvent partitioning on*C. benthamiana* methanol extract

The methanol extract of *C. benthamiana*leaf (192 g) was suspendedin methanol - water (75:25) and solvent-partitioned successively in a separating funnel, with each ofn-hexane anddichloromethane (DCM) respectively. Thepartitioned fractions (hexane, dichloromethane and aqueous)were separately concentrated to dryness *in vacuo* to obtain 47.21g of hexane, 38.63g of dichloromethane and 101.00 g of aqueousfractions respectively.

3.7.2. Solvent-solvent partitioning on*C. racemosum*methanol extract

The same procedure stated in section 3.7.1 above was carried out on the methanol extract of *C. racemosum*root (175 g) to obtain a yield of 25.57 g of hexane, 47.98 g of dichloromethane and 90.05 g of aqueousfractions respectively.

3.7.3. Thin layer chromatographyon fractions of *C. benthamiana*

Analytical thin-layer chromatography (TLC) was carried out separately on the methanol extract and solvent-partitioned fractions of *C. benthamiana* leafusing analytical TLC pre-coated plates.Suitable solvent systems(hexane, ethyl acetate and methanol) were used in the process. Visualisation of spots on the TLC plates was achieved with the aid of UV light (254 nm and 365 nm). R_f values for the separated spots were determined.

3.7.4.Thin layer chromatographyof fractions of *C. racemosum*

The same procedure stated in section 3.7.3 above was carried out on the methanol extract and partitioned fractions of *C. racemosum*.

3.7.5.Isolation of compounds from *C. benthamiana*

3.7.5.1. Column chromatographic purification of dichloromethane fraction of *C. benthamiana*

Gravity column chromatography (GCC)was performed for the purification of *C. benthamiana*dichloromethane (DCM) fraction. Chromatographic glass column of (internal diameter = 3.5 cm, length = 80.0 cm) was used in the purification process. A portion of the DCM fraction (20 g)of the leaf extract of *C benthamiana* was loaded into the gravity coloumn (80.0 x 3.5 cm) packed with a bed of 120 g of silica gel (80-200 mesh size) in *n*-hexane and successfully eluted with solvent mixtures of increasing polarity starting from hexane (non-polar) to medium polar (ethyl acetate) to 100% methanol (polar) (Table 3.2). A total of 349 fractions were obtained and pooled to 21 fractions based on analytical TLC profile (Solvent system: petroleum ether/acetone/ethyl acetate; 7: 3: 1).

3.7.5.2. Preparative thin layer chromatography (PTLC)

Preparative thin layer chromatography was used for the purification of compounds from *C. benthamiana*.Pre-coated glass prep TLC plates(0.5 – 2.0 mm thickness) purchased from Fischer's scientific UK was used for the isolation of compounds in theDCM fractions of*C. benthamiana*. From 21 pooled fractions obtained from gravity coloumn chromatography preformed as stated in section 3.7.5.1 above,pooled fraction 7 (2.18 g), (65% - 35% to 40% - 60% hexane: ethyl acetate), which crystallized on standing, was further purified with Prep-TLC, solvent system (petroleum ether: ethyl acetate: acetone 8: 2: 1) to give compound (**CB1**), a UV active single band, eluted with 100% ethyl acetate.Pooled fraction 8 (0.92 g), (60% - 40% to 55% - 45% hexane: ethyl acetate), on further purification using prep-TLC glass plates, solvent system (petroleum ether: ethyl acetate: acetone 8: 2: 1) indicated three different bands, which on elution with 100% ethyl acetate, produced four UV active; off white oily crystal substance (**CB5**), black viscous solid substance (**CB6**) and greenish brown oily paste substance (**CB7**). Pooled fraction 10 (1.66 g), (45% - 55% to 40% - 60% hexane: ethyl acetate), was subjected to short column(internal diameter = 1.5 cm, length = 40.0 cm) chromatography to obtain 45 fractions,solvent system (petroleum ether: ethyl acetate: acetone 8: 2: 1), which was pooled to 6 fractions. Pooled fraction 4 (0.14 g), (50% - 50% to 40% - 60% hexane: ethyl acetate), was further purified with prep-TLC plates, solvent system (petroleum ether: ethyl acetate: acetone 8: 2: 1), three UV active bands

Table 3.2: Column chromatographic fractionation of dichloromethane fraction of*C.benthamiana*

Fraction no	Mobile phase in percentage (%)
1-6	Hexane; 100
7-12	Hexane/Ethyl acetate; 95/5
13-18	Hexane/Ethyl acetate; 90/10
19-24	Hexane/Ethyl acetate; 85/15
25-32	Hexane/Ethyl acetate; 80/20
33-40	Hexane/Ethyl acetate; 75/25
41-51	Hexane/Ethyl acetate; 70/30
52-77	Hexane/Ethyl acetate; 65/35
78-90	Hexane/Ethyl acetate; 60/40
91-104	Hexane/Ethyl acetate; 55/45
105-122	Hexane/Ethyl acetate; 50/50
123-137	Hexane/Ethyl acetate; 45/55
138-153	Hexane/Ethyl acetate; 40/60
154-168	Hexane/Ethyl acetate; 35/65
169-192	Hexane/Ethyl acetate; 30/70
193-217	Hexane/Ethyl acetate; 25/75
218-239	Hexane/Ethyl acetate; 20/80
240-259	Hexane/Ethyl acetate; 15/85
260-283	Hexane/Ethyl acetate; 10/90
285-299	Hexane/Ethyl acetate; 5/95
300-315	Hexane/Ethyl acetate; 100
316-322	Hexane/Ethyl acetate; 95/5
323-324	Ethyl acetate/Methanol; 90/10
325-327	Ethyl acetate/Methanol; 80/20
328-338	Ethyl acetate/Methanol; 70/30
339-349	Methanol; 100

gave; a yellowish brown viscous substance (**CB2**), a brownish black viscous substance (**CB3**) and a brownish black viscous substance**(CBD4).**

3.7.6.Isolation of compounds from *C. racemosum*

3.7.6.1. Columnchromatographic purification of dichloromethame fraction of *C. racemosum*

Gravity column chromatography (GCC)was performed for the purification of *C. benthamiana* dichloromethane (DCM) fraction. Chromatographic glass column of (internal diameter = 3.5 cm, length = 80.0 cm) was used in the purification process. A portion of the DCM fraction (12 g) of the root bark extract of *C. racemosum* was introduced into the gravity coloumn (80.0 x 3.5 cm) packed with a bed of 100 g of silica gel (80-200 mesh size) in *n*-hexane and successfully eluted with solvent mixtures of increasing polarity starting from hexane (non-polar) to medium polar (ethyl acetate) to 70% methanol (polar) (Table 3.3).A total of 215 fractions were obtained and pooled to 6 fractions based on analytical TLC profile (solvent system;toluene/ethyl acetate/ethanol; 7: 2: 1).

3.7.6.2. Preparative thin layer chromatography (PTLC)

Preparative thin layer chromatography was used for the purification of compounds. Pre-coated glass preparative TLC plates(0.5 – 2.0 mm thickness) purchased from Fischer's scientific UKwas used for the isolation of compounds in theDCM fraction of*C. racemosum*. From 6 pooled fractions obtained from gravity coloumn chromatography preformed as stated in section 3.7.6.1 above, pooled fraction 2(0.13 g), (95% - 5% to 75% - 25% hexane:ethyl acetate), which crystallized on standing, was further purified with Prep-TLC, solvent system(toluene: ethyl acetate: ethanol 6: 3: 1), to give a greenish yellow oily compound **(CRD 1)**. Pooled fraction 3(0.69 g), (70% - 30% to 5% - 95% hexane:ethyl acetate), pooled fraction 4 (0.54 g) (95% - 5% hexane:ethyl acetate to 90% - 10% ethyl acetate: methanol), and pooled fraction 5 (0.86 g) (85% - 15% ethyl acetate:methanol) were combined together based on analytical TLC profile,solvent system(toluene: ethyl acetate: ethanol 6: 3: 1)and subjected to short column (internal diameter= 1.5 cm, length = 40.0 cm) chromatography to obtain 150 fractions, which was pooled to 8 fractions. Pooled fraction 6 (1.41 g) (54% - 46% to 44% - 56% hexane:ethyl acetate), was further purified with prep-TLC plates, solvent system(toluene: ethyl acetate: ethanol 6: 3: 1),

Table 3.3: Column chromatographic fractionation of dichloromethane fraction of*C.racemosum*

Fraction no	Mobile phase in percentage (%)
1-3	Hexane; 100
4-8	Hexane/Ethyl acetate; 95/5
9-12	Hexane/Ethyl acetate; 95/5
13-16	Hexane/Ethyl acetate; 90/10
17-22	Hexane/Ethyl acetate; 85/15
23-27	Hexane/Ethyl acetate; 80/20
28-34	Hexane/Ethyl acetate; 75/25
35-41	Hexane/Ethyl acetate; 70/30
42-47	Hexane/Ethyl acetate; 65/35
48-54	Hexane/Ethyl acetate; 60/40
55-61	Hexane/Ethyl acetate; 55/45
62-70	Hexane/Ethyl acetate; 50/50
71-79	Hexane/Ethyl acetate; 45/55
80-85	Hexane/Ethyl acetate; 40/60
86-92	Hexane/Ethyl acetate; 35/65
93-99	Hexane/Ethyl acetate; 30/70
100-105	Hexane/Ethyl acetate; 25/75
106-111	Hexane/Ethyl acetate; 20/80
112-118	Hexane/Ethyl acetate; 15/85
119-126	Hexane/Ethyl acetate; 10/90
127-132	Hexane/Ethyl acetate; 5/95
133-136	Hexane/Ethyl acetate; 100
137-148	Hexane/Ethyl acetate; 95/5
149-167	Ethyl acetate/Methanol; 90/10
168-182	Ethyl acetate/Methanol; 85/15
183-206	Ethyl acetate/Methanol; 80/20
107-215	Methanol; 100

two UV active bands gave two brown solid substances**(CRD 3)** and **(CRD 4)** respectively.

3.8.Spectroscopic characterisationsof isolatedcompounds

Mass spectrometric data of the isolated compounds were made by the atmospheric pressure chemical ionisation mass spectrosmetry(APCI-MS), (Thermo Scientific, UK). Infrared spectra of the isolated compounds were performed on a PerkinElmer FTIR Spectrum BX spectrometer. ^{1}H and ^{13}C Spectra of the isolated compounds were performed using Bruker Avance^{TM} III HD and Bruker Ascend^{TM} Spectrometersand data were recorded at 500 MHz/150 MHz, and 400 MHz/100 MHz, respectively. Quaternary, methane, methylene and methyl carbons were differentiated by DEPT experiment. Also, ^{1}H homonuclear bondingwas identified by the COSY experiment. While the HSQChelped to determine^{1}H- ^{13}C bound connectivity and HMBChelped to determinetwo and three ^{1}H- ^{13}C and ^{13}C - ^{13}C bond connectivity.Deuterateddimethyl sulfoxide (DMSO), chloroform, methanol and acetone were used in recording the spectra.

3.9.Summary of the work flow

Ethnobotanical survey

Preliminary phytochemical screening (Powdered sample ofplants collected from the survey)

Preliminary *in vitro*cytotoxicity assay (Crude extracts of plants collected from the survey)

Selected plants crude methanol extracts (*Caesalpinia benthamiana* and *Combretum racemosum*)

Solvent-solvent partitioning (Methanol crude extracts)

*In vitro*cytotoxicityassay on partitioned fractions (Hexane, dichloromethaneand aqueousfractions)

In vitro cyquant direct and caspase-3/7 green detection reagent assays(2 most active fractions)

Most active fractions (Dichloromethane fractions of *C. benthamiana* and *C. racemosum*)

Column chromatography (Monitored by TLC)

Preparative thin layer chromatography

Isolated compounds

In vitro cytotoxicity assay (MTT assay on isolated compounds)

Spectroscopic analysis

Scheme 3.1: Work flow chart adopted in this study

CHAPTER FOUR
RESULTS

4.1. Ethnobotanical survey

4.1.1. Demographic data of respondents

The result of demographic data of respondents are presented in Table 4.1.A total of thirty-eight (38)respondents(TMPs) were interviewed, most of them were herbalist (39.4%), followed by Diviner (21.1%), and only one (2.6%) of the respondent was a nurse.Mojority of the respondents (60.5%) were above 50 years, 5.3% was recorded for 21-30 years, while none of them were below 20 years.Majority of the respondentswere male (68.4%), while 31.6% were female.Most (47.4%) of the respondent hadformal education, while only 7.8% were not educated. Christainity (57.9%) was the major religion of the respondent, followed by African Traditional Religion (ATR) (31.6%), and Islam (10.4%). Majority (76.3%) of the respondents were married, widow (13.2%), widower (10.5) and none of them were either divorced or single (0%).

4.1.2. Source of knowledge and duration of practice of respondents

The means through which the respondents acquired their knowledge and the duration of their practice are shown in Table 4.2. It was observed that 47.4% of the respondents acquired their medicinal knowledge through their parents/grandparent, 15.8% got their medical knowledge via training, while only three (7.8%) of the respondents received their inspiration through dream. It was observed that most (39.5%) of the respondents had more than fourty years of cancer treatment experience, while only two (5.3%) of the respondents had less than ten years experience.

4.1.3.Causes, diagnosis and treatments of cancer by the respondents

Different factors responsible for the development of cancer, diagnosis and treatments as mentioned by the respondents are reported in Table 4.3. It was observed that the majority (50.0%) of the respondents believed that spiritual attack is the main

factorresponsible forcancer, 31.6% indicated consumption of can foods as the cause of cancer, while only three (7.9%) of the respondents cited heredity as cause of cancer.

Table 4.1. Demographic survey of respondents

Parameter	**Number**	**Percentage (%)**
Primary occupation		
Diviners	8	21.1
Herbalists	15	39.4
Herb-sellers	6	15.8
Traditional birth attendants	3	7.9
Peasant farmer	2	5.3
Hunter	3	7.9
Nurse	1	2.6
Age (Years)		
< 20	0	0.0
21-30	2	5.3
31-40	4	10.5
41-50	9	23.7
> 50	23	60.5
Sex		
Male	26	68.4
Female	12	31.6
Educational level		
Primary	18	47.4
Secondary	12	31.6
Tertiary	5	13.2
None (Informal)	3	7.8
Religion		
Christianity	22	57.9
Islam	4	10.5
African Traditional Religion	12	31.6
Marital status		
Single	0	0.0

Married	29	76.3
Divorced	0	0.0
Widow	5	13.2
Widower	4	10.5

Table 4.2.Source of knowledge and duration of practice of respondents

Parameter	**Number**	**Percentage**
Source of Knowledge		
Inheritance	18	47.4
Training	6	15.8
Inheritanceand Training	11	29.0
Dream	3	7.8
Duration of practice (year)		
< 10	2	5.3
11-20	3	7.8
21-30	8	21.1
31-40	10	26.3
> 40	15	39.5

Table 4.3. Causes, diagnosis and treatment of cancer by the respondents

Parameter	Number	Percentage
Causes		
Spiritual attack	19	50.0
Committing abominable act	4	10.5
Consumption of can foods	12	31.6
Heredity	3	7.9
Diagnosis		
Symptoms (chronic state of illness)	16	42.1
Ifa oracle (divination)	9	23.7
Presence of swollen and hard growth on the skin	5	13.2
Hospital confirmation	8	21.0
Treatment		
Herbal preparation only	18	47.3
Divination solution only	8	21.1
Incantation only	4	10.5
Herbal preparation + incantation	6	15.8
Herbal preparation + animal parts	2	5.3

4.1.4. Medicinal plants used in the treatment of different types of cancer amongst people of Gbonyin, Moba and Efon LGAs of Ekiti State, southwestern Nigeria

Fifty-seven different medicinal plantsused in the herbal formulation for the treatment of cancer disease amongst the traditional healersin the study area are shown inTable 4.4. Majority of the plants were tree 21 (38%), followed by herb 11 (20%), grass and clumping were 1% each (Figure 4.1).The morphological part of the surveyed plants most frequently used was the leaf (33%) followed by the stem bark (26%), while the bulb and seed were 2% each (Figure 4.2). The family Euphobiaceae, hadthe highest number of plants; 8(14%), followed by Annonaceae; 3(5%), while others are represented by one plant each (Table 4.4).

4.1.5. Herbal recipes, preparations and dosagesusedfor cancer therapy

Thirty-one herbal recipe formulations with different methods of preparation which includes: decoctions (38.2%), tincture (19.4%), powder (12.9%), soup (6.5%), charring (6.5%), paste (6.5%), soap (3.2%), infusion (3.2%) and ointment (3.2%) were cited by the respondents. The mode of administration was observed to be either oral (87.0%), topical (6.5%) or both (6.5%). Theherbal formulations were taken in shot glass twice daily, half tea cup 2-3 times daily, tea cup two or three times daily, a tea spoon once daily among others. These were indicated by the herbal healers of the selected LGAs as been used for their formulations for treatment of cancer (Table 4.5).

4.2.Percentage yield of plant extracts

The percentage yield of the nine plant crude extractsshowed that*Lagenaria breviflora* fruit gave the highest yield of 11.63% followed by *Caesalpinia benthamiana*leaf and *Enantiachloranthas*tem bark with yield of9.60% and 9.59%respectively. *Euphorbia opuntioides*whole plant (3.08%) gave the lowest yield(Table 4.6).

4.3.Preliminary phytochemical screening of the plantsamples

Different amounts of phytochemicals were observed in the plants. It was observed that*Combretum racemosum* had phenol and anthraquinone in abundanceand lacked steroid. In addition, *Euphorbia opuntioides, Nymphaea lotus and Olax subscorpioidea*all had phenol in abundance. Also,*Caesalpiniabenthamiana* possessed tannin and phenol in moderate amountsand lack only saponins, while *Ritchiealongipedicellata*had only saponins and steroids in trace amounts (Table 4.7).

Table 4.4.Medicinal plants used inthe treatment of different types of canceramongst the people of Gbonyin, Moba and Efon LGAs of Ekiti State, southwestern Nigeria.

S/N	Plant name	Family name	Common name	Local name	Habit	Part used	Use Mentionindex (UMi)
1	*Acanthus montanus* (Nees) T. Anders.	Acanthaceae	Thorny weed/ Leopard's tongue	Irunmarungbo/ Ahon ekun	Herb	Whole Plant	0.026
2	*Aframomum melegueta* K. Schum.	Zingiberaceae	Guinea pepper	Atare	Herb	Fruit	0.078
3	*Alchornea laxiflora* (Benth.)Pax & K.Hoffm.	Euphorbiaceae	Lowveld bead-string	Iya/Pepe/ Opoto	Shrub	Fresh leaf	0.026
4	*Allium cepa* L.	Amaryllidaceae	Bulb onion	Alubosa	Herb	Bulb	0.052
5	*Anacardium occidentale*L.	Anacardiaceae	Cashew	Kaju/Kasu	Tree	Stem bark	0.026
6	*Annona muricata* L.	Annonaceae	Prickly custard apple	Abo	Tree	Fruit	0.184
7	*Antiaris africana*Engl.	Moraceae	Sacking tree	Oriro	Tree	Stem bark	0.026
8	*Aristolochia ringens*Vahl	Aristolochiaceae	Snake work	Akoigun/ Areogun	Herb	Whole Plant	0.052
9	*Azadirachta indica*A.Juss.	Meliaceae	Neem tree	Dongoyaro	Tree	Stem bark	0.026
10	*Boerhavia coccinea*Mill.	Nyctaginaceae	Hogweed	Etupalola	Herb	Leaf	0.052
11	*Boerhavia diffusa* L.	Nyctaginaceae	Spreading hogweed	Etupalola	Herb	Leaf	0.026
12	*Bridelia micrantha*(Hochst.) Baill.	Phyllanthaceae	Mitseeri,	Arasan	Tree	Leaves	0.026
13	*Caesalpiniabenthamianus* Baill.	Leguminosae	Caesalpinia	Amuranju	Straggling shrub	Leaf	0.078

14	*Calliandra portoricensis*(Jacq.) Benth.	Leguminosae	Corpse awakener	Tude	Shrub	Root	0.10

Table 4.4.Medicinal plants used inthe treatment of different types of cancer amongst the people of Gbonyin, Moba and Efon LGAs of Ekiti State, southwestern Nigeria (cont')

S/N	Plant name	Family name	Common name	Local name	Habit	Part used	Use Mentionindex (UMi)
15	*Ceiba pentandra*(L.) Gaertn.	Malvaceae	Kapok	Araba	Tree	Stem bark	0.052
16	*Chasmantheradependens* Hochst.	Menispermaceae	-	Atoo	Climber	Root	0.052
17	*Chenopodium ambrosioides* L.	Chenopodiaceae	American Wormseed	Arupale	Herb	Leaf	0.026
18	*Cissampelos owariensis* P.Beauv. ex DC.	Menispermaceae	Velvet leaf/ Lungwort	Jenjoko/ Jokojee	Climber	Leaf amd Root	0.052
19	*Citrus aurantifolia* (Cristm.) Swingle	Rutaceae	Key lime	Osan wewe	Shrub	Fruit	0.026
20	*Combretum racemosum*P. Beauv	Combretaceae	Bush Willows	Arunbiesuru/Okan	Climber	Root	0.10
21	*Croton lobatus* L.	Euphorbiaceae	Croton	Aru	Herb	Leave	0.026
22	*Cucurbita maxima* Duchesne.	Cucurbitaceae	Pumpkin	Elegede	Climber	Leave	0.052
23	*Cymbopogon citratus*(DC.) Stapf	Poaceae	Lemon grass	Ewe tea	Grass	Leave	0.052
24	*Elaeis guineensis* Jacq.	Arecaceae	Oil palm	Igi ope	Tree	Stem bark	0.052
25	*Enantia chlorantha*Oliv.	Annonaceae	African yellow wood	Dokita igbo	Tree	Stem bark	0.078
26	*Euphorbia lateriflora*	Euphorbiaceae	Blind-eye	Oro enukokure	Shrub	Latex	0.026

	Schumach. & Thonn.						
27	*Euphorbia sp* (*opuntioides*)Mill.	Euphorbiaceae	-	Oro alagogo	Shrub	Latex	0.078

Table 4.4.Medicinal plants used inthe treatment of different types of cancer amongst the people of Gbonyin, Moba and Efon LGAs of Ekiti State, southwestern Nigeria (cont')

S/N	Plant name	Family name	Common name	Local name	Habit	Part used	Use Mentionindex (UMi)
28	*Euphorbia poissonii* Pax	Euphorbiaceae	Candle plant	Oro adete	Shrub	Latex	0.052
29	*Ficus mucuso* Welw. ex Ficalho	Moraceae	Bush fig/ Wild fig	Eepo obobo	Tree	Stem bark	0.026
30	*Fimbristylisdichotoma* (L.) Vahl	Cyperaceae	Common fringe sedge	Fidi	Clumping	Leaf	0.052
31	*Hymenocardia acida* Tul	Phyllanthaceae	Heart fruit	Orunpa	Tree	Stem bark	0.078
32	*Jatrophacurcas* L.	Euphorbiaceae	Barbados nut	Lapalapa	Shrub	Latex	0.026
33	*Jatropha gossypiifolia*L.	Euphorbiaceae	Bellyace bush	Botuje pupa	Shrub	Latex	0.052
34	*Khaya grandifoliola*C. DC.	Meliaceae	Khaya	Oganwo	Tree	Stem bark	0.026
35	*Lagenaria breviflora*(Benth.) Roberty	Cucurbitaceae	Wild colocynth	Tagiri	Climber	Fruit	0.052
36	*Nymphaealotus* L.	Nymphaeaceae	Egyptian white water-lily	Osibata	Herb	Leaf	0.026
37	*Olax subscorpioides* Oliv.	Olacaceae	Subcorpioides	Ifon	Tree	Stem	0.078
38	*Parquetina nigrescens* (Afel.) Bullock	Asclepiaceae	African parquentina	Ogbo	Climber	Leaf	0.052
39	*Petiveriaalliacea* L	Phytolaccaceae	Guinea henweed	Awogba arun	Herb	Leaf	0.026

40	*Piper guineense* Schumach. & Thonn.	Piperaceae	West African pepper	Iyere	Climber	Leaf	0.078
41	*Pistiastratiotes* L.	Araceae	Water cabbage	Ojuoro	Herb	Leaf	0.052
42	*Plukeneta conophora* Mill. Arg.	Euphorbiaceae	African walnut	Ausa	Climber	Leaf and Stem bark	0.078

Table 4.4.Medicinal plants used inthe treatment of different types of canceramongst the people of Gbonyin, Moba and Efon LGAs of Ekiti State, southwestern Nigeria (cont')

S/N	Plant name	Family name	Common name	Local name	Habit	Part used	Use Mentionindex (UMi)
43	*Plumbagozeylanica* L.	Plumbaginaceae	Ceylon Leadwort or Doctorbush	Inabiri	Straggling shrub	Root	0.078
44	*Psidium guajava* L.	Myrtaceae	Guava	Goaba	Tree	Stem bark	0.026
45	*Psychotria viridis* Ruiz & Pav.	Rubiaceae	Chacrona	Ewe afintoto	Shrub	Leave	0.052
46	*Pterocarpus osun* Craib	Leguminosae	African padauk	Arosu/Osun	Tree	Stem bark	0.026
47	*Pycnanthus angolensis*(Welw.) Warb.	Myristicaceae	African nutmeg	Eepo akomu	Tree	Root bark	0.052
48	*Ritchiea longipedicellata*Gilg	Capparaceae	Witaro (Nig.)	Logbekiya	Undershrub	Leaf	0.052
49	*Rouwolfia vomitoria*Afzel.	Apocynaceae	Swizzle stick	Igi asofeyeje	Tree	Root and Stem bark	0.026
50	*Sarcocephalus latifolius*(Sm.) E.A.Bruce.	Rubiaceae	African peach	Egbesi/ Ogbesi	Shrub	Root	0.131
51	*Securidacalongipedunculata* Fresen.	Polygalaceae	Violet tree	Ipeta	Small tree	Root	0.026
52	*Solenostemon monostachyus* (P. Beauv.) Briq.	Lamiaceae	-	Olojongbodu	Herb	Leaf	0.026
53	*Spondias mombin* L.	Anacardiaceae	Hog plum	Okikan	Tree	Stem bark	0.052
54	*Terminalia superba* Engl. &	Combretaceae	Whute afara	Afara	Tree	Stem bark	0.052

	Diels.						
55	*Tetraplueratetraptera*(Schum. & Thonn.) Taub.	Leguminosae	Aidan tree	Aidan	Tree	Fruit	0.052
56	*Trema orientalis* (L.) Blume.	Cannabaceae	Charcoal tree	Afefe	Tree	Stem bark	0.026
57	*Xylopia aethiopica*(Dunal) A. Rich.	Annonaceae	Ethiopia pepper	Arunje/ Aruje	Tree	Root bark and Seed	0.10

Table 4.5. Herbal recipes used for the treatment of different typesof cancer among the people of Gbonyin, Moba and Efon Local Government Areas of Ekiti State, Southwestern Nigeria

S/N	Recipe formulation	Solvent used	Types of preparation	Mode of administration	Dosage
1	The dried fruits of *A. muricata* and *S. latifolius* are dried and ground.	Pap	Powder	Oral	Tea spoon, once daily
2	Leaves of *P. stratiotes, N. lotus, P. guineese* and fruits of *X. aethiopica*, all in equal quantity are boiled with water in a local pot.	Water	Decoction	Oral and Topical (breast cancer patient cover herself with it).	Tea cup, twice daily
3	Roots of *P. zeylanica, C. portoricensis* and leaves of *C. benthamiana* are macerated in alcohol 12 to 24 h. .	Alcohol	Tincture	Oral	Shot glass, twice daily for twenty days to one month.
4	The leaves of *E. poissoni*, stem of *E. opuntioides* and *E. lateriflora* are pounded and mixed with 'obu otoyo' and 'adin.'	Local oil	Paste	Topical	Apply with cotton wool on the breast as often as possible
5	Leaves of *P. stratiotes*, bulb of *A. cepa* and fruits of *T. tetraptera* are soaked in alcohol for 12 to 24 h.	Alcohol	Tincture	Oral	Short cup, twice daily
6	Roots and leaves of *R. vomitoria,*roots and leaves of *C. owariensis* are boiled with water in a local pot.	Water	Decoction	Oral	Shot glass, twice daily.
7	Leaves of *P.guineense, R. longipedicellata* androots	Red oil	Soup	Oral	To be taken once

	of *P. conophora* all in equal quantity are cooked with snail, salt, local pepper, locust beans, red oil and water in a local pot.	and water			daily
8	Leaves of *J. gossypiifolia*, leaves and roots of *P. conophora* are boiled with pap water in a local pot that contains 'ako okuta' underneath.	Pap water	Decoction	Oral	Tea cup, three times daily

Table 4.5. Herbal recipes used for the treatment of different types of cancer among the people of Gbonyin, Moba and Efon Local Government Areas of Ekiti State, Southwestern Nigeria (cont')

S/N	Recipe formulation	Solvent used	Types of preparation	Mode of administration	Dosage
9	Leaves of *F. dichotoma* and seeds of *A. breviflorus* all in equal quantity are boiled with water in a local pot.	Water	Decoction	Topical ('Iya ose' and decocted water is use to bath the patient's breast very early in the morning when she has not greeting anyone).	As much as possible
10	The fresh leaves of *A. laxiflora* are touched three times very early in the morning, incantations are recited, then leaves collected, dried and powdered.	Hot pap	Powder	Oral	Tea spoon, once daily
11	Leaves of *P. nigrescens,* stem bark of *L. cupanioides*, root of *S. latifolius* and stem bark of *H. acida*, all in equal quantity are boiled with water.	Water	Decoction	Oral	Tea cup, three times daily
12	Fruits of *T. tetraptera, X. aethiopica*, and root of *S. longipedunculata* and bulb of *A. cepa* are soaked in alcohol for 12 to 24 h.	Alcohol	Tincture	Oral	Shot glass, twice daily
13	Roots of *P. conophora* are pounded, water	Water	Soap	Topical	Birth the patient

	extracted and latex of *J. curcas* are mixed together with local soap (ose dudu).	and Latex			regularly especially breast cancer
14	Stem bark of *A. africana* and *C. pentandra* all in equal quantity are boiled in water in a local pot.	Water	Decoction	Oral	Tea cup, twice daily

Table 4.5. Herbal recipes used for the treatment of different types of cancer among the people of Gbonyin, Moba and Efon Local Government Areas of Ekiti State, Southwestern Nigeria (cont')

S/N	Recipe formulation	Solvent used	Types of preparation	Mode of administration	Dosage
15	Stem barks of *K. grandifoliola, E. chlorantha,* and *P. angolensis*, all in equal quantity are boiled with water in a local pot.	Water	Decoction	Oral	Tea cup, twice daily
16	Leaves of *C. ambrosioides, S. monostachyus* and fruits of *C. aurantifolia*, the first two in equal quantity with little *C. aurantifolia* are boiled with water in a local pot.	Water	Decoction	Oral	Tea cup, three times daily
17	Fresh leaves of *A. muricata, B. diffusa* and*P.alliacea* are boiled with water in a local pot.	Water	Decoction	Oral	Tea cup, three times daily
18	Roots of *C. racemosum* and *P. zeylanica* are soaked with alcohol for 12 to 24 h.	Alcohol	Tincture	Oral	Shot glass, twice daily
19	Fruits of *S. latifolius* and*X. aethiopica* are dried, burnt and ground.	Hot pap	Sharing	Oral	Teaspoon, once daily
20	The leaves and fruits of *A. muricata* and stem bark of *P. angolensis* are boiled with water.	Water	Decoction	Oral	Tea cup, two to three times daily
21	Leaves and seeds of *P. guineense*, fruits of *X. aethiopica* and roots of *C. dependens*, all in equal quantity are	Alcohol	Tincture	Oral	Shot glass, twice daily

	soaked in alcohol for 12 to 24 h.				
22	Stem bark of *F. mucuso, E. chlorantha* and fruits of *A. muricata* all in equal quantity are boiled with water in a local pot.	Water	Decoction	Oral	Tea cup, taken regularly by the patient
23	Leaves of *P. guineense, C. benthamiana, R.longipedicellata*and roots of *O. subscorpioides* are boiled with water in a local pot.	Water	Decoction	Oral	Tea cup, two to three times daily

Table 4.5. Herbal recipes used for the treatment of different types of cancer among the people of Gbonyin, Moba and Efon Local Government Areas of Ekiti State, Southwestern Nigeria (cont')

S/N	Recipe formulation	Solvent used	Types of preparation	Mode of administration	Dosage
24	Roots of *C. dependens* are pounded and soaked in 'adin' for a week.	Oil	Ointment	Oral and Topical	Tea spoon, twice daily
25	Roots of *C. racemosum,* fruits of *P. guineense* and*S. latifolius* are dried, burnt and grounded	Hot pap	Charring	Oral	Tea spoon, once daily
26	Leaves of *L. breviflorus, C. owariensis* and *F. dichotoma* all in equal quantity are boiled with pap water in a local pot	Pap water	Decoction	Oral	Tea cup, once daily (very early in the morning).
27	Leaves of *A. montanus, A. occidentale* and whole plant of *A. ringens* are dried and ground.	Hot pap	Powder	Oral	Tea spoon, once daily (very early in the morning).
28	Stem bark of *E. guineensis and H. acida*, as well as root of *p. nigrescens* are dried, powdered and ground.	Hot pap	Powder	Oral	Tea spoon, once daily
29	Seed of *C. maxima, T. superba* and *P. osun* are soaked in alcohol with the leaves of *P. viridis* and *T. orientalis* for 48 h	Alcohol	Tincture	Oral	Shot glass, twice daily
30	Fresh leaves of *B. coccinea, C. owariensis*, *C. citratus*	Hot	Infusion	Oral	Tea cup, three times

	and C. lobatus, all in equal quantity are put inside a local pot, added hot water for 10 – 15 minutes.	water			daily
31	Fresh roots of *P. guajava, S. mombin, T. superba* and *C. lobatus* are pounded and soaked in local oil for a week; the extract is mixed with small local soap and potash.	Local oil	Paste	Oral	Tea spoon, twice daily

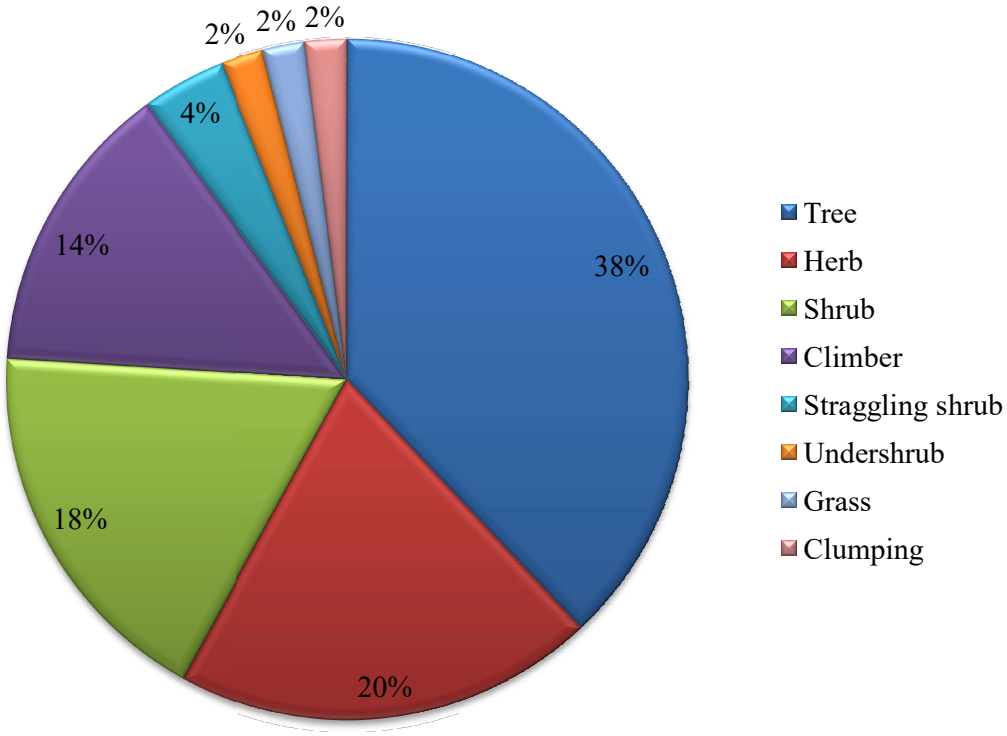


Figure 4.1.Proportion of plant habit reportedly used in cancer recipepreparation

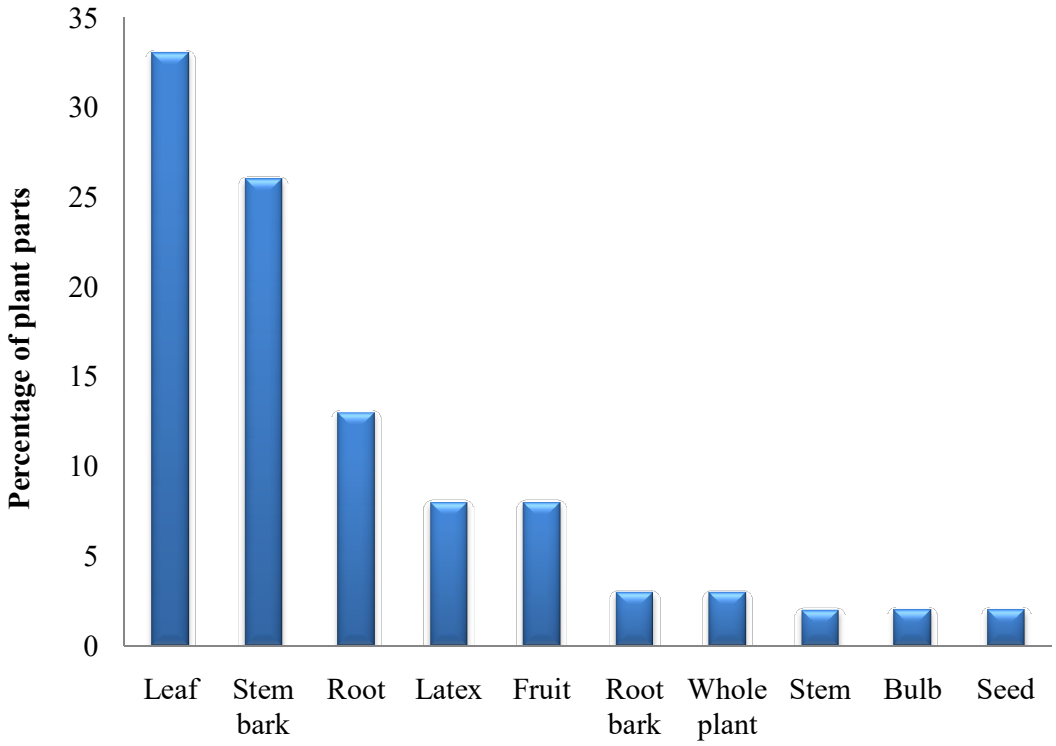


Figure 4.2.Proportion of plant part reportedly used in cancer recipe preparation

Table 4.6.Percentage yield of plant extracts

S/N	Plant names	Plant part	Solvent used	Weight of plant(g)	Weight of extract (g)	% Yield
1	*C. benthamiana*	Leaf	100% methanol	700	67.21	9.60
2	*C. dependens*	Root	100% methanol	1000	69.71	6.97
3	*C. racemosum*	Root bark	100% methanol	1500	79.00	5.26
4	*E.chlorantha*	Stem bark	100% methanol	700	67.17	9.59
5	*E. opuntioides*	Whole plant	100% methanol	1500	46.28	3.08
6	*L. breviflora*	Fruit	100% methanol	700	81.42	11.63
7	*N.lotus*	Leaf	100% methanol	900	55.89	6.21
8	*O. subscorpioides*	Root bark	100% methanol	700	56.89	8.12
9	*R.longipedicellata*	Leaf	100% methanol	1200	65.22	5.43

Table 4.7.Preliminary phytochemical screening of the plants extracts

		Phytochemicals							
S/N	**Plant names**	**Alkaloid**	**Saponin**	**Tanin**	**Flavoniod**	**Steroid**	**Phenol**	**Cardiac glycoside**	**Anthraquinone**
1	*C. benthamiana*	+	_	++	+	+	++	+	+
2	*C. dependens*	+	+++	+	_	_	_	_	_
3	*C. racemosum*	+	+	++	+	_	+++	+	+++
4	*E.chlorantha*	++	+	+	_	++	_	_	_
5	*E. opuntioides*	++	+	++	_	+	+++	_	_
6	*L. breviflora*	_	++	+	_	_	_	+	_
7	*N.lotus*	+	++	++	+	+	+++	+	_
8	*O. subscorpioides*	+	+	+	_	+	+++	+	_
9	*R.longipedicellata*	_	+	_	_	+	_	_	_

Key:+++=Concentrated, ++=Present, += Trace, -=Absent

4.4.Cytotoxicity studies

4.4.1.Preliminary cytotoxicity screening of the nine plant extracts

Table 4.8 shows the result of the preliminary cytotoxic effect of the nine plant extracts against human breast adenocarcinoma (MDA-MB-453) cell lineand the normal kidney (HEK-293) cell. *Combretum racemosum*(CR) (IC_{50} of 0.71 and 0.26µg/mL) and *Caesalpinia benthamiana*(CB) (IC_{50} of 3.21and 0.09 µg/mL)were observed as the two most active plant against the two cells, respectively having the lowest IC_{50} values.Methotrexate, the reference drughadIC_{50} of 0.94and 0.02 µg/mL, respectively. (Values were means ± SEM). The preliminary screening indicated the plant to further work on.

4.4.2.Viability assay (MTT) on fractions

4.4.2.1.Effect of fractions of *C. benthamiana*and *C. racemosum*on MCF-7 cells

Table 4.9 shows the cytotoxic effects of fractions of *C. benthamiana*(CB) leaf and *C. racemosum* (CR) root against human breast adenocarcinoma cells (MCF-7).TheIC_{50} were derived from a sigmoidaldose-response curve obtained from curve-fitting of data obtainedforeach of the fractions (Figure 4.3). It was observed that the fractions displayed significant cytotoxic activity in comparison with the standard reference. Dichloromethane fractions of *C. benthamiana*(CBD) and *C. racemosum*(CRD) wereobserved to be the most active against MCF-7 with IC_{50} of 10.46 µg/mL and goodness of fit (R^2 = 0.97) and IC_{50} of 19.49 µg/mL and goodness of fit (R^2 = 0.99), respectively.Cytotoxic activity of the DCM fractions were in comparison with the cytotoxic activitity of the reference drug, methotrexate, with IC_{50} of 12.11 µg/mL, (R^2= 0.97).

4.4.2.2.Effect of fractions of *C. benthamiana*and *C. racemosum* on A549 cells

Table 4.10 shows the cytotoxic effects of fractions of *C. benthamiana*(CB) leaf and *C. racemosum* (CR) root against human lung adenocarcinoma cells (A549). The$IC_{50}$was derived from a sigmoidal dose-response curve obtained from curve-fitting of data for each of the fractions (Figure 4.4). It was observed that the fractions displayed significant cytotoxic activity in comparison with the standard. Dichloromethane fraction of *C. benthamiana*(CBD) and *C. racemosum*(CRD) wereobserved to be most active against A549 with IC_{50} of 8.45 µg/mL and goodness of fit (R^2 = 0.97) and IC_{50} of 9.76 µg/mL and goodness of fit (R^2 = 0.97), respectively.Cytotoxicity activity of the

DCM fractions were in comparison with the cytotoxicity of the reference drug, methotrexate, with IC_{50} of 9.97 µg/mL, (R^2= 0.98).

Table 4.8.Preliminarycytotoxicity screening of the plant extracts on two human cells[a]

S/N	Plant names	IC_{50} (µg/mL, n=3)[b]	
		Human breast cancer cell (MDA-MB-453)	**Normal human kidney cell (HEK-293)**
1	*C. benthamiana*	3.28±0.09	0.09±0.08
2	*C. dependens*	11.19±0.16	3.68±0.17
3	*C. racemosum*	0.71±0.11	0.26±0.20
4	*E.chlorantha*	27.0±0.20	3.26±0.21
5	*E. opuntioides*	31.79±0.12	15.82±0.18
6	*L. breviflora*	40.07±0.16	8.94±0.22
7	*N.lotus*	18.03±0.13	3.37±0.21
8	*O. subscorpioides*	59.22±0.13	55.42±0.19
9	*R.longipedicellata*	59.26±0.08	9.23±0.12
10	Methotrexate	0.94±0.30	0.02±0.13

Key:[a]MDA-MB-453;Human breast adenocarcinoma cell; HEK-293;Human normal kidney cell,[b]MTT assay;(means ± SEM, n= 3)

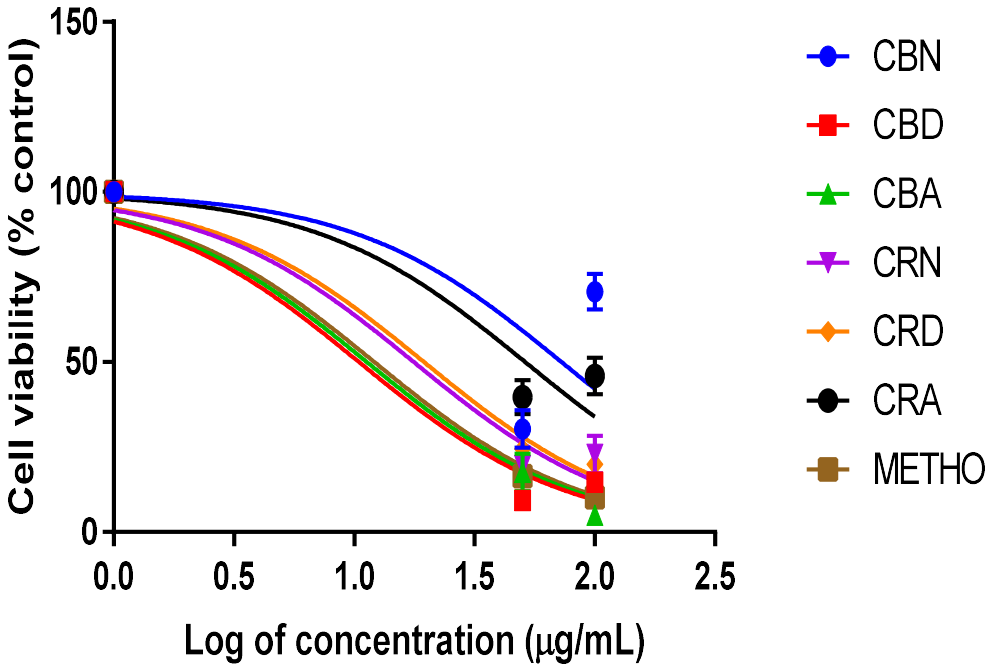


Figure 4.3. Sigmoidal dose-response curve for*C. benthamiana* and *C. racemosum* fractions with methotrexate (reference drug) againstMCF-7adenocarcinoma cells. Absorbance values are in triplicate and the data were analysed at $p<0.05$. Anti-log of concentration at 50% viability was taken as half-maximal inhibitory concentration (IC_{50}) for each of the fractions

Table 4.9. Cytotoxic effect of fractions of *Caesalpinia benthamiana*and *Combretumracemosum* onMCF-7 cell line[a]

Fractions	IC_{50} (µg/mL)[b]	95% CI	R^2
CBN	72.75±0.18	22.18 to 244.9	0.3194
CBD	10.46±0.10	6.136 to 16.67	0.9677
CBA	11.28±0.08	7.322 to 16.47	0.9677
CRN	17.67±0.08	11.09 to 26.07	0.9630
CRD	19.49±0.04	15.33 to 24.24	0.9877
CRA	51.35±0.08	32.4 to 79.31	0.8632
METHO	12.11±0.06	8.726 to 16.24	0.9865

Key: [a]MCF-7;Human breast adenocarcinoma cell,[b]MTT assay; (means ± SEM, n= 3), CBN;*Caesalpinia benthamiana* hexane fraction, CBD;*Caesalpinia benthamiana* dichloromethane fraction, CBA;*Caesalpinia benthamiana* aqueous fraction, CRN;*Combretum racemosum* hexane fraction, CRD;*Combretum racemosum* dicloromethane fraction, CRA;*Combretum racemosum* aqueous fraction, R^2; Goodness of fit

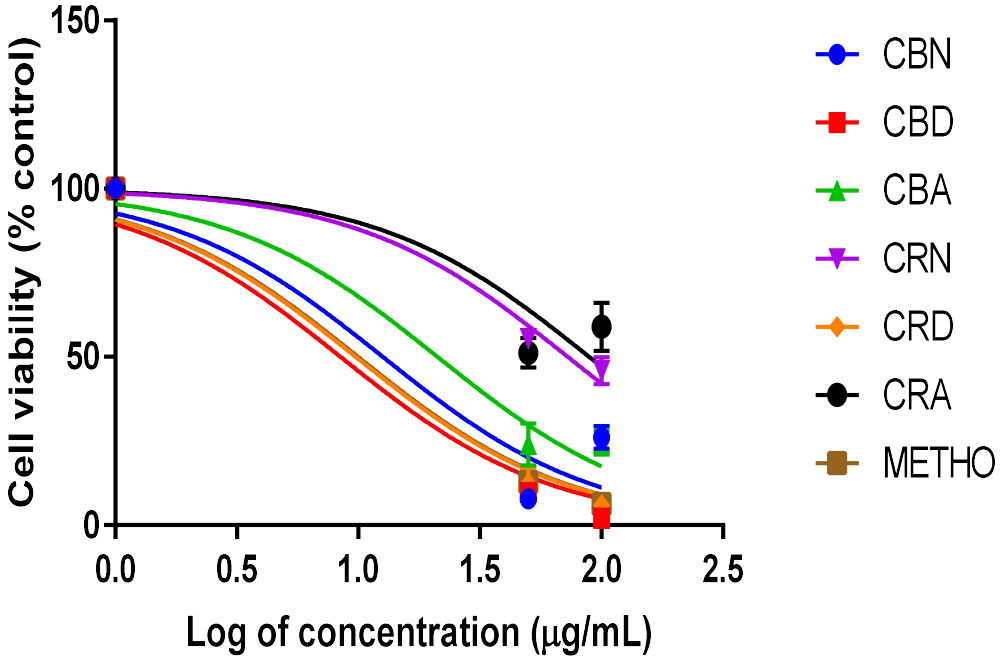


Figure 4.4.Sigmoidal dose-response curve for*C. benthamiana* and *C. racemosum* fractions with methotrexate (reference drug) against A549adenocarcinoma cells. Absorbance values are in triplicate and the data were analysed at $p<0.05$. Anti-log of concentration at 50% viability was taken as half-maximal inhibitory concentration (IC_{50}) for each of the fractions

Table 4.10.Cytotoxic effect of fractions of *Caesalpinia benthamiana*and *Combretum racemosum* on A549 cell line[a]

Fractions	IC_{50} (µg/mL)[b]	95% CI	R^2
CBN	12.61±0.16	5.134 to 25.42	0.9085
CBD	8.45±0.11	5.07 to 13.52	0.9717
CBA	21.17±0.07	14.06 to 30.03	0.9637
CRN	72.75±0.03	61.94 to 85.47	0.9735
CRD	9.76±0.09	6.2 to 14.67	0.9773
CRA	89.10±0.08	55.81 to 145.6	0.7524
Methotrexate	9.97±0.08	6.576 to 14.52	0.9806

Key: [a]A549;Human lung adenocarcinoma cell, [b]MTT assay; (means ± SEM, n= 3), CBN;*Caesalpinia benthamiana* hexane fraction, CBD;*Caesalpinia benthamiana* dichloromethane fraction, CBA;*Caesalpinia benthamiana* aqueous fraction, CRN;*Combretum racemosum* hexane fraction, CRD;*Combretum racemosum* dicloromethane fraction, CRA;*Combretum racemosum* aqueous fraction, R^2; Goodness of fit

4.4.2.3.Effect of fractions of *C. benthamiana*and *C. racemosum* on PC3 cells

Table 4.11 shows the cytotoxic effects of fractions of *C. benthamiana*(CB) leaf and *C. racemosum* (CR) root against human prostateadenocarcinoma cells (PC3). The half-maximal inhibitory concentration (IC_{50}) was derived from sigmoidaldose-response curve obtained from curve-fitting of data foreach of the fractions (Figure 4.5). It was observed that the fractions displayed significant cytotoxic activity in comparison with the standard. Aqueous fraction of *C. benthamiana*(CBD) and hexane fraction of *C. racemosum*(CRD) were the most active against PC3 with IC_{50} of 16.60 µg/mL and goodness of fit (R^2 = 0.98) and IC_{50} of 25.15 µg/mL and goodness of fit (R^2 = 0.99), respectively.Cytotoxic activity of the DCM fractions were in comparison with the cytotoxic activitity of the reference drug, methotrexate, with IC_{50} of 12.98 µg/mL and goodness of fit (R^2= 0.98) (Table 4.11).

4.4.3.Cyquant direct cell proliferation assay

4.4.3.1.Effect ofdichloromethane fractions of *C. benthamiana*and *C. racemosum* on MCF-7 cells

Figure 4.6 shows the effects of dichloromethane fractionsof *C. benthamiana* and*C. racemosum*on theproliferationofhuman breast adenocarcinoma cells (MCF-7)treated with 50µg/mL and 100 µg/mL for 24 h using x2 reagent cyquant direct cell proliferation assay. A significant reduction of viable cancer cells (green colour) proliferation was observed in treated compared to untreated (DMSO only). Images shownwere representativesof five of such fields of view per sample. Cells imaging were acquired on a standard FITC filter set microscope (x100 magnification)using scale bar = 100µm.

4.4.3.2. Effect ofdichloromethane fraction of *C. benthamiana*and *C. racemosum* on A549 cells

Figure 4.7shows the effects ofdichloromethane fractionsof *C. benthamiana* and *C. racemosum*on theproliferationofhuman lung adenocarcinomacells (A549) treated with 50 µg/mL and 100 µg/mL for 24 h using x2 reagent cyquant direct cell proliferation assay. A significant reduction of viable cancer cells (green colour) proliferation was observed in treated compared to untreated (DMSO only). Images shownwere representativesof five of such fields of view per sample. Cells imaging were acquired on a standard FITC filter set microscope (x100 magnification)using scale bar = 100µm.

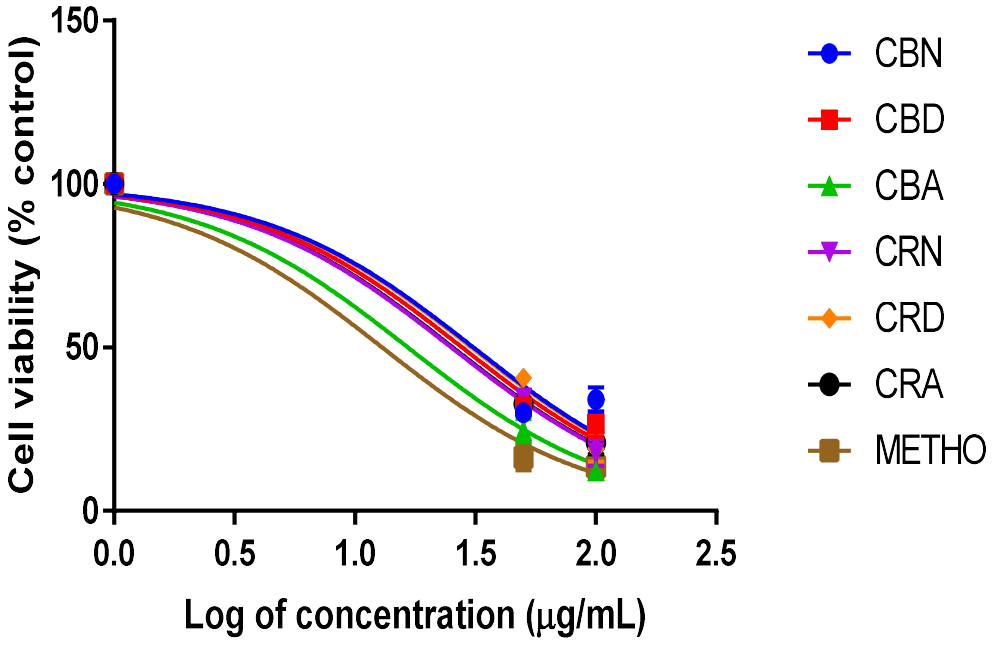


Figure 4.5. Sigmoidal dose-response curve for*C. benthamiana* and *C. racemosum* fractions with methotrexate (reference drug) againstPC3adenocarcinoma cells. Absorbance values are in triplicate and the data were analysed at $p<0.05$. Anti-log of concentration at 50% viability was taken as half-maximal inhibitory concentration (IC_{50}) for each of the fractions

Table 4.11.Cytotoxic effect of fractions of *Caesalpinia benthamiana*and *Combretum racemosum* on PC3 cell line[a]

Fractions	IC_{50} (µM) [b]	95% CI	R^2
CBN	31.07±0.07	20.26 to 45.27	0.9352
CBD	27.90±0.04	22.16 to 34.53	0.9821
CBA	16.60±0.04	12.98 to 20.74	0.9896
CRN	25.15±0.03	21.22 to 29.51	0.9915
CRD	30.47±0.03	24.95 to 36.76	0.9854
CRA	25.49±0.02	21.97 to 29.34	0.9932
Methotrexate	12.98±0.07	8.851 to 18.14	0.9809

Key: [a]PC3;Human prostate adenocarcinoma cell, [b]MTT assay; (means ± SEM, n= 3), CBN; *Caesalpinia benthamiana* hexane fraction, CBD;*Caesalpinia benthamiana* dichloromethane fraction, CBA;*Caesalpinia benthamiana* aqueous fraction, CRN;*Combretum racemosum* hexane fraction, CRD;*Combretum racemosum* dicloromethane fraction, CRA;*Combretum racemosum* aqueous fraction, R^2; Goodness of fit

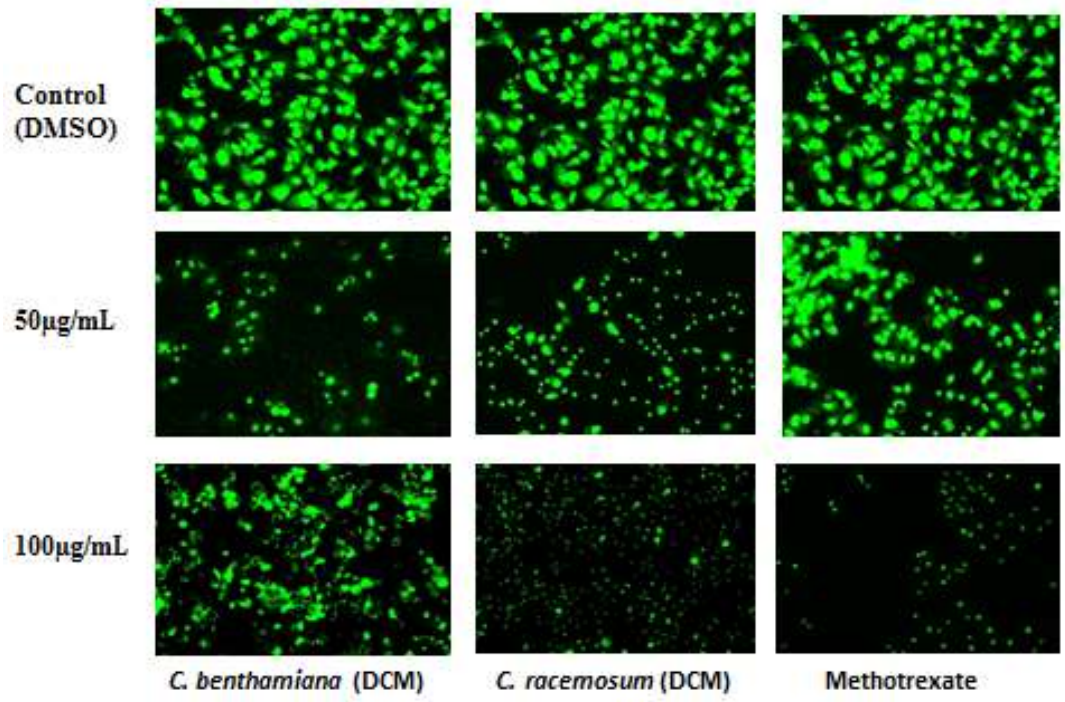


Figure 4.6.Photomicrographs showing the effect of the fractions(50 µg/mLand 100 µg/mL) on theproliferationofMCF-7adenocarcinoma cells using cyquant direct cell proliferation assay, where green colours were evidence of reduction of viable cells proliferation. Images shown are representatives of at least five of such fields of view per sample. Cells imaging were acquired on a standard FITC filter set microscope (x100 magnification). Scale bar = 100µm

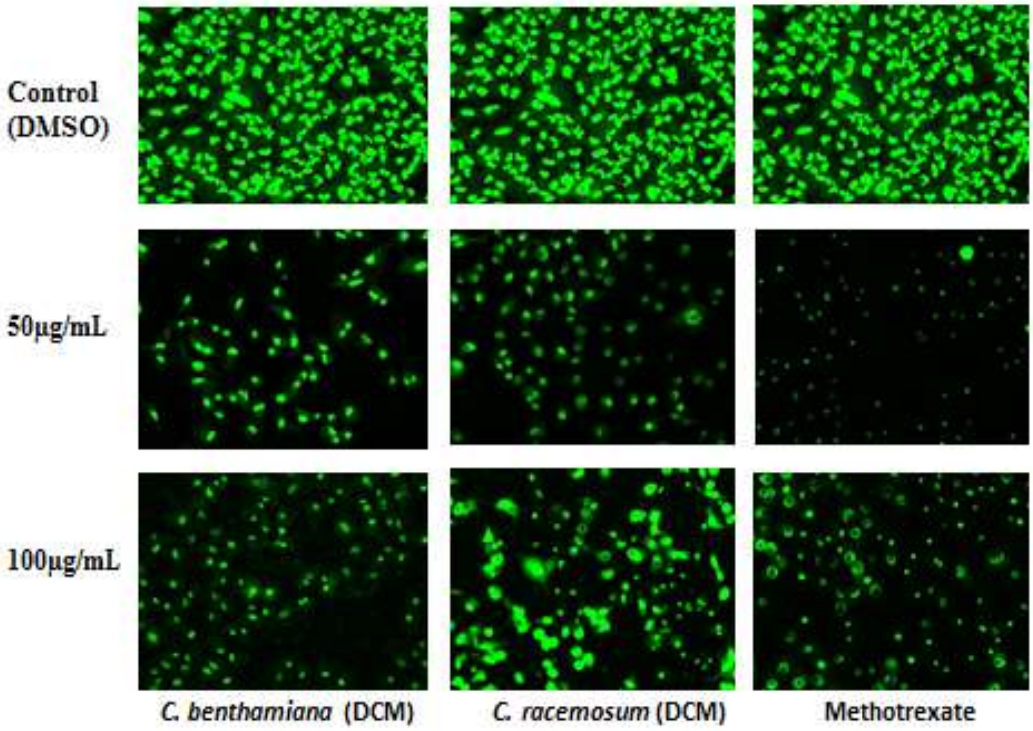


Figure 4.7.Photomicrographs showing the effect of the fractions(50 µg/mLand 100 µg/mL) on theproliferationofA549adenocarcinoma cells using cyquant direct cell proliferation assay, where green colours were evident of reduction of viable cells proliferation. Images shown are representatives of at least five of such fields of view per sample. Cells imaging were acquired on a standard FITC filter set microscope (x100 magnification). Scale bar = 100µm

4.4.3.3. Effect ofdichloromethane fraction of *C. benthamiana*and *C. racemosum* on PC3 cells

Figure 4.8 shows the effects of dichloromethane fractionsof *C. benthamiana* and *C. racemosum* on theproliferationof human prostate adenocarcinoma cells (PC3) treated with 50 µg/mL and 100 µg/mL for 24 h using x2 reagent cyquant direct cell proliferation assay. A reduction of viable cancer cells (green colour) proliferation was observed in treated compared to untreated (DMSO only). Images shown are representativesof five of such fields of view per sample. Cells imaging were acquired on a standard FITC filter set microscope (x100 magnification)using scale bar = 100µm.

4.4.4.Caspase-3/7 green detection reagentassay

4.4.4.1. Effect of dichloromethane fraction of *C. benthamiana* and *C. racemosum* on MCF-7 cells

Figure 4.9 werephotomicrographs that show the effects of dichloromethane fractionsof *C. benthamiana* and *C. racemosum* on human breast adenocarcinoma cells (MCF-7) treated with 50 µg/mL and 100 µg/mL for 24 h using caspase-3/7 assay.A bright, fluorogenic green colourobserved in treated group was evident of dead cancer cells compared to the untreated (DMSO only). Images shown are representatives of five of such fields of view per sample. Cells imaging were acquired on a standard FITC filter set microscope (x100 magnification)using scale bar = 100µm.

4.4.4.2. Effect of dichloromethane fraction of *C. benthamiana* and *C. racemosum* on A549 cells

Figure 4.10werephotomicrographs that show the effects of dichloromethane fractionsof *C. benthamiana* and *C. racemosum* on human lung adenocarcinoma cells (A549) treated with 50 µg/mL and 100 µg/mL for 24 h using caspase-3/7 assay.A bright, fluorogenic green colourobserved in treated group was evidence of dead cancer cells compared to the untreated (DMSO only). Images shown are representatives of five of such fields of view per sample. Cells imaging were acquired on a standard FITC filter set microscope (x100 magnification)using scale bar = 100µm.

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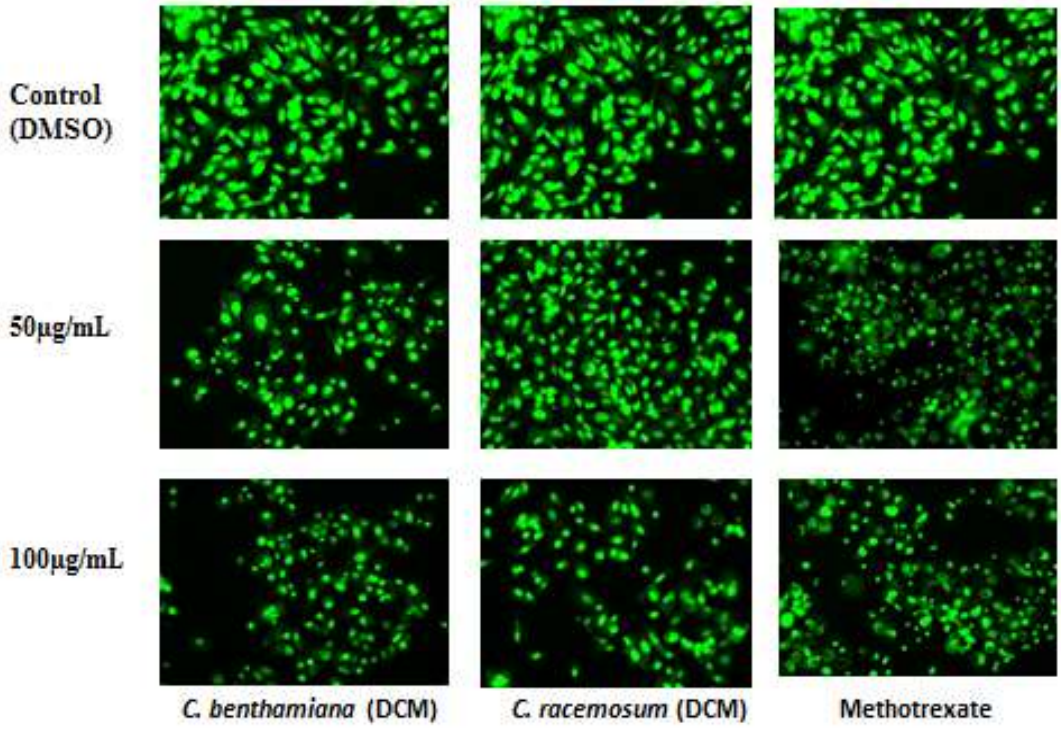


Figure 4.8.Photomicrographs showing the effect of the fractions(50 µg/mLand 100 µg/mL) on theproliferationofPC3 adenocarcinoma cells using cyquant direct cell proliferation assay, where green colours were evidence of reduction of viable cells proliferation. Images shown are representatives of at least five of such fields of view per sample. Cells imaging were acquired on a standard FITC filter set microscope (x100 magnification). Scale bar = 100µm

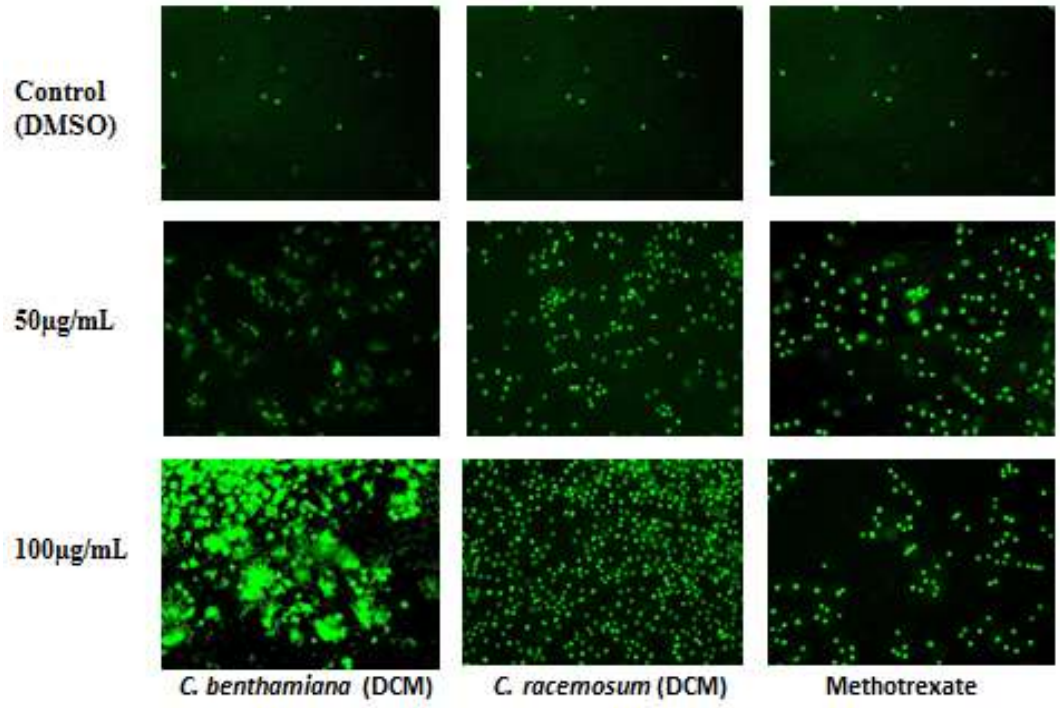


Figure 4.9.Photomicrographs showing the effect of the fractions(50 µg/mLand 100 µg/mL) on MCF-7adenocarcinoma cells using caspase-3/7 assay, where bright fluorogenic green colours were evidenceof dead cells. Images shown are representatives of at least five of such fields of view per sample. Cells imaging were acquired on a standard FITC filter set microscope (x100 magnification). Scale bar = 100µm

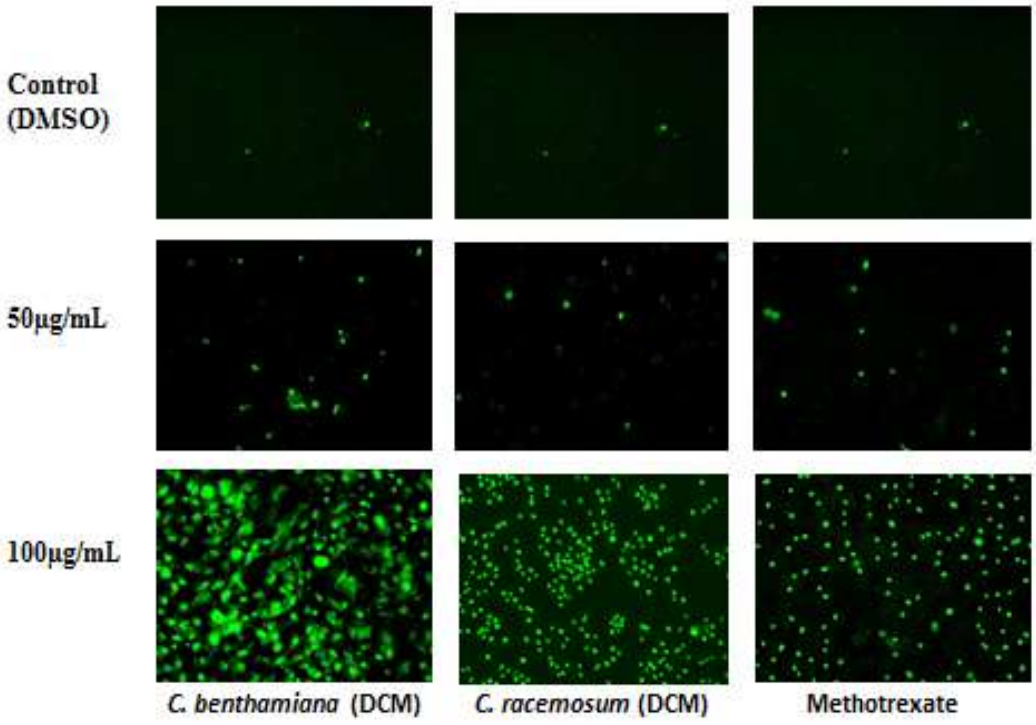


Figure 4.10.Photomicrographs showing the effect ofthe fractions(50 µg/mLand 100 µg/mL) on A549adenocarcinoma cells using caspase-3/7 assay, where bright fluorogenic green colours were evidenceof dead cells. Images shown are representatives of at least five of such fields of view per sample. Cells imaging were acquired on a standard FITC filter set microscope (x100 magnification). Scale bar = 100µm

4.4.4.3. Effect of dichloromethane fraction of *C. benthamiana* and *C. racemosum*

on PC3 cells

Figure 4.11werephotomicrographs that shows the effects of dichloromethane fractionsof *C. benthamiana* and *C. racemosum* on human prostate adenocarcinoma cells (PC3) treated with 50 µg/mL and 100 µg/mL for 24 h using caspase-3/7 assay.A bright, fluorogenic green colours observed in treated group were evident of dead cancer cells compared to the untreated (DMSO only). Images shown are representatives of five of such fields of view per sample. Cells imaging were acquired on a standard FITC filter set microscope (x100 magnification) using scale bar = 100µm.

4.5. Chromatographic purification and characterisation of compounds

4.5.1. Isolated compounds from *C. benthamiana*

Seven compounds CB1 to CB7 were isolated from *C. benthamiana*.Each compound was identified accordingly.However, compound CB4 isolated from *C. benthamiana*was not fully characterised in this study due to insufficient spectroscopic data, Appendix 5a-cshows theMS, ^{1}H and DEPT spectra.

4.5.2.Isolated compounds from *C. racemosum*

Three compounds (CR1, CR3 and CR4) were isolated from *C. racemosum*.Compound CR1 was characterized as racemonoate. Whereas, compounds CR3 and CR4 isolated from *C. racemosum*were not fully characterised in this study due to insufficient spectroscopic data. Appendixes 10a – 10e and 11a – 11e show the IR, ^{1}H,^{13}CNMR, DEPT and HSQC spectraof the two compounds, respectively.

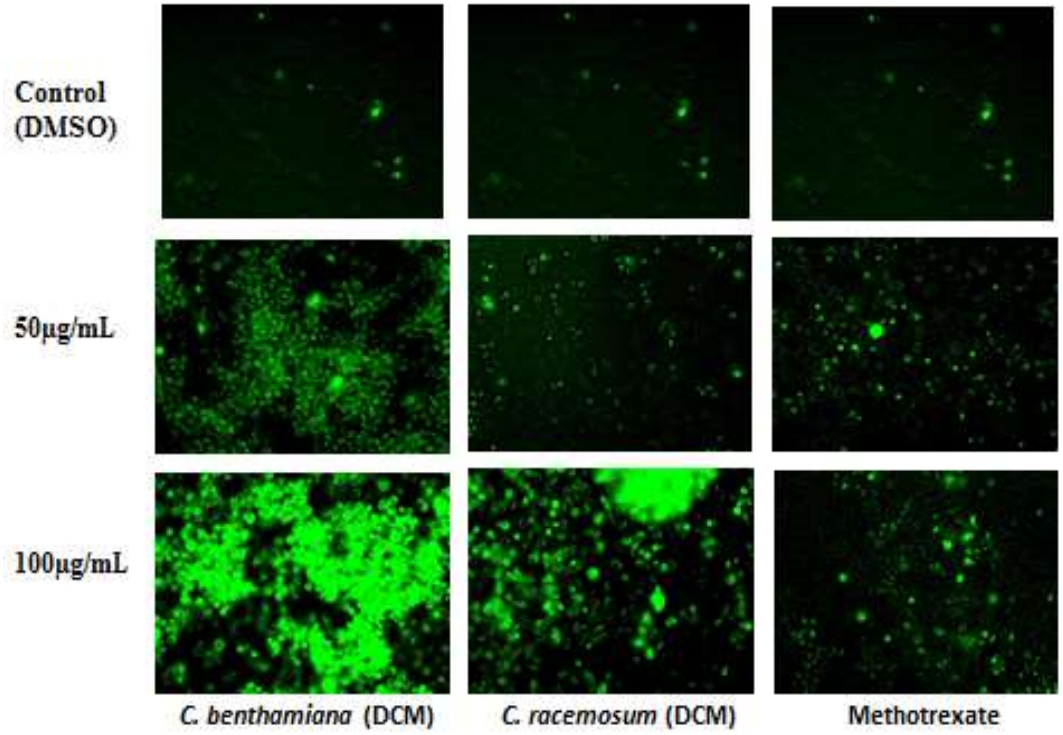


Figure 4.11.Photomicrographs showing the effect of the fractions(50 µg/mLand 100 µg/mL) on PC3adenocarcinoma cells using caspase-3/7 assay, where bright fluorogenic green colours were evidenceof dead cells. Images shown are representatives of at least five of such fields of view per sample. Cells imaging were acquired on a standard FITC filter set microscope (x100 magnification). Scale bar = 100µm

4.5.1.1. Characterisation of CB1

Compound CB1 (900 mg) was isolated as a white/off-white crystalline powder, R_f 0.28 (petroleum ether/acetone/ethyl acetate 7: 3: 1) with a melting point of 201°C (Lit m.pt 198–200°C), (Sanchez *et al*., 2013) and was identified as Methyl 3, 4, 5-trihydroxylbenzoate (Methyl gallate) and classified as a phenolic acid(Figure 4.12), with molecular formula $C_8H_8O_5$. The mass from APCI-MS m/z (% relative intensity) was 184.9 $[M+H]^+$, Calculated for$C_8H_8O_5$ 184.1491. ^{1}H NMR spectra data (DMSO, 500 MHz) and ^{13}C NMR spectra data (DMSO, 150 MHz)are shown in Table 4.12.Assignments were confirmed by DEPT, COSY, HMBC and HSQC experiments (Appendix 2c-2f).

4.5.1.2. Characterisation of CB2

Compound CB2 (130 mg) was isolated as a viscous/oily yellowish brown sample, R_f0.42 (petroleum ether/acetone/ethyl acetate 7: 3: 1) with a melting point of 55°C and identified as 1-(3, 4, 5 - trihydroxyphenyl)ethyl 2-hydroxy-3-(hydroxylmethyl) benzoate and named as benthamianoate (new compound) (Figure 4.13) with molecular formula $C_{16}H_{16}O_7$. The mass from APCI-MS m/z (% relative intensity) was 320.09 $[M+H]^+$. Calculated for$C_{16}H_{16}O_7$320.28964. ^{1}H NMR spectra data (DMSO, 500 MHz) and ^{13}C NMR spectra data (DMSO, 150 MHz)are shown in Table 4.13.Assignments were confirmed by DEPT, COSY, HMBC and HSQC experiments (Appendix 3c-3e).It was classified as tannin.

4.5.1.3. Characterisation of CB3

Compound CB3 (200 mg) was isolated as brownish black viscous substance, R_f0.69 (petroleum ether/acetone/ethyl acetate 7: 3: 1) with a melting point of 45°Cand identified as 3-amino-4-hydroxy-3-methyldihydrofuran-2-one and named as benthamiacone (new compound) (Figure 4.14), with molecular formula $C_5H_9NO_3$. The mass of benthamiacone from APCI-MS m/z (% relative intensity) was 132.98 $[M+H]^+$. Calculated for$C_5H_9O_5$ 131.13166. The ^{1}H NMR spectra data (DMSO, 500 MHz) and ^{13}C NMR spectra data (DMSO, 150 MHz)are shown in Table 4.14.Assignments were confirmed by DEPT, HMBC, HSQC andNOESY experiments (Appendix 4c-4f).It was classified as a dihydrofuran.

Table 4.12.[1]H-and [13]C- (500 and 150 MHz in DMSO) NMR data for compound **CB1**

		CB1 data			Reported data (Sanchez *et al*., 2013)	
S/N.	**Types of C**	δ_H	δ_C	**HMBC**	δ_H	δ_C
1	CQ	-	136.87	-	-	119.32
2	CH=	6.94 (1H, s, H-2)	109.06	136.87, 146.03, 109.06	6.94, s	107.86
3	CQ	-	146.03	-	-	144.10
4	CQ	-	136.87	-	-	136.99
5	CQ	-	146.03	-	-	144.10
6	CH=	6.94 (1H, s, H-6)	109.06	136.87, 146.03, 109.06	6.94, s	107.86
7	C=O	-	166.78	-	9.21, s	165.67
7a	CH_3-O	3.75 (s)	52.15	166.78	3.74, s	50.36

Key:CQ; Quaternary carbon, C=O; Carbonyl carbon, CH=; Methine carbon, CH_3-O; Methoxyl carbon, $\delta_{H;}$ [1]H chemical shift,δ_C; [13]C chemical shift, HMBC; Heteronuclear Multiple Bond Correlation

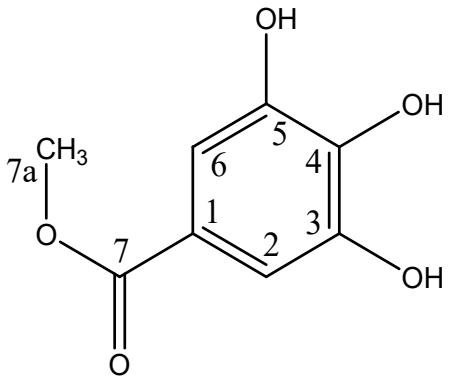


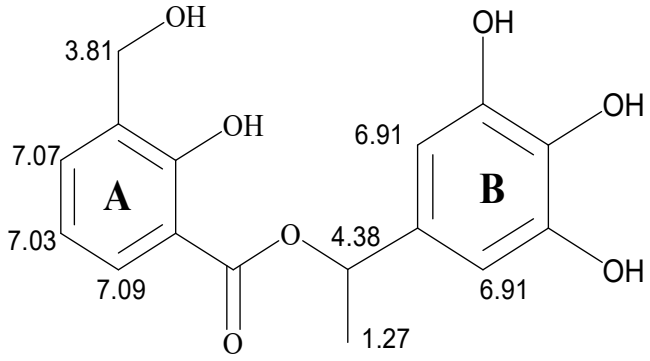
Figure 4.12. Chemical structure ofcompound **CB1**(methyl gallate) - **Known Class of compound:**Phenolic acid

Table 4.13.[1]H-and [13]C- (500 and 150 MHz in DMSO) NMR data for compound**CB2**

S/N	Types of C	δ_H	δ_C	HMBC
1	CQ	-	131.60	-
2	CH=	6.91 (s)	109.20	73.10, 131.60, 138.40, 146.10
3	CQ	-	146.10	-
4	CQ	-	138.40	-
5	CQ	-	146.10	-
6	CH=	6.91 (s)	109.20	73.10, 131.60, 138.40, 146.10
1°	CH-	4.36-4.38 (m)	73.10	167.96
1b	CH_3-	1.27 (d)	21.74	73.10, 167.96
1'	CQ	-	119.50	-
2'	CQ	-	151.0	-
3'	CQ	-	121.0	-
3'a	CH_2-	3.75-3.81 (d)	71.97	-
4'	CH=	7.07 (d)	118.8	71.97, 114.0, 116.10
5'	CH=	7.03 (dd)	116.10	118.8, 114.0
6'	CH=	7.09 (d)	114.0	116.10, 118.8
1′a	C=O	-	167.96	-

Key: CQ; Quaternary carbon, C=O; Carbonyl carbon, CH=; Methine carbon, CH_2-; Methylene carbon, CH_3-; Methyl carbon, $\delta_{H;}$ [1]H chemical shift,δ_C; [13]C chemical shift, HMBC; Heteronuclear Multiple Bond Correlation

^{1}H NMR



^{13}C NMR

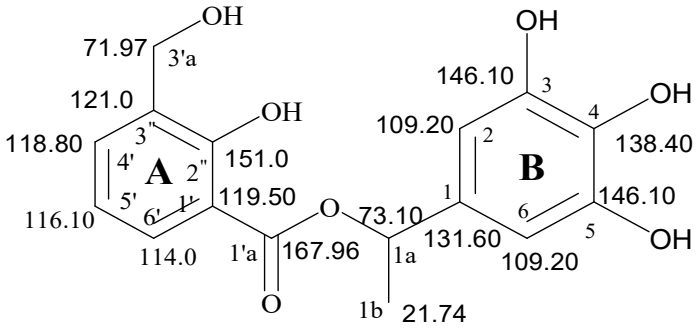


Figure 4.13. Chemical structure ofcompound **CB2**(benthamianoate) - **New Class of compound:**Tannin

Table 4.14.[1]H-and [13]C- (500 and 150 MHz in DMSO) NMR data for compound**CB3**

S/N	Types of C	δ_H	δ_C	HMBC
-	-	-	-	-
2	C=O	-	178.70	-
3	CQ	-	72.03	-
3°	CH_3-	1.25 (d)	21.74	72.03, 178.70, 73.17
4	CH-OH	4.37 (t)	73.17	72.03, 62.91
5	CH_2-	4.01 (m)	62.91	73.17, 178.70

Key: C=O; Carbonyl carbon, CH-; Methine carbon, CH_2-; Methylene carbon, CH_3-; Methyl carbon, $\delta_{H;}$ [1]H chemical shift,δ_C; [13]C chemical shift, HMBC; Heteronuclear Multiple Bond Correlation

^{13}C NMR^{1}HNMR

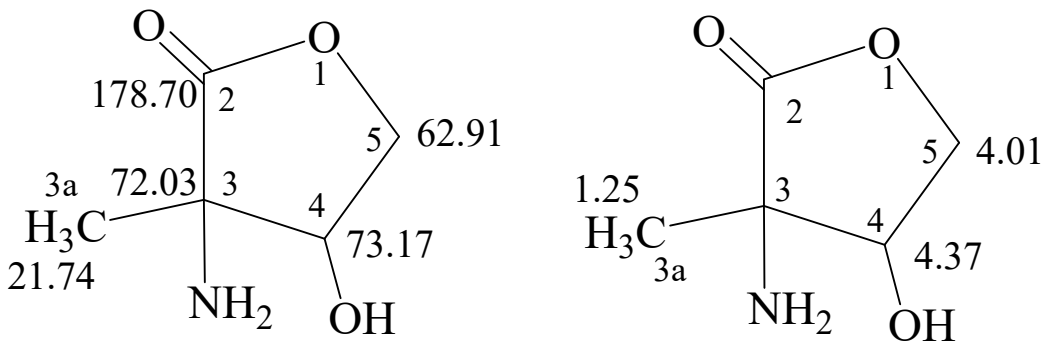


Figure 4.14. Chemical structure ofcompound **CB3**(benthamiacone) - **New Class of compound:**Dihydrofuran

4.5.1.4. Characterisation of CB5

Compound CB5 (180 mg) was isolated as off white oily crystal, R_f0.43 (petroleum ether/acetone/ethyl acetate 7: 3: 1) with a melting point of 14°Cand identified as 2-methoxyacrylic acid and classified as an unsaturated monocarbonxylic acid(Figure 4.15), with molecular formula $C_4H_6O_3$. The IR υ_{max} cm-^{1} showed bands at 3461.5, 3286.5, 3087.1 (O-H), 2951.8 (CH_3), 2850.0 (CH_2), 1691.8 (C=O) cm-^{1}. The ^{1}H NMR spectra data (CD_3OD / Acetone, 400 MHz) and ^{13}C NMR spectra data (CD_3OD / Acetone, 100 MHz)are shown in Table 4.15.Assignments were confirmed by DEPT and COSY experiments (Appendix 6d-6f). The MS m/z (% relative intensity) was 102.0 $[M+H]^+$. Calculated for$C_4H_6O_3$102.09044.

4.5.1.5. Characterisation of CB6

Compound CB6 (20 mg) was isolated as yellowish brown crystals, R_f0.20 (petroleum ether/acetone/ethyl acetate 7: 3: 1) with a melting point of 17°Cand identified as 1-aminohexane-1-ol and named as benthamianine (new compound) (Figure 4.16), with molecular formula $C_6H_{15}NO$. The IR υ_{max} cm-^{1} showed bands at 3362.3 (O-H), 2923.8 (CH_3), 2853.6 (CH_2), 1512.4, 1443.9 (C-N), 908.9 and 729.6 (C-H) cm-^{1}. The ^{1}H NMR spectra data (CD_3OD, 400 MHz) and ^{13}C NMR spectra data (CD_3OD, 100 MHz)are shown in Table 4.16.Assignments were confirmed by DEPTand COSY experiments (Appendix 7d-7f).The MS m/z (% relative intensity) was 117.12 $[M+H]^+$. Calculated for$C_6H_{15}NO$117.19.It was classified as an amine.

4.5.1.6. Characterisation of CB7

Compound CB7 (10 mg) was isolated as greenish brown oily paste substance, R_f 0.36 (petroleum ether/acetone/ethyl acetate 7: 3: 1) with a melting point 153°C and identified as 4-(hexadecahydro-3-hydroxy-6,10,13,15-tetramethyl-1*H*-cyclopenta[*a*]phenanthren-17-yl)-5-hydroxy-5-methylfuran-2(5*H*)-one and named as benthamianol (new compound) (Figure 4.17),with molecular formula $C_{26}H_{40}O_4$. The IR υ_{max} cm-^{1} showed bands at 2916 (CH_3), 2849 (CH_2), 1734 (C=O), 1437 (CH-OH), 1373, 1251 (C-O-C)913, 881, 757 (C-H) cm-^{1}. The ^{1}H NMR spectra data (CD_3OD, 400 MHz) and ^{13}C NMR spectra data (CD_3OD, 100 MHz)are shown in Table 4.17.Assignments were confirmed by DEPT, COSY, HMBC, HSQC and NOESY experiments (App. 8d-8h).The MS m/z (% relative intensity) was 416.29 $[M+H]^+$. Calculated for$C_{26}H_{40}O_4$416.5878.It was classified as a triterpenoid.

Table 4.15.[1]H-and [13]C- (400 and 100 MHz in CD_3OD and Acetone) NMR data for compound**CB5**

S/N	Types of C	δ_H	δ_C
1	C=O	-	168.27
2	QC	-	145.18
2°	CH_3-O	3.89 (s)	50.10
3	CH_2=	5.50 - 5.52 (m)	108.86

Key:CQ; Quaternary carbon, C=O; Carbonyl carbon, CH_2-; Methylene carbon, CH_3-O; Methoxyl carbon, $\delta_{H;}$ [1]H chemical shift,δ_C; [13]C chemical shift

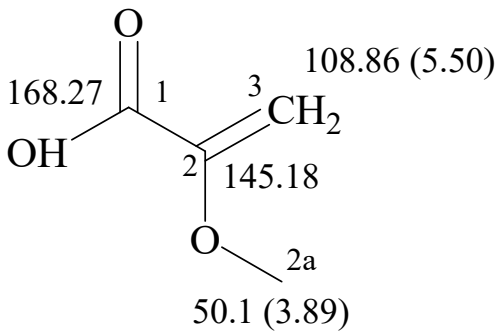


Figure 4.15. Chemical structure ofcompound **CB5**(2-methoxyacrylic acid) - **Known Class of compound:**Unsaturated monocarbonxylic acid

Table 4.16.[1]H-and [13]C- (400 and 100 MHz in CD_3OD) NMR data for compound**CB6**

S/N	Types of C	δ_H	δ_C	COSY
1	CH-	3.50 (m)	76.71	H-2, H-3
2	CH_2-	1.57-1.73 (m)	31.94	H1, H-3, H-4
3	CH_2-	1.14-1.35 (m)	29.37	H-1. H-2, H-4, H-5
4	CH_2-	1.14-1.35 (m)	29.71	H-2, H-3, H-5
5	CH_2-	1.32-1.35 (m)	22.71	H-3, H-4, H-6
6	CH_3-	0.97 (t)	14.22	H-4, H-5

Key: CH-; Methine carbon, CH_2-; Methylene carbon, CH_3-; Methyl carbon, $\delta_{H;}$ [1]H chemical shift,δ_C; [13]C chemical shift, COSY; Correlation spectroscopy

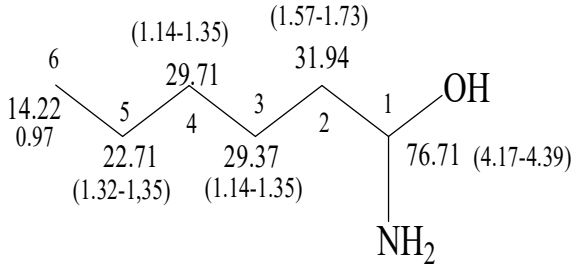


Figure 4.16. Chemical structure ofcompound **CB6**(1-aminohexane-1-ol) - **New Class of compound:**Amine

Table 4.17.[1]H-and [13]C- (400 and 100 MHz in CD_3OD) NMR data for compound **CB7**

S/N.	Types of C	δ_H	δ_C	HMBC
1	CH_{2-}	1.23-1.57 (m)	31.9	23.7, 43.6, 75.1
2	CH_{2-}	1.65-1.92 (m)	23.7	31.9, 30.0, 75.1
3	CH-OH	3.19 (bs)	75.1	30.0, 23.7, 31.9, 44.5
4	CH_{2-}	1.57-1.92 (m)	30.0	44.5, 75.1, 43.6, 41.3, 23.7
5	CH-	1.65-1.92 (m)	44.5	75.1, 30.0, 41.3, 43.6, 27.7
6	CH-	1.31-1.58 (m)	41.3	30.0, 44.5, 43.6, 27.7, 37.3
7	CH_{2-}	1.65-1.92 (m)	27.7	44.5, 41.3, 37.3, 49.8
8	CH-	1.31-1.58 (m)	37.3	41.3, 43.6, 27.7, 49.8, 50.9, 37.0
9	CH-	1.31-1.58 (m)	49.8	43.6, 27.7, 37.3, 31.9
10	CQ-	-	43.6	-
11	CH_{2-}	1.27-1.61 (m)	22.2	43.6, 49.8, 37.3, 36.3
12	CH_{2-}	1.23-1.57 (m)	36.3	22.2, 49.8, 36.7, 50.9, 49.7
13	CQ-	-	36.7	-
14	CH-	1.23-1.57 (m)	50.9	27.7, 37.3, 36.7, 49.7, 31.6
15	CH-	1.65-1.92 (m)	37.0	37.3, 36.7, 50.9, 49.7, 31.6, 14.5
16	CH_{2-}	1.31-1.58 (m)	31.6	36.7, 50.9, 49.7, 37.0, 14.5
17	CH-	2.12-2.17 (m)	49.7	115.6, 170.1, 108.0, 36.3, 36.7, 31.6
18	CH_{3-}	1.23 (s)	17.6	36.7, 49.7
19	CH_{3-}	1.25 (s)	11.9	43.6, 49.8, 31.9
20	CH_{3-}	1.18 (m)	14.5	50.9, 37.0, 31.6
21	CH_{3-}	1.16 (d)	14.2	44.5, 41.3, 27.7
1′	-	-	-	-
2′	C=O	-	171.4	-
3′	CH=	5.8 (s)	115.6	170.1, 108.0, 171.4, 49.7
4′	CQ-	-	170.1	-
5′	CQ-	-	108.0	-
5′a	CH_3-	1.81 (s)	21.2	170.1, 108.0

Key:CQ; Quaternary carbon, C=O; Carbonyl carbon, CH-; Methine carbon, CH_2-; Methylene carbon, CH_3-; Methyl carbon, $\delta_{H;}$ [1]H chemical shift,δ_C; [13]C chemical shift, HMBC; Heteronuclear Multiple Bond Correlation

^{13}C NMR^{1}HNMR

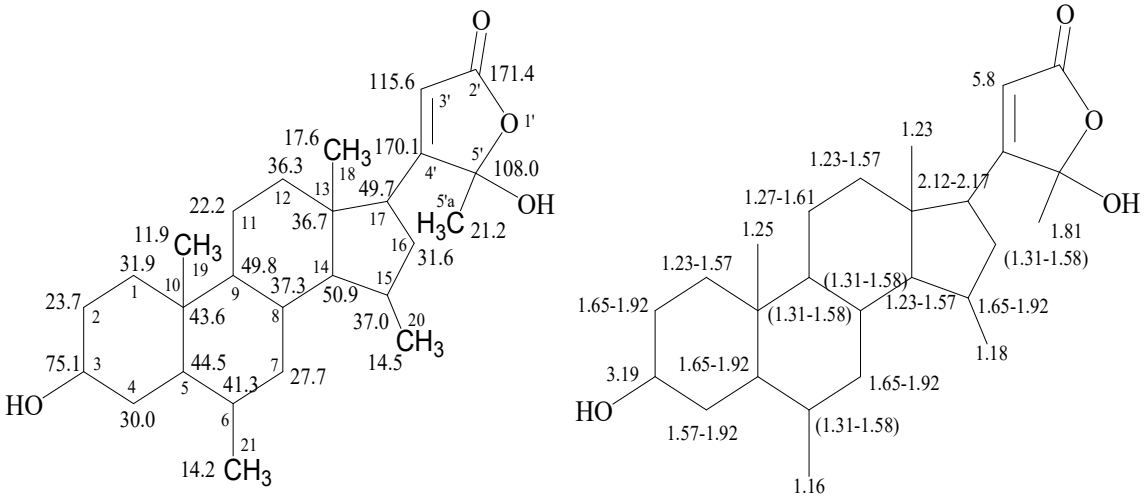


Figure 4.17. Chemical structure ofcompound **CB7** (benthaminol) - **New**

Class of compound: Triterpenoid

4.5.2.1. Characterisation of CR1

Compound CR1 (70 mg) was isolated as yellowish oily paste substance, R_f0.69 (toluene/ethyl acetate/ethanol 7: 2: 1) with a melting point of 168°C and identified as 3-hydroxy-2-(hydroxymethyl) nonadec-15-enoate and named as racemonoate (new compound) (Figure 4.18), with molecular formula $C_{20}H_{38}O_4$. The IR υ_{max} cm-^{1} showed bands at 2916 (CH_3), 2818 (CH_2), 2380, 2342 (CH), 1733, 1689 (C=O), 1461 (C-OH), 1367, 1313.3, 1251 (C-O-C), 966, 718 (C-H)cm-^{1}. The ^{1}H NMR spectra data (CD_3OD, 400 MHz) and ^{13}C NMR spectra data (CD_3OD, 100 MHz)are shown in Table 4.18.Assignments were confirmed by DEPT, COSY, HMBC, HSQC and NOESY experiments (Appendix 9d-9h).The MS m/z (% relative intensity) was 342.2 $[M+H]^+$. Calculated for$C_{20}H_{38}O_4$342.50792. It was classified as a long chain fatty acid.

4.6. Cytotoxicity of compounds

4.6.1.Effect of the isolated compounds on MCF-7 carcinoma cell line

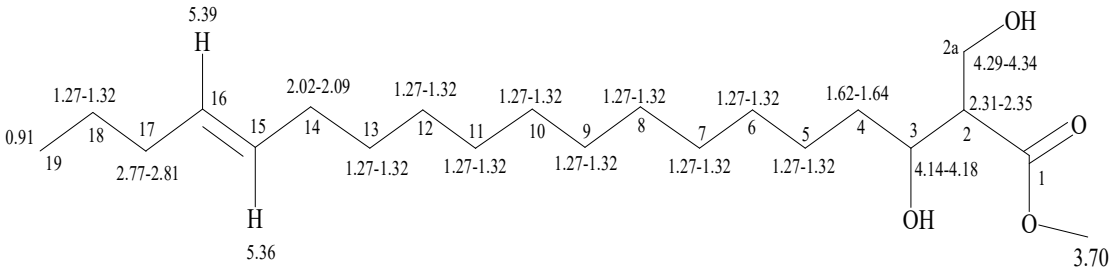
Table 4.19 shows the cytotoxic effectof isolated compoundsfrom*C. benthamiana*(CB) leaf and *C. racemosum* (CR) root against MCF-7(breastadenocarcinoma). The half maximal inhibitory concentration (IC_{50}) was derived from a sigmoidal dose-response curve obtained from the curve-fitting of data for each of the isolated compounds (Figure 4.19). It was observed that the compounds displayed significant cytotoxic activity against the tested cell line. Four of the isolated compounds: CB3 (benthamiacone), CB4 (unidentified), CB5 (2-methoxyacrylic acid) and CR1 (racemonoate) displayed significant cytotoxic activity in comparison with the reference drug (methotrexate). Benthamiacone had IC_{50} of 21.97±0.06 µg/mL with goodness of fit (R^2 = 0.83), CB4 (unidentified) had IC_{50} of 25.72±0.8 µg/mL; (R^2 = 0.70), 2-methoxyacrylic acid had IC_{50} of 31.37±0.70 µg/mL; (R^2 = 0.76) and racemonoate had inhibitory concentration of 30.89±0.05µg/mL; (R^2 = 0.85), respectively. Methotrexate, the reference drug had IC_{50} of 8.261±0.08 µg/mL and goodness of fit (R^2= 0.86). Compounds CB1 (methyl gallate) and CB2 (benthamianoate) were moderately active with IC_{50} of 13.44±0.17; (R^2= 0.18) and26.44±0.12µg/mL;(R^2= 0.33), respectively.

Table 4.18.^{1}H-and ^{13}C- (400 and 100 MHz in CD_3OD) NMR data for compound **CR1**

S/N.	Types of C	δ_H	δ_C	HMBC
1	C=O	-	173.3	-
1°	CH_3-O	3.7 (s)	51.5	173.3
2	CH-	2.31-2.35 (m)	34.2	173.3
2°	CH_2-OH	4.29-4.34 (m)	62.1	173.3, 68.9
3	CH-OH	4.14-4.18 (m)	68.9	173.3, 62.1
4	CH_2-	1.62-1.64 (m)	31.5	24.9, 31,5, 173.3
5	CH_2-	1.27-1.32 (m)	24.9	34.0, 31,5, 29.6
6	CH_2-	1.27-1.32 (m)	29.7	34.0, 29.5, 29.6, 24.9
7	CH_2-	1.27-1.32 (m)	29.6	29.5, 29.4, 31.5, 24.9
8	CH_2-	1.27-1.32 (m)	29.5	29.4, 29.6, 31.5, 29.3
9	CH_2-	1.27-1.32 (m)	29.4	29.5, 29.6, 29.3, 29.7
10	CH_2-	1.27-1.32 (m)	29.3	29.5, 29.4, 29.7
11	CH_2-	1.27-1.32 (m)	29.7	29.4, 29.3, 29.8
12	CH_2-	1.27-1.32 (m)	29.7	29.3, 29.8, 31.9
13	CH_2-	1.27-1.32 (m)	29.8	29.7, 31.9
14	CH_2-	2.021-2.09 (m)	31.9	29.8, 29.7, 130.2, 127.9
15	CH=	5.36 (m)	130.2	29.8, 31.9, 127.9
16	CH=	5.39 (m)	127.9	31.9, 130.2
17	CH_2-	2.77-2.81 (m)	31.9	127.9, 130.2
18	CH_2-	1.27-1.32 (m)	22.7	14.1
19	CH_3-	0.91 (d)	14.1	22.7, 34.2

Key:CQ; Quaternary carbon, C=O; Carbonyl carbon, CH-; Methine carbon, CH_2-; Methylene carbon, CH_3-; Methyl carbon, $\delta_{H;}$ ^{1}H chemical shift,δ_C; ^{13}C chemical shift, HMBC; Heteronuclear Multiple Bond Correlation

^{1}H NMR



^{13}C NMR

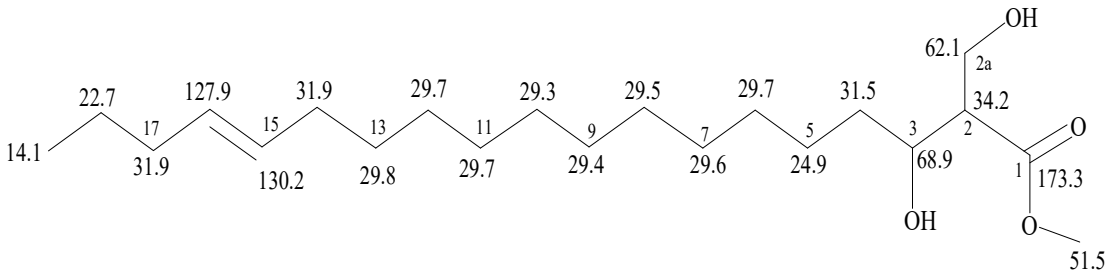


Figure 4.18. Chemical structure of compound **CR1**(racemonoate) - **New Class of compound:** Long chain fatty acid

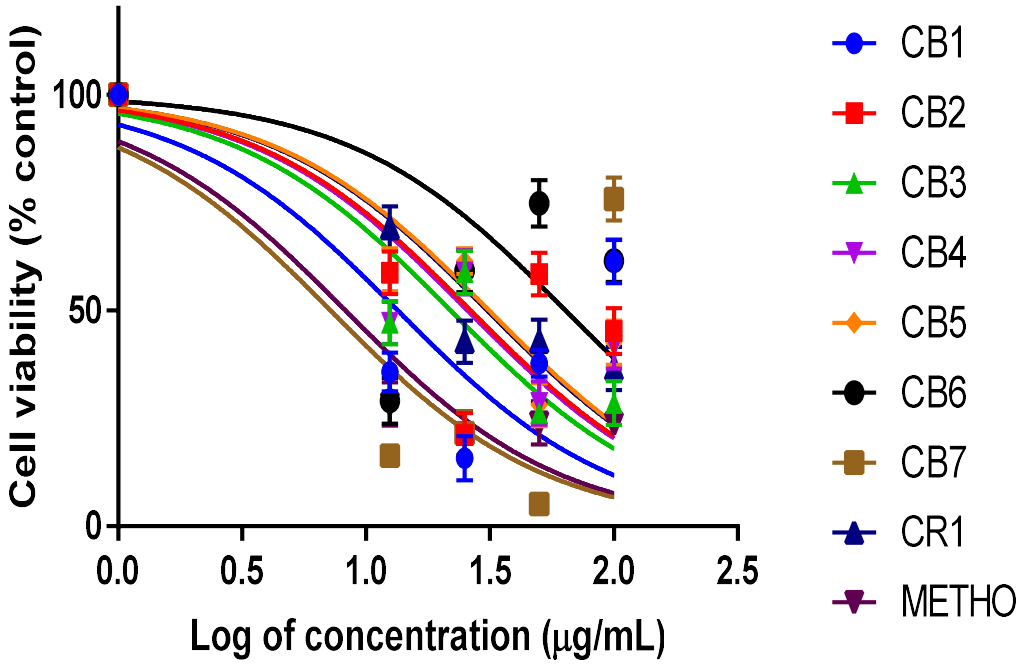


Figure 4.19. Sigmoidal dose-response curve of isolated compounds with methotrexate (reference drug) againstMCF-7adenocarcinoma cells. Absorbance values are in triplicate and the data were analysed at $p<0.05$. Anti-log of concentration at 50% viability was taken as half-maximal inhibitory concentration (IC_{50}) for each of the compounds

Table 4.19Cytotoxic effect of isolated compounds against MCF-7 cell line

	MCF-7[a]		
Compounds	**IC_{50} (µg/mL) [b]**	**95% CI**	**R^2**
CB1	13.44±0.17	4.512 to 35.11	0.1794
CB2	26.44±0.12	12.59 to 54.77	0.33
CB3	21.97±0.06	15.36 to 31.01	0.83
CB4	25.72±0.8	15.99 to 40.82	0.6582
CB5	31.37±0.70	21.69 to 45.29	0.7628
CB6	63.87±0.17	21 to 221	0.4924
CB7	7.197±0.25	1.666 to 24.23	0.2209
CR1	30.89±0.05	23.2 to 41.1	0.8513
METHO	8.261±0.08	5.267 to 12.42	0.8571

Key: [a]MCF-7;Human breast adenocarcinoma cell, [b]MTT assay; (means ± SEM, n= 3), CB1; Methyl gallate, CB2; Benthamianoate, CB3; Benthamiacone, CB4; unidentified, CB5;2-methoxyacrylic acid, CB6; Benthamianin, CB7; Benthamianol, CR1; Racemonoate, METHO; Methotrexate (reference drug), R^2; Goodness of fit

4.6.2.Effect of the isolated compounds onA549 carcinoma cell line

Table 4.20 shows the cytotoxic effects of isolated compounds from *C. benthamiana* (CB) leaf and *C. racemosum* (CR) root against A549 (lung adenocarcinoma).The IC_{50}were derived from asigmoidaldose-response curve (Figure4.20).It was observed that the compounds displayed significant cytotoxic activity against the tested cell line.Four of the isolated compounds: CB2 (benthamianoate), CB3 (benthamiacone), CB6 (benthamianin) and CR1 (racemonoate) displayed significant cytotoxic activity in comparison with the reference drug(methotrexate). Benthamianoate had IC_{50} of 16.16±0.10 µg/mL with goodness of fit (R^2 = 0.76), benthamiacone had IC_{50} of 13.46±0.07 µg/mL; (R^2 = 0.87), benthamianin had IC_{50} of 16.33±0.10 µg/mL; (R^2 = 0.73) and racemonoate had inhibitory concentration of 5.878±0.09 µg/mL; (R^2 = 0.88), respectively. Methotrexate, the reference drug had IC_{50} of 9.734±0.15 µg/mL with goodness of fit (R^2 = 0.72). CB5 (2-methoxyacrylic acid)and CB1 (methyl gallate)had the least activity with IC_{50} of 22.09±0.14 and 28.97±0.09 µg/mL, respectively. While CB7 (benthamianol) and CB4 (unidentified) were moderately active with IC_{50} of 10.9±0.14; (R^2= 0.49)and 19.1±0.14 µg/mL; (R^2= 0.39), respectively (Table 4.20).

4.6.3.Effect of the isolated compounds onPC3 carcinoma cell line

Table 4.21 shows the cytotoxic effects of isolated compounds from *C. benthamiana* (CB) leaf and *C. racemosum* (CR) root against PC3 (prostate adenocarcinoma). The IC_{50} were derived from a sigmoidaldose-response curve (Figure4.21). It was observed that the compounds displayed significant cytotoxic activity against the tested cell line. Six of the isolated compounds:CB1 (methyl gallate), CB3 (benthamiacone), CB4 (unidentified), CB6 (benthamianin), CB7 (benthamianol) and CR1 (racemonoate) displayed significant cytotoxicity in comparableto the reference drug(methotrexate). Methyl gallate had IC_{50} of 8.44±0.05 µg/mL; (R^2 = 0.95), benthamiacone had IC_{50} of 13.23±0.10 µg/mL; (R^2 = 0.70), CB4 (unidentified) had IC_{50} of 13.13±0.09 µg/mL; (R^2 = 0.75) benthamianin had IC_{50} of 10.78±0.10 µg/mL; (R^2 = 0.97), benthamianol had IC_{50} of 10.29±0.04 µg/mL; (R^2 = 0.88) and racemonoate had inhibitory concentration of 18.59±0.09 µg/mL; (R^2 = 0.70). Methotrexate, the reference drughad IC_{50} of 7.054±0.08 µg/mL with goodness of fit (R^2 = 0.89). Compound CB5 (2-methoxyacrylic acid) was moderately active against prostate carcinoma cell with IC_{50} of 16.71±0.13 µg/mL; (R^2= 0.62), while compounds CB2 (benthamianoate)had the least cytotoxic activity(IC_{50} of 11.13±0.18 µg/mL; (R^2= 0.24) (Table 4.21).

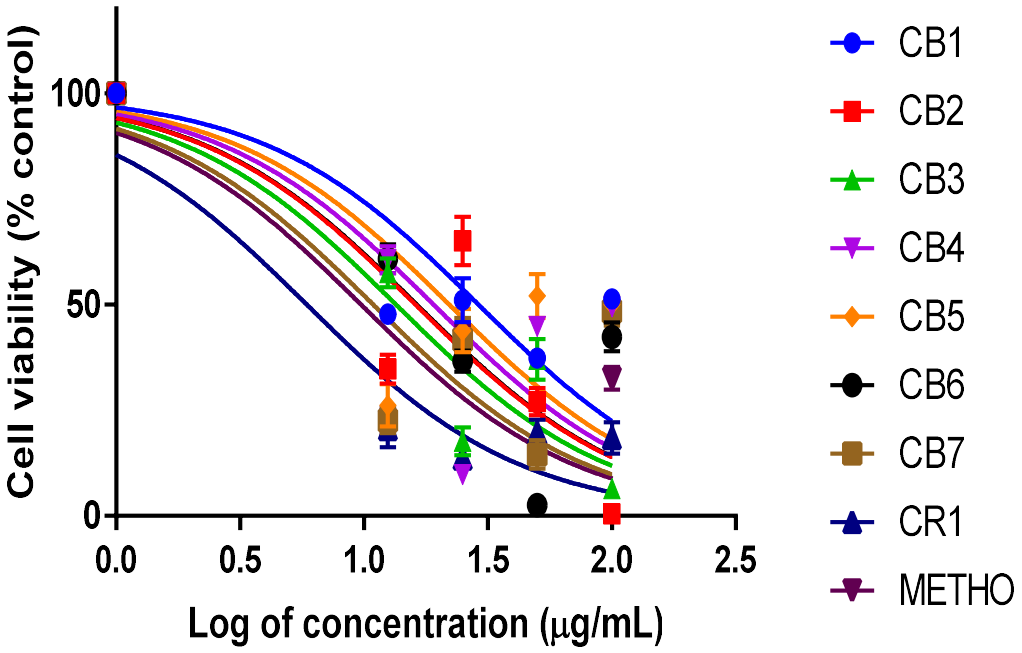


Figure 4.20. Sigmoidal dose-response curve of isolated compounds with methotrexate (reference drug) againstA549adenocarcinoma cells. Absorbance values are in triplicate and the data were analysed at $p<0.05$. Anti-log of concentration at 50% viability was taken as half-maximal inhibitory concentration (IC_{50}) for each of the compounds

Table 4.20.Cytotoxic effects of isolated compounds against A549cellline

A549[a]			
Compounds	**IC_{50} (µg/mL)** [b]	**95% CI**	**R^2**
CB1	28.97±0.09	16.45 to 50.63	0.4333
CB2	16.16±0.10	9.144 to 26.98	0.7631
CB3	13.46±0.07	9.115 to 19.35	0.8746
CB4	19.1±0.14	8.516 to 40.89	0.3903
CB5	22.09±0.14	8.203 to 55.37	0.02751
CB6	16.33±0.10	9.519 to 26.92	0.7322
CB7	10.9±0.14	4.483 to 23.33	0.4872
CR1	5.878±0.09	3.727 to 8.917	0.8806
METHO	9.734±0.15	5.106 to 17.14	0.7163

Key: [a]A549;Human lung adenocarcinoma cell, [b]MTT assay; (means ± SEM, n= 3), CB1; Methyl gallate, CB2; Benthamianoate, CB3; Benthamiacone, CB4; unidentified, CB5;2-methoxyacrylic acid, CB6; Benthamianin, CB7; Benthamianol, CR1; Racemonoate, METHO; Methotrexate (reference drug), R^2; Goodness of fit

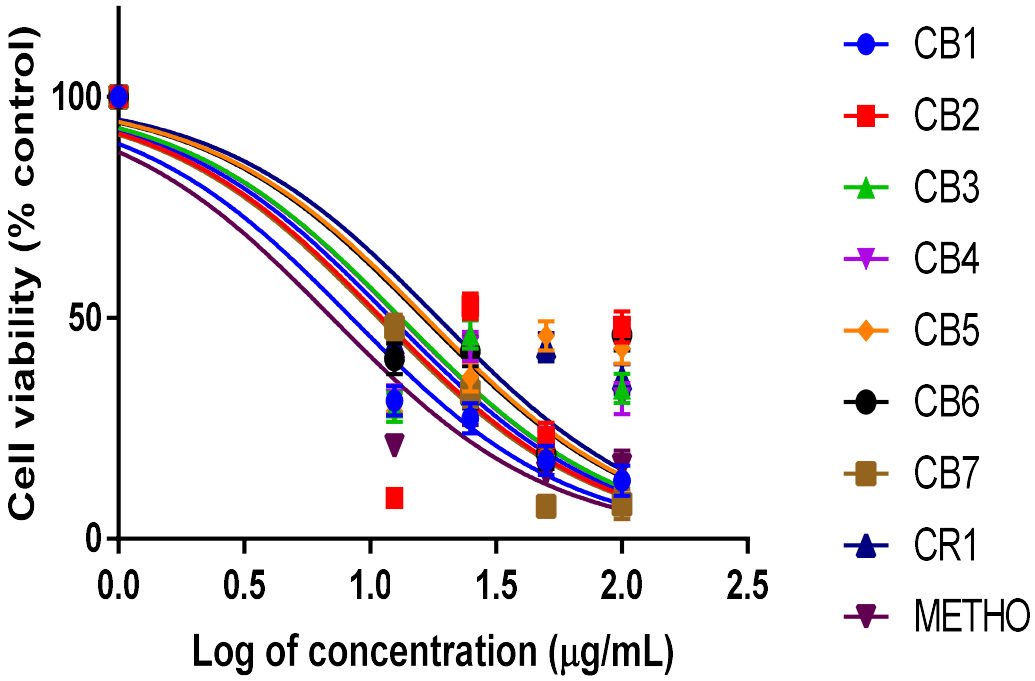


Figure 4.21 Sigmoidal dose-response curve of isolated compounds with methotrexate (reference drug) againstPC3adenocarcinoma cells. Absorbance values are in triplicate and the data were analysed at $p<0.05$. Anti-log of concentration at 50% viability was taken as half-maximal inhibitory concentration (IC_{50}) for each of the compounds

Table 4.21.Cytotoxic effects of isolated compounds against PC3 cell line

PC3[a]			
Compounds	**IC_{50} (µg/mL)** [b]	**95% CI**	**R^2**
CB1	8.44±0.05	6.429 to 10.9	0.9471
CB2	11.13±0.18	3.432 to 29.69	0.23883
CB3	13.23±0.10	7.426 to 23.3	0.7018
CB4	13.13±0.09	7.858 to 20.97	0.7533
CB5	16.71±0.13	8.994 to 23.32	0.6185
CB6	10.78±0.10	8.645 to 13.3	0.9627
CB7	10.29±0.04	8.237 to 12.65	0.8826
CR1	18.59±0.09	11.06 to 30.36	0.6509
METHO	7.054±0.08	4.661 to 10.3	0.8945

Key: [a]PC3;Human prostate adenocarcinoma cell, [b]MTT assay; (means ± SEM, n= 3), CB1; Methyl gallate, CB2; Benthamianoate, CB3; Benthamiacone, CB4;unidentified, CB5;2-methoxyacrylic acid, CB6; Benthamianin, CB7; Benthamianol, CR1; Racemonoate, METHO; Methotrexate (reference drug), R^2; Goodness of fit

4.7.Statistical analysis on cytotoxicity of isolatedcompounds compared with the reference drug

In addition to the sigmoidal dose-response curve analysis, the statistical difference in activities of the isolated compounds compared with the standard reference, methotrexate was assessed by ANOVA with dunnett's multiple comparison tests conducted at$\alpha_{0.05}$(Appendices 14 -16). Differences in the activity at $\alpha_{0.05}$were considered statistically significant. The analysis revealed that there was no statistically significant difference in cytotoxicity of the isolated compounds and methotrexate on MCF-7 cells (p=0.3774). Dunnett's multiple comparison tests on MCF-7 cells also comfirmed that there was nostatistically significant difference in cytotoxicity of each of the isolated compounds and methotrexate (P>0.05) (Appendices 14a-14b). The cytotoxicity of isolated compounds and methotrexate against A549 cells showed no statistically significant difference (p=0.4528), it was also observed with Dunnett's multiple comparison tests on A549 cells that there was nostatistically significant difference in cytotoxicity of each of the isolated compounds and methotrexate (P>0.05)(Appendices 14c-14d). On the PC3 cells also, there was no statistically significant difference in the cytotoxicity of the isolated compounds and methotrexate (p=0.2752), Dunnett's multiple comparison tests on PC3 cells also showed that there was nostatistically significant difference in cytotoxicity of each of the isolated compounds and methotrexate (P>0.05) (Appendices 14e-14f).

CHAPTER FIVE
DISCUSSION AND CONCLUSION

5.1.Discussion

5.1.1.Ethnobotanical survey

Ethnobotanical survey carried out in Gbonyin, Moba and Efon Local Government Areas (LGAs) of Ekiti State, Southwestern Nigeria in this study, has helped in documentation of indigenous knowledgeand identification of medicinal plants used amongst the TMPs (respondents)in the Statefor the management of different types of cancer disease. The choice of the LGAs used in this study was based on the prevalence of cancer incidence and death statictics in the State as reported by previous workers (Awololaet *al*., 2011; Babatundee*t al*., 2013). In addition, each of the three LGAs selected for this study represented each of the three geo-political zones:Gbonyin (Ekiti South), Moba (Ekiti North) and Efon (Ekiti Central). This was based on an attempt to have anoverview of indigenous knowledge of cancer management by the TMPs found in the state. Hence, the survey revealed that the indigenous knowledge of the use of medicinal plants for cancer managementamong the respondents in the selected LGAs was thesame.

The demographic results from the survey indicated 38 respondents made up of herbalist (47.4%), herb seller (15.8%), diviners (23.7%), traditional birth attendants (5.3%)and others (7.8%). Sixty-eight percent (68%) of the respondents were male; this may be because of the cultural opinion of the people, that males are the successors of the family inheritance, thereby, in traditional medicinepractice; the knowledge of plantuse is transferred from generation to generation to themale child. In addition, the rigors inthe preparation of medicinal plants in traditional medicine have restricted the trainee to be mainly males(Tesfaye*et al*., 2009). More so, various forms of dangers associated with the collection of plants from the wild have made the practice lessattractive to the female counterpart (Tesfaye*et al*., 2009).

Christianity (58%) and traditional African religion (32%) were the predominant religions of the respondents, while only a very small percentage of the people were Islamic worshipers Sixty-one percent (61%) of the respondents were above 50 year of age; this clearly shows that the elderly are the main repository of indigenous knowledge about the local use of plants than any other age population (Teklehaymanot, 2017). The educational status of the respondents was very low. However, the percentage that had basic education (primary) (47%) is incomparable with just (8%) that had no formal education. Larger percentage (40%) of the respondents claimed to have been practising for more than 40 years, with majority (47%) inheriting the practice from their grandparents and parents, but few of them learned through informal training and dream. Similar findings on the traditional practitioner's demographic data such as age, educational level and source of the traditional knowledge have been reported by (Adebo and Alfred, 2011). Majority (63%) of the respondents interviewed were operating from their homes while 21%had offices/clinics and another 18% were consulting at the market place. Only nine (9) of the respondents had their practice registered with corporate affairs commission, while some were affiliated to National association of traditional medicine practitioners in Ekiti State.This may not be unconnected with the failure of the Federal Government of Nigeria to incorporate herbal medicine to the Nation's health care delivery system.

Respondents were found to have knowledge about cancer disease and its treatment as they are able to recognise cancer as '*aisan jejere*' in Ekiti dialect. They understand that cancer has the ability to spread from its original location and migrate to other parts of the body. They indicated that cancer can affect all parts of the body and cancers of the breast, skin, and prostate were the specific types mentioned. The respondents recognised some of the symptoms of cancer such as general weakness of the body, weight loss and severe pain of the affected region. Their ability to identify the disease is very important otherwise, information provided on the treatment can be misleading (Ngulde *et al*, 2015). Fifty percent (50%) of the respondents specified that spiritual attack is the main cause of cancer, while superstitious beliefs such as committing abominable act in the family was statedby (11%) of the respondents. Meanwhile, thirty percent (32%) of the respondents indicated diet like consumption of canned food as the cause of cancer and seven percent (7%) cited hereditary as the factor responsible for

cancer development. Similar observation has been reported by Segun *et al*. (2018)among the Ijebus in Ogun State.

The respondents diagnose cancer by the manifestation of lumps or thickening on breast (42%), swollen and hard growth on the skin (solid tumours) (24%), and spiritual divinations such as consultation of the '*Ifa*' oracle in some cases (24%). The respondents also stated that many of the cancer sufferers may have been confirmed with the disease in the hospital first (21%) but, because of insufficient fund, decided to undergo a traditional healer treatment, as it is less expensive. Ashidi *et al*. (2010) reported a similar case of this nature. Majority of the respondents (47%) said they use herbs only for their preparations, 21% said they use divination only, 16% said they use both herbs and incantation, 11% said they use incantation only, while only 2% admitted the use of other materials including animal parts alongside with herbs. Some other materials reportly used by the respondents include snail (*Archatina archantina*), honey, palm cannel oil ('adin agon') local gin, cow bile (*Bos taurus*), and minerals like alum and potash.

Documented plant parts used by the respondents in their herbal formulations for cancer treatment, as observed in this study consist of majorly leaves (33%), followed by the stem bark (26%) and root (13%).Similar studies in Nigeria, (Abubakar *et al*., 2007; Sonibare *et al*., 2009; Ogbole and Ajaiyeoba, 2010; Segun*et al*., 2018) have also reported similar observations in Ogun State. This was supported by Bhat *et al*. (2013), who reported that in traditional medicines for cancer therapy in some cultures such as India and Kenya, the plant part most exploited are the leaves. This may not be unconnected with their easy process of harvesting as well as their less effect on plant conservation. In addition, respondents indicated their preference for leaves in ethnomedicinal formulations due to its collection having the least threat on plant species life cycle and its all-round the year relative availability (Moshi *et al*., 2012). Leaves and barks appeared as medicinally active agents/compoundsrepository, thus, they are given more preference in herbal recipes formulation(Adnan *et al*., 2014). Roots on the other hand are the part of the plants that are mostly rich in secondary metabolites, and are commonly used by respondents (Adnan *et al*., 2014). Although, root anchors the plant to the soil, excessive harvesting may results into the death of the plant, therefore caution must be taken when harvesting (Verma *et al*., 2007).

Thirty-one (31) different herbal recipes, majorly multicomponent in nature were indicated by the respondents of the selected local government areas of Ekiti State for the treatment of cancer. Decoction (38%) was the most dominant formulation method, which can be attributed to the fact that water is easily accessible to use in herbal formulation and its extraction is exhaustive. In addition, the majority (90%) of the respondents admitted no side effect for recipes prepared with water. While other preparations such as tincture (19%), powder (13%), soup (7%), sharing (7%), paste (7%) soap (3%), infusion (3%) and ointment (3%) were also used (Augustine and Alex, 2017). Majority of the recipes were found to be polyherbal, and the respondents prescribe the polyherbal to patients as they claimed and believed that it usually exhibited increased efficacy as against a single plant preparation. This can be explained by the buffering and synergy principles offered by the different herbs when combined to make herbal recipe (Pal and Shukla, 2003). This was also supported by Chen *et al*. (2014), who reported that in Chinese traditional medicinal system, for ages, the use of polyherbal therapies is a common practice.The respondents also claimed that the use of other materials such as 'iya ose', 'obu otoyo', 'adin eyan', 'ako okuta', 'ose dudu' and honey in some herbal recipes made for the treatment of cancer, in some cases served as preservatives as well as additives.

Respondents claimed that the plants were either collected fresh from the forest (where available) or purchased from herb markets. The tree which accounted for 38% of the life form was mostly collected for used by the respondents in their anticancer recipes preparations. In most cases, the herbal preparations are usually administered orally while in some, it can be applied topically and the most commonly mentioned quantities and frequency of administration were teacup (approximately 250 mL) taken for two or three times daily, shot glass (approximately 60 mL) taken twice daily, table spoon (15 mL) taken twice daily and teaspoon (5 mL) taken once or twice daily. Similar findings have been documented by Keter and Mutiso, (2012). The need for treatment for up to 6 months and above, between 3-6 months, between 1-3 months and below one month as the case may be is usually recommended to the patients by some of the respondents.

The respondents, however, indicated refrigeration of recipes as their main method of preservation, especially water-based prepared recipes, while others stated that regular warrning (every 3 days) of the recipe helps in the preservation. The respondents were generally aware of possible toxicity from phytomedicinesand majority (90%) of the

respondents said no “occurrence of side effects” after patient’s use of their herbal preparations. However, some of the respondents said some patients may experience nausea, feel drowsy or even sleep for a prolonged period of time after taking their recipe due to the presence of alcohol in some of the recipes. Some of them reported advising their patients against the use of their herbal medications with orthodox medicines to avoid drug interaction as well as complications(Keter and Mutiso, 2012).

The recipes comprised of fifty-seven (57) different plants species from 38 families, belonging to 53 genera, with Ephorbiaceae (14%), Leguminosae (7%) and Anonnaceae (5%), as the frequently cited of all. The use mention index (UMI) analysis of the plants recorded in the study revealed thatthe most cited plant species was *A. muricata*(0.184). Other anticancer plants well-mentioned include*S. latifolius* (0.131);*C portoricensis* (0.10), *C. racemosum*(0.10)and *X. aethiopica*(0.10). Ethnobotanical surveys regarding cancer disease management had been conducted across different parts of Nigeria by previous workers. A similar work carried out by Soladoye *et al*. (2010) in Ogun State, Southwestern region of Nigeria revealed seventy-three plants used in various cancer disease treatment. This study also reported 19 of such plants. A recent study conducted by Segun *et al*. (2018) in Ogun State, Southwestern Nigeria also reported90 medicinal plants used by Ijebus in the management of various cancers; 31 of the plants were also reported in this study.In addition, Abubakar *et al*. (2007), in their investigation of medicinal plants used by the Hausa ethnic group people of Northwestern Nigeria in the management of various cancers, reported 72 plants; this study reported 6 of those plants. More so, in another study conducted by Ngulde *et al*. (2015) in North Eastern part of Nigeria, Askira/Uba local government area, 65 different plants were reportedly used in various cancer types management. This study also reported 4 of the plants.

Differences in the vegetation type as well as the different cultural practices between Southern and Northern parts of Nigeria may explain the wide variation observed in plant species similarities. However, *E. lateriflora, S. longipedunculata, P. guineense and X. aethiopica* were medicinal plants common amongst the studies conducted across the country’s different regions (Abubakar *et al*., 2007; Soladoye *et al*., 2010; Ngulde *et al*., 2015;Segun *et al.*, 2018). Some of the plants implicated in this study have also been reportedly used in folklore medicine of other cultures for the treatment of cancer. Such species include; *A. occidentale* in India (Jain and Sharma, 2013), *B.diffusa* in India and South America (Mumtaz *et al*., 2015), *B. micrantha* in Kenya

(Ochwangi *et al*., 2014), *C. maxima* in Iran (Tonekaboni, 2011) and *A. ringens* in Algeria (Benarba and Meddah, 2014). The use of the same plants species in different cultures in the treatment of cancer may strongly suggest their effectiveness against cancer. It is important to mention that some of the plants species implicated in this survey are becoming threatened.

Screening of the selected plants obtained from ethnobotanical survey for the presence of phytochemicals such as alkaloids, tannins, flavonoids, saponins, steroids and cardiac glycosides revealed that all the nine plants contained chemicals in different amount and proportions, with the two major plants (*C. benthamiana* and *C. racemosum*) having almost all the phytochemicals. The result of phytochemical analysis supported the work of Onocha *et al.* (2005) who reported the presence of flavonoids, alkaloids, steroids, tannins, saponins, terpenes and cardiac glycosides in the extracts of *C. racemosum*. However, Borokiniand Omotayo (2012) reported the absence of steroids in *C. racemosum*. Also, Mbagwu and Adeyemi (2008) and Fayemi and Oso (2012) reported the presence of saponins in *C. benthamiana*whichis absent in this study.These compounds contained therein are substances responsible for the therapeutic effects observed in the plants (Kienle *et al*., 2011).

The presence of alkaloids in the root and leaf extracts of plants indicates their usefulness in alleviating some symptoms associated with pains (Egunyomi *et al*., 2009). Saponins are for haemolytic activities and are also expectorants, and cough suppressants (Okwu, 2005). Tannins are well known for their anti-inflammatory and diuretic potentials, anti-microbial and anti-oxidant properties, as well as skin regeneration and soothing relief (Okwu and Okwu, 2004). Egunyomi *et al.* (2009) reported that the presence of tannins in the root and leaf extracts of plants made them suitable in cleansing/bathing skin ulcers surface, which may have developed due to cancer disease. Flavonoids and its derivatives are secondary metabolites widely distributed with antiradical and antioxidant properties (Nakayoma and Yamada, 1995). The presences of flavonoids in the root and leaf extracts of plants are evidence that they may be useful in the treatment of constipation cases in cancer patients (Egunyomi *et al*., 2009). Cardiac glycoside exerts their effect on the muscles of the heart and promotes urine production (Manikandan *et al*., 2006). The presence of cardiac glycosides may indicate cardiac insufficiency; coughs and circulatory problems cure (Ejele *et al*., 2012). Steroids possess anti-inflammatory properties, protein metabolism

and regulate carbohydrate. In addition, terpenes or terpenoids have hepatoprotective properties, thus may help prevent liver damage due to inflammation caused by tumour cells (Borokini and Omotayo, 2012).

5.1.2. Cytotoxicity of extracts/ fractions of *C. benthamiana* and *C. racemosum*

MTT assay was used for the preliminary screening of the plant crude extracts. According to the criteria by America National Cancer Institute (NCI), for crude extracts to be considered active in a preliminary assay, its IC_{50}must be <30 µg/mL after 72 h of exposure time (Rajkapoor *et al.*, 2007). IC_{50}(the half maximal inhibitory concentration) is a measure of potency of a substance in inhibiting a specific biological or biochemical function.It is the concentration of an inhibitor (drug) where the response (or binding) is reduced by half (50%). The IC_{50} derived in this study was considered with its 95% confidence interval (CI) and goodness of fit (R^2)of the observed data for its inhibitory potential. Both CI and R^2 are known to be a function of the reliability of the IC_{50}generated from the set of data-fitting to a dose-response curve(Labelle*et al*., 2019). In other words, the closer the R^2 to 1, the more accurate the data obtained. Although, some of the data obtained in this study does not gave a perfect R^2. However, the calculated inhibitory concentration obtained from the sigmoidal dose-response curve clearly demonstrated the cytotoxic potentials of the extracts, fractions and isolated compounds investigated in this study as compared with the standard reference, methotrexate.

The crude extracts of *C. benthamiana* and *C. racemosum* were the twoplants extracts, out of the nine that most met this criterion, with the lowest IC_{50}values (the lower the IC_{50}, the higher the activity). Crude methanol extract of *C. racemosum* exhibited the highest cytotoxic effects with $IC_{50}$of 0.71±0.11 µg/mL against the human breast adenocarcinoma cell (MDA-MB-453) and 0.26±0.20 µg/mL against the normal human kidney cell (HEK-293), followed by *C. benthamiana* with $IC_{50}$of 3.21±0.09 µg/mL and 0.09±0.08 µg/mL against the human breast adenocarcinoma cell (MDA-MB-453) and normal human kidney cell (HEK-293), respectively.While the reference drug, methotrexate had $IC_{50}$of 0.94±0.30 µg/mL and 0.02±0.13 µg/mL against the human breast adenocarcinoma cell (MDA-MB-453) and normal human kidney cell (HEK-293), respectively. Although, the lower the inhibitory concentration (IC_{50}), the higher the activity. However, the slightly higher IC_{50} observed with the *C. benthamiana*

(IC_{50}= 0.09±0.08) and *C. racemosum* (IC_{50}= 0.26±0.20) against the normal kidney cell compared to the standard (IC_{50}= 0.02±0.13)might indicate their selective ability on tested cancer cell over the reference drug, methotrexate.The two plants were investigated further for cytotoxic potentials in this study based on their activity displayed in the preliminary screening using three human cancer cell lines as against the two human cell lines used in the preliminary screening.Although, little or no report of the cytotoxicity of the methanol extracts of *C. benthamiana* and *C. racemosum* has been reported in literature, however, the cytotoxicity of the methanolic extracts of the roots and leaves of a species of *Combretum*, *Combretum molle*R.Br. ex G.Don (Combretaceae) has been reported to show strong cytotoxic effects against T-24 bladder cancer cells (Fyhrquist *et al*., 2006).

The cytotoxic activity of the leaf fractions of *C. benthamiana*and root bark fraction of*C. racemosum* wereinvestigated, using the MTT assay against human breast adenocarcinoma (MCF-7), human prostate adenocarcinoma (PC3) and human lung adenocarcinoma (A549) cell lines.The choice of use of these three carcinoma cells in this study was basedon their high occurrence and the need to develop safe and effective medication for them. More importantly, they are the most mentioned cancer cell in ethnobotanical survey. In addition, they are the commonest cause of human death among different types of cancer disease(Hannah and Max, 2018). Breast cancer for example, is one of the commonest form of cancer in females, while the prostate cancer on the other hand, represents cancer type most commonly diagnosed in males epecially in Nigeria(Popoola *et al*., 2013). More so, lung cancer is the commonest cause of cancer mortality wordwide every year. For instance in 2012, lung cancer accounted for 1.7 million deaths (18.2%) of the total cancer mortality (Torre *et al*., 2015), similarly, cancer of the lung is the leading cancer death in 2018, accounted for 1.8 million deaths (18.4%) of the total (WHO, 2018).

Further purification of the constituentsassumed to have accounted for the cytotoxic effect observed with the*C. benthamiana*plant extract, through fractionation showedthat out of the three fractions (hexane, dichloromethane and aqueous) tested, thedichloromethane(DCM) fraction of *C. benthamiana*exhibited the highest cytotoxic activity with greatest effect on the lung carcinoma cells (IC_{50} = 8.45±0.11µg/mL) and the lowest effect on the prostate carcinoma cells (IC_{50} = 27.90±0.04µg/mL), while the least activity was observed with its hexane fraction with IC_{50} of 72.75±0.18µg/mL,

12.61±0.16µg/mL, and 31.07±0.07µg/mL on breast, lung and prostate cancer cells, respectively. The aqueous fraction of *C. benthamiana* was moderately active(IC_{50} ranges from11.28±0.08 to 21.17±0.07µg/mL) across the panel of cancer cell lines tested.

In the same way, further purification of the constituents assumed to have accounted for the cytotoxic effect observed with the *C. racemosum*plant extractthrough fractionation, indicated that dichloromethane (DCM)fraction was found to displaythe highest cytotoxic activity amongst the fractions (hexane, DCM and aqueous) tested.The DCM fraction had greatest effect on the lung carcinoma cells (IC_{50} = 9.76±0.09µg/mL) and exhibited lowest activity against prostate carcinoma cells (IC_{50} = 30.47±0.03µg/mL), while the least activity was observed with the aqueous fraction of the plant with IC_{50} of 51.35±0.08µg/mL, 89.10±0.08 µg/mL and 25.49±0.02µg/mL on breast, lung and prostate cancer cells respectively. However, moderate to low cytotoxic activity (IC_{50} ranges from17.67±0.08 to 72.75±0.03 µg/mL) was observed with the hexane fraction of *C racemosum* across the panel of cancer cell lines tested.The cytotoxic activity observed with the fractions of the two plants investigated in this study could not be supported with reported informations, as there was no report on their cytotoxicity in literature. Hence, further investigation was conducted on the DCM fractions of *C benthamiana* and *C. Combretum* in this study. The cytotoxicity of the fractions in this study was higher than the cytotoxicity of two fractions (fraction A and fraction B) obtained from the ethanol extract of a species in this family, *Eriosema robustum*Baker(Leguminasae),investigated againstmonkey kidney Vero cellswith IC_{50}values of 17.91and93.76 µg/mL, respectively (Awouafack*et al*., 2013).

Further establishment of the cytotoxic activity of the dichlorometane fractionsof *C benthamiana* (CBD)and *C. Combretum* (CRD),evaluated at 50µg/mL and 100 µg/mL for 24 h againstthe threehuman adenocarcinoma cells (MCF-7, A549 and PC3), using cyquant® direct assay as described by Abraham *et al*. (2008), indicated a significant reduction inthe viability of the carcinoma cells. The assay principle was based on the ability of its binding dye to permeate the DNA of the cells, in blend with a background suppression reagent. The dye is irresponsive to dead cells as well as cells with compromised cell membranes, thereby causing only healthy cells to be stained. The photomicrograph acquired with a standard FITC filter set microscope at x100 magnificationfrom at least five different views per sample after the experiment, showed that there was a significantreduction in the viability of the carcinoma cells

(green colour) and a progressive loss of cell membrane integrity across the panels of tested cell lines,and in some cases, atotal rupture of the cell membrane in some of the cell lines was observed as compared to the untreated group (DMSO only),where a greater number of viable carcinoma cells were living evident. Theresults signify the visualisation importance of this dye in evaluation of antiproliferative and cytotoxicity of a test drug on cancer cellsin cell studies (Quent*et al*., 2010). Its measurement was independent of the metabolic state of the cells and not normally affected by factors which can influence metabolic activity of the cells(Quent*et al*., 2010).

The caspase assay, performed as described by Cobanoglu *et al*. (2016),which helps to indicate/analyse the rate at which the test fractions induced apoptosis (cell death) in the treated cells revealed that a significant number of dead carcinoma cells,were recorded in this study after the experiment. The assay reagent contains a nucleic acid binding dye, which after activation in apoptotic cells, bind to DNA, and produces a bright, fluorogenic response. The dichloromethane fractions of *C. benthamiana*and *C. racemosum*evaluated at 50µg/mL and 100 µg/mL for 24 h againstthree human adenocarcinoma cells (MCF-7, A549 and PC3), using caspase-3/7 green detection showed significant number of dead carcinoma cells. It was shown that the photomicrographs obtained, with a standard FITC filter set microscope at x100 magnification,from at least five different views per sample after the experiment,that there were significantlybright, fluorogenic green colour cells (dead cells) in the treated group across the panel of the carcinoma cells,compared tothe untreated group (DMSO only),where very few green colour cells (dead cells) were seen.This shows the significance of the reagent in analysis of apoptotic cells in cytotoxicity studies, as it is essential in visualization of event of cell deathfrom induced apoptosis(Payne*et al*., 2013).The caspase 3/7 detection reagent assay was however used by Huang*et al.* (2011)to monitorpardaxin induced apoptotosis in HT-1080cancer cells,the results indicated caspase was a key regulators in pardaxin-induced apoptosis in the cell, suggesting that pardaxin-induced apoptosis is dependent on caspase activity.Unlike this study, where the reagent was used to visualise the apoptic cells. The cells apoptosis induction, leading to the significant increase in the number of death cells observed with the caspase 3/7 assay in this study is in line with the activity observed in cell viability experiments.

5.1.3.Characterisation of isolated compounds

Compound CB1 (900 mg) isolated as a white/off-white crystalline powder was identified as methyl 3, 4, 5-trihydroxylbenzoate (methyl gallate), with a melting point of 201°C (Lit m.pt 198–200°C).APCI-mass spectrometry (APCI-MS) exhibited the molecular ion peak at m/z 184.9 9 (M^++H) compatible with a molecular formula $C_8H_8O_5$.The ^{1}H NMR and ^{13}CNMR spectraof compound CB1 revealed the presence of an aromatic system exhibiting signals due to the presence of a pyrogallol ring at 6.94, (2H, s) which was assigned to the chemically equivalent protons H-2 /H-6 placed on carbons C-2 / C-6 (δc 109.06) in the ring. The presence of a methoxyl group at position C-7 was confirmed by unambiguous HMBC correlation of a singlet OCH_3 proton at 3.75 Hz (δc 52.15) to the carbonyl group, C- 7 (δc 166.78) (Appendix 2e). The ^{13}C NMR spectrum revealed the presence of three phenolic groups attached to C-3, C-4 and C-5. The signal of chemically equivalent C-3 and C-5 appeared more downfield at (δ 146.0) compared with phenolic carbon at C-4 (δ 138.67) due to the combination of inductive and anisotropic effects of the ring on the carbon atom. Based on the above evidence, **CB1** was concluded to be methyl gallate which was in agreement with the reported literature values (Sanchez *et al*., 2013.).

Compound CB2 (130 mg) isolated as a viscous/oily yellowish brown sample has an exact mass of 320.09 corresponding to a molecular formula $C_{16}H_{16}O_7$. Its ^{1}H NMR and ^{13}C NMR spectra data revealed that it is a benzoate derivative. Total assignment of this molecule was possible on studying its HMQC and HMBC data (Appendix 3e). The ^{1}H NMR spectra showed five aromatic protons; two with a chemical shift at 6.99 2H, (s) which was assigned to the chemically equivalent protons H-2 /H-6 placed on carbons C-2 / C-6 (δC 109.20) in ring (B), the other three at δ 7.07 (d, 5.75 Hz, H-4′); 7.03 (s, H-5′); and 7.09 (d, 5.75 Hz, H-6′) was found on carbons C-4′, C-5′ and C-6′ (δC 118.8, 116.10 and 114.0), respectively in ring A.The ^{1}H NMR spectrum further showed the presence of a non aromatic oxygenated methylene at δ_H 3.81 (δc 71,97) attached to C-3′) of ring A, a methyl group at δ_H 1.27 (3H, d) (δc 21.74) attached to a non aromatic oxygenated methine at δ_H 4.36 - 4.39attached to C-1a (δc 73.10)(Appendix 3b).

The ^{13}C NMR spectrum showed the carbonyl carbon at δC 167.96 attached to C-1′ (δc 119.50) of ring A. In the ^{13}C NMR, the six signals (δc 119.50, 151.0, 121.0, 118.80, 116.10 and 114.0)were attributable to the carbons 1′, 2′, 3′, 4′, 5′ and 6′ of ring A, while a chemical shift at δc 131.6 was assigned to C-1, δc 109.20 assigned to the

chemically equivalent carbons (C-2 / C-6), δc 146.10 assigned to another chemically equivalent carbons (C-3 / C-5) and δc 138.40 assigned to C-4 of ring B.The Proton/Carbon Correlations- HSQC spectrum gave clear correlations of the carbon atoms with the protons directly attached to them.In the HMBC spectrum, the position of the methyl group at C-1b was confirmed by the correlation of the methyl protons at 1.27 ppm with carbon C-1a (δc 73.10) and C-1′a (δc 167.96) attributed to the carbonyl group. The chemical shift signal δ_H 7.07 (H-4′) was similarly situated with observed correlations of the aromatic proton with oxygenated methylene signal at C-3′a (δc 71.97) and C-5′ (δc 116.10), C-6′ (δc 114.0). In addition, H-2 and H-6 (δc 109.20) showed HMBC correlations with carbons C-1, C-3, C-4, C-5 and C-1a (δc 131.60, 146.10, 138.40 and 73.10). These assignments were clearly confirmed by COSY, HSQC, HMBC and DEPT experiments (Appendix 3di-3e). On the basis of this information, the compound was identified as 1-(3, 4, 5-trihydroxyphenyl)ethyl 2-hydroxy-3-(hydroxymethyl)benzoate (benthamianoate)**(CB2).**

Compound CB3 (200 mg) isolated as brownish black viscous substance was identified as 3-amino-4-hydroxy-3-methyldihydrofuran-2-one,with an accurate mass of 132.0, while the calculated mass was 131.13 corresponding to a molecular formula $C_5H_9NO_3$.The mass spectrum showed no diagnostic fragmentation and this could be due to the method of ionization utilized in the analysis which is APCI-MS. The molecular ion peak obtained from the MS revealed a mass of 132.9 (M^++H). TheDEPT spectrum (Appendix 4c)indicated that there were 5 carbon atoms present. The signal identified as the carbonyl group and the most desheilded carbon atom (δ 178.60) is assignable to C-2. TheDEPT spectrum also revealed the presence of a non-aromatic oxygenated methylene at δc 62.91 assignable to C-5, a non-aromatic oxygenated methine carbon at δc 73.17 assignable to C-4, a non-aromatic quaternary carbon at δc 72.03 (C-3) which is attached to a primary amine and a methyl group at δc 21.74assignable to C-3a as confirmed by the HSQC and HMBC spectra (Appendix 4d and 4e), respectively.

The ^{1}H NMR spectrum of CB3 showed three non-aromatic protons environment viz: a methyl group at δ_H 1.25 attached to C-3; an aliphatic oxygenated methine proton at δ_H4.01 attached to C-4 and an aliphatic oxygenated methylene proton at δ_H 4.37 attached to C-5. The HSQC spectrum gave correlations of the carbon atoms with the protons directly attached to them. In the HMBC spectrum, the position of the methyl

group at 3a was confirmed by the correlation of the methyl protons at 1.25 ppm with carbon C-3 (δc 72.03); C-4 (δc 73.17); and C-2 (δc 178.10). These assignments were clearly confirmed by COSY, HSQC, HMBC and DEPT experiments (Appendix 4c-4e). On the basis of this information, the compound was identified as 3-amino-4-hydroxy-3-methyldihydrofuran-2-one(benthamiacone) (**CB3**).

Compound CB5 (180 mg) isolated as off white oily crystals, was identified as 2-methoxyacrylic acid, with an accurate mass of 102.0, while the calculated mass was 102.09 which corresponding to a molecular formula $C_4H_6O_3$.The ^{13}CNMR spectrum indicated 4 carbon signals viz: two (2) quaternary carbon signals resonating at δc 168.27 and δc 145.18 assignable to C-1 and C-2 respectively; one (1) methoxy carbon with a chemical shift at δc 50.10; and one (1) sp2 methylene carbon signal at δc 108.86. The ^{1}HNMR spectrum (500 MHz, CD_3OD) revealed the presence of a singlet methoxy proton at δ_H 3.89 and a geminal proton signals at δ_H5.50-5.52 (m) located on carbon C-3 (δc 108.86) as confirmed by the DEPT, and COSY spectra (Appendix 6d and 6e). The presence of the carbonyl, methylene, methyl and hydroxyl groups was further confirmed by the IR spectrum with signal stretch at 1691 cm^{-1}2850cm^{-1}, 2951$cm^{-1}$and 3087 cm^{-1}, respectively. On the basis of these spectra data, the compound was identified as 2-methoxyacrylic acid **(CB5)**.

Compound CB6 (20 mg) isolated as yellowish brown crystals was identified as 1-aminohexane-1-ol (benthamianin), with an accurate mass of 117.12, while the calculated mass was 117.19corresponding to a molecular formula $C_6H_{15}NO$. The ^{13}CNMR and DEPT spectra showed 6 carbon signals composed of a methyl, CH_3 (1) at C-6 (δc 14.22); four non-aromatic methylene CH_2 (4) at C-5 (δc 22.71), C-4 (δc 29.71), C-3 (δc 29.37) and C-2 (δc 31.94); non-aromatic oxygenated methine,HC-OH (1) at position C-1 (δc 76.71) attached to a primary amine group.The observed ^{1}H - ^{1}H COSY indicated the correlations of H-1 to CH_2 of H-2, H-3; H-2 to H-3, H-4 and H-5; H-3 to H-4, H-5 and H-6; H-4 to H-5 and H-6; H-5 to H-6; H-6 cross peak H-5; H-5 cross peak H-4); H-4 cross peak H-3); H-3 cross peak H-2); and H-2 cross peak H-1. These assignments were further confirmed by the IR signals at 3362, 2923, 2853,1512 and 1443, 908 and 729 cm-^{1} which are characteristics of O-H,CH_3, CH_2, C-N and C-H, respectively (Appendix 7a). ^{1}HNMR spectrum showed a strong downfield signal at δ 3.50 (m), which could be attributed to the electronegativity environment.

Other signal from the ^{1}HNMR spectrum are δ 1.62 (m), δ 1.27 (m) δ 1.27 (m), δ 1.27 (m), and δ 0.89 (m).

Compound CB7 (10 mg) isolated as greenish brown oily paste substance has a mass (m/z: 416.29) corresponding to a molecular formula $C_{26}H_{40}O_4$.The^{1}H NMR and ^{13}C NMR spectra showed one olefinic proton as a singlet at δ_H 5.80 (δc 115.8) assignable to C-3′, a non-aromatic oxygenated methine, δH 3.19 (δc 75.1) assignable to C-3, doubly oxygenated carbon (hemiketal) assignable to C-5′ (δc 108.0), a quaternary carbonyl at δc 171.4 assignable to C-2′.The ^{13}CNMR and DEPT NMR spectra showed 26 carbon signals composed of CH_3 (5), CH_2 (7), non-olefinic CH (7), non-aromatic oxygenated methine, CHOH (1), olefinic CH (1) and quaternary carbons (5).

The HMBC spectrum also showed the diagnostic connectivity of the olefinic proton at δ_H 5.80 (H-3′) to C-2′(δc 171.4), C-4′ (δc 170.1), C-5′ (δc 108.0), C-17 (δc 49.7). Crucial is also the HMBC connectivity of H-3 (δ_H 3.19) to C-2 (δc 23.7), C-1 (δc 31.9), C-4 (δc 30.0) and C-5 (δc 44.5). The presence of the CH_3, CH_2, C=O, C-OH, C-O-Cand C-H were further confirmed in the IR spectrum with absorption at V_{max}2916.6 (CH_3), 2849.0 (CH_2), 1734.2(C=O), 1437.3 (CH-OH), 1373.8 and 1251.7 (C-O-C)913.0, 881.9 and 757.6 (C-H) cm-^{1}. The identity of **CB7** as 4-(hexadecahydro-3-hydroxy-6,10,13,15-tetramethyl-1*H*-cyclopenta[*a*]phenanthren-17-yl)-5-hydroxy-5-methylfuran-2(5*H*)-one (benthamianol) was confirmed by the DEPT, HSQC and HMBC spectra (Appendix 8d-8g) which are here reported isolated from the plant for the first time.

Compound CR1 (70 mg) isolated as yellowish oily paste substance has a mass (m/z: 342.2) corresponding to a molecular formula $C_{20}H_{38}O_4$.Its ^{1}H NMR and ^{13}CNMR spectra showed two diagnostic olefinic protons as a doublet of doublet at δ_H 5.36 (δc 130.2) and δ_H 5.39 (δc 127.9) assignable to C-15 and C-16, respectively; analytical aliphatic oxygenated methine, as a multiplet at δH 4.14-4.18 (δc 68.9) assignable to C-3 and diagnostic aliphatic oxygenated methylene as a multiplets at δH 4.29-4.34 (δc 62.1) assignable to C-2a; an indicative aliphatic non-oxygenated methine as multiplet at δ_H2.31-2.35 (δc 34.2) assignable to C-2. The ^{13}CNMR spectrum further revealed an analytical quaternary carbonyl at δc 173.3 assignable to C-1 and a methoxyl proton as a singlet at δ_H 3.7 (δc 51.5) assignable to C-1a.

The ^{13}CNMR and DEPT NMR spectra showed 21 carbon signals composed of methyl,CH_3 (1), methoxyl, CH_3OH (1), aliphatic methylene, CH_2 (13), non-olefinic methine, CH (1), non-aromatic oxygenated methine, CHOH (1), aliphatic oxygenated methylene, CH_2OH (1), olefinic CH (2) and quaternary carbonyl carbon (1). The HMBC spectrum also showed the diagnostic connectivity of the olefinic proton at δ_H 5.36 (H-15) to C-16 (δc 127.9), C-14 (δc 31.9), C-13 (δc 29.8). Diagnostic is also the HMBC connectivity of H-2 (δ_H2.31-2.35) to C-1 (δc 173.3). Furthermore, the HMBC spectrum showed connectivity of H-3 at δ_H4.14-4.18 (m) to C-1 (δc 173.3) and C-2a (δc 62.1). In addition, the aliphatic oxygenated methylene protons at δ_H 4.29-4.34 (H-2a) cross peaks with C-1 (δc 173.3) and C-3 (δc 68.9). The singlet methoxyl proton at δ_H 3.7 (H-1a) equally cross peaks with C-1 (δc 173.3). The presence of the CH_3, CH_2, CH, C=O, C-OH, C-O-C and C-H were further confirmed in the IR spectrum with absorption at V_{max}2916.2 (CH_3), 2818.6 (CH_2), 2380.4 and 2342.0 (CH), 1733.3 and 1689.8 (C=O), 1461.6 (C-OH), 1367.1, 1313.3 and 1251.7 (C-O-C), 966.4 and 718.0 (C-H)cm-^{1}. The identity of **CR1** as methyl 3-hydroxy-2-(hydroxymethyl) nonadec-15-enoate (racemonoate) was confirmed by the DEPT, HSQC and HMBC spectra (Appendix 9d-9g) which are here reported isolated from the plant for the first time.

5.1.4. Cytotoxicity ofthe isolated compounds

The isolated compounds CB1 – CB7 and CR1 investigated against three human cancer cell lines (MCF-7, A549 and PC3), where methotrexate and DMSO vehiclewere used as the positive and negative controls, respectivelywere found to display significant cytotoxic activity across the panel of the cancer cells.The isolated compounds wereclassified as follows; phenolic acid (CB1), tannin (CB2), dihydrofuran (CB3), monocarbonxylic acid (CB5),amine (CB6), triterpenoid(CB7)and fatty acid (CR1).Amongst the isolated compounds,the dihydrofuran and triterpenoid displayed potent to moderate cytotoxicity with IC_{50} value ranging from13.23±0.10 to21.97±0.06 µg/mL (CB3) and 7.197±0.25 to 10.9±0.14 µg/mL (CB7) against the carcinoma cells investigated. The phenolic acid (CB1) (IC_{50} valvefrom 8.44±0.05 to28.97±0.09 µg/mL), tannin (CB2)(IC_{50} value from11.13±0.18 to26.44±0.12 µg/mL), monocarbonxylic acd(CB5)(IC_{50} value from16.71±0.13 to 31.37±0.70 µg/mL) andfatty acid (CR1)(IC_{50} value from5.878±0.09 to 18.59±0.09 µg/mL)were potent to moderately activeactivities against the carcinoma cells investigated, while the amine

(CB6) displayed moderate to low cytotoxic activity with IC_{50} value ranges from 10.78±0.10 to 63.87±0.17 µg/mL against the carcinoma cells investigated.

In this study,the cytotoxicity of the isolated compoundsagainst MCF-7 carcinoma cells revealed that benthamiacone (CB3), unidentified compound (CB4), 2-methoxyacrylic acid (CB5) and racemonoate (CR1) were the most active compounds in comparison to the cytotoxicity of the reference drug,methotrexate, Benthamiacone (CB3) was found to display the highest cytotoxic activity (IC_{50} = 21.97±0.06 µg/mL, R^2 = 0.83) on MCF-7 carcinoma cells. On A549 carcinoma cells however, benthamianoate (CB2), benthamiacone (CB3), benthamianine (CB6) and racemonoate (CR1) were found to have significant cytotoxic activity comparable to the cytotoxicity of the reference drug, methotrexate. Racemonoate (CR1) displayed the highest cytotoxic activity (IC_{50} = 5.878±0.09 µg/mL, R^2 = 0.88) against A549 carcinoma cells. Six of the isolated compounds including methyl gallate (CB1), benthamiacone (CB3), unidentifiedcompound (CB4), benthamianine (CB6), benthamianol (CB7) and racemonoate (CR1) were found todisplay significant cytotoxic activity against PC3 carcinoma cells in comparison to the cytotoxicity of methotrexate. The methyl gallate (CB1) showed the highest cytotoxic activity (IC_{50} =8.44±0.05 µg/mL, R^2 = 0.94) against PC3 carcinoma cells.

Although, all the isolated compoundsshowed significant cytotoxic activity, however, the dihydrofuran (CB3) (IC_{50} value = 21.97±0.06 µg/mL; R^2 = 0.83), 13.46±0.07 µg/mL; R^2 = 0.87 and13.23±0.10 µg/mL; R^2 = 0.70) and long chain fatty acid (CR1) (IC_{50} value = 7.197±0.25 µg/mL; R^2 = 0.83, 5.878±0.09 µg/mL; R^2 = 0.88 and 18.59±0.09 µg/mL; R^2 = 0.70) were the highest cytotoxic activity against breast, lung and prostate carcinoma cells investigated in this study, respectively. The cytotoxicity displayed by these two compounds are in comparison with the cytotoxicity of the reference drug, methotrexate (IC_{50} value = 8.261±0.08 µg/mL; R^2 = 0.86), 9.734±0.15 µg/mL; R^2 = 0.72) and 7.054±0.08 µg/mL; R^2 = 0.89) against breast, lung and prostate carcinoma cells investigated, respectively. The cytotoxic activity of the dihydrofuran, benthamiacone (CB3) may be attributed to the presence of the carbonyl, amino and hydroxyl functional groups located at position 2, 3 and 4, respectively in its chemical structure, respectively. Generally, terpenoids and their derivatives are known as one of the classes of compound with potent cytotoxic/antiproliferative activities (Rabi and Bishayee, 2009). On the other hand, the long chain fatty acid, racemonoate (CR1)

cytotoxic activity may be attributed to the presence of carbonyl and hydroxyl functional groups located at position 1, 2a and 3, respectively as well as the presence of *pi* bond coupled with a long carbon skeleton contained in its chemical structure. Phenolic compounds are generally knownas a class of compound that exhibit potent antiproliferative activity (Miranda *et al*., 1999).

To the best of the knowledge of available literature, this is the first report, of the isolation of these compounds from *C. benthamiana* as well as the first report of the cytotoxicity of the phenolic acid; methyl gallate (CB1), tannin; benthamianoate (CB2), dihydrofuran; benthamiacone (CB3), momncarbonxylic acid; 2-methoxyacrylic acid (CB5), amine; benthamianine (CB6)and triterpenoid; benthamianol (CB7) from *C. benthamiana*. The methyl gallate, a polyphenol compound found naturally in plant, has been reported to have potent antioxidant, anticancer, and anti-inflammatory activities (Sanchez *et al*., 2013).Reported polyphenol compounds with anticancer activity includes; catechin, apigenin, luteolin, kaempherol, myricetin, quercetin, rutin, orientin, vitexin, cyanidin, ellagic acid, resveratrol and curcumin (Batra and Sharma, 2013).

The cytotoxic activity of methyl gallate (CB1), observed in this work was high (IC_{50} value from 5.87 to 18.59 µg/mL)compared to the work of Kamathama *et al*., (2015), where the growth of A431 cells was significantly reduced by methyl gallate, with IC_{50} value of 11 mg/mL and 43 mg/mL at 24 h and 48 h, respectively in an *in vitro* cytotoxicity study of methyl gallate against human epidermoid carcinoma A431 cell lines. In another study by Lee and his co-worker, methyl gallate reported to reduce tumour growth both *in vitro* and *in vivo* in tumour-bearing mice by decreasing $CD4^+CD25^+$ Treg cell migration and reducing the suppressive function of effector T cells (Lee *et al*., 2010). In addition to the cytotoxic activity of the compound, methyl gallate has been shown to possess strong antimicrobial activity with MBC values of 30 ± 1 and 50 ± 1 mg/mL against *Vibriocholerae* strains 569-B and 1837 respectively, in a study reported by Sanchez *et al*. (2013). Methyl gallate has been involvedin the inhibition of neutrophil recruitment, production of inflammatory mediators, and activation of macrophages in an experimental model of arthritis (Correa *et al*., 2016). It has also been shown to be a potent anti-herpetic compound (Cynthia *et al*., 1988).

Similarly, the cytotoxic activity displayed by the isolated compoundsfrom *Caesalpiniabenthamiana*in this study is in line with reported antiproliferative/cytotoxic

activity of caesalpinolida A and caesalpinolida B isolated from a species of the genus*Caesalpinia*, *Caesalpinia bonduc*(L.) Roxb. (Leguminosae) against breast cancer (MCF-7 and MDA-MB-231), endometrial (Ishikawa) and cervical (Hela) cell lines. It was reported that caesalpinolida A and caesalpinolida B showed significant inhibition againstMCF-7 cancer cell with IC_{50} of 12.8 μM and 6.1 μM, respectively (Yadav *et al*., 2009). In addition, the cytotoxic activity of brazilein, a compound isolated from another *Caesalpinia* species, *Caesalpinia sappan* L. (Leguminosae) has been reported to display cytotoxic activity against human cancer cell lines, such as HepG2 and Hep3B (liver), MDA-MB-231 and MCF-7 (breast), A549 (pulmonary), and CA9-22 (gingival) (Yen*et al*., 2010). One other compound (CB4) isolated from *C. benthamiana*in this study with significant cytotoxic activity was not identified due to insufficient spectroscopic data (Appendix 5a-c).

Cytotoxic activity (IC_{50} values range from 5.878±0.09 to 18.59±0.09 μg/mL) of the isolated compound, racemonoate(CR1), the only compound characterised in *C.racemosum*in this study, is in line witha similar work conducted by Gossan and co-worker, who reported cytotoxicity of three known compounds (3-0-β-acetyl-ursolic acid, betulinic acid and quadranoside II) from *C. racemosum* with IC_{50} values ranging from 13 to 50 μMagainst promyelocytic leukemia HL-60 and human erythromyeloblastoid leukemia K562 cell lines (Gossan *et al*., 2016).The cytotoxicity of racemonoate (CR1),a fatty acid compound, is been reported in this study for the first time, and from all indications, this is the first report of the isolation of the compound from *C. racemosum*. However, two other compounds (CR3 and CR4) isolatedfrom *C. racemosum*in this study both had low cytotoxic activity and goodness of fit (Appendix 13a-c) across the three (MCF-7, A549 and PC3) carcinoma cells investigated, therefore, they were notincluded in the cytotoxicity analysis. In addition, they were both unidentified in this study due to insufficient spectroscopic data (Appendices 10a-e and 11a-e), respectively.

The present study revealed that the crude methanol extract of *C. benthamiana* and *C. racemosum* displayed the highest cytotoxic activity among the nine plants screened in the preliminary study; it was observed that all the fractions and test compounds investigated elicited reduction in activity against all the human carcinoma cells investigated, although with varying potencies as observed by their IC_{50} values. In addition, all the fractions and test compounds investigated exhibited cytotoxic activity

as the extent of morphological damage to the cells increased with prolonged exposure period. This can be affirmed in the observed photomicrographs reported on the fractions of the two plants.As the treatment time increased, a progressive loss of normal elongated shape as the cells shrank to smaller round cells was observed, which eventually resulted into loss of cell viability. Compound (CB1),out of the seven isolated compounds has been reported in literature by previous workers (Lee *et al*., 2010; Kamathama *et al*., 2015). In addition, compound CB5 has been synthesized by one author (Keiko *et al*., 1973), while compounds (CB2, CB3, CB6, CB7 and CR1) have not been reported anywhere in literature to the best of my knowledge, hence, they are new compounds. Similarly, only compound CB1 has been reported for cytotoxic activity, while the cytotoxicityof compounds CB2, CB3, CB5, CB6, CB7 and CR1 are being reported in this study for the first time.

In addition to the dose-response curve analysis, one-way analysis of variance with dunnett's multiple testconducted at $p<0.05$ revealed that the cytotoxicity of the reference standard, methotrexatewere not significantly different from the cytotoxicity of the isolated compounds across the carcinoma cells investigated. On MCF-7 carcinoma cells, thecytotoxicity of the reference standard, methotrexate was not statistically different (p = 0.65, 0.28, 0.46, 0.25, 0.24, 0.25 0.99 and 0.17) from the cytotoxicity of the isolated compounds CB1, CB2, BC3, CB4, CB5, CB6,CB7 and CR1, respectively(Appendix 14). Also, the inhibitory ability of methotrexate against A549 carcinoma cells was not statistically significant (p = 0.11, 0.99, 0.99, 0.91, 0.49, 0.96 0.86 and 0.70) fromthe isolated compounds CB1, CB2, BC3, CB4, CB5, CB6, CB7 and CR1, respectively(Appendix 15). On PC3 carcinoma cells however, the inhibitory activity of the standard, methotrexate and the isolated compounds CB1, CB2, BC3, CB4, CB5, CB6, CB7 and CR1 were not statistically different (p = 0.97, 0.70, 0.15, 0.09, 0.25, 0.23 0.99and 0.19), respectively(Appendix 16). It was also observed that there were slight variations in the activities elicited by differentisolated compoundsagainst each of the three (MCF-7, A549 and PC3)carcinoma cells investigated.

5.2. Conclusion

The present study revealed 57 plants species, belonging to 38 families, used by the traditional healers in three selected Local Government Areas of Ekiti State, Southwestern Nigeria, for the management of cancer disease. Two of the plants, *C. benthamiana* and *C. racemosum* displayed significant cytotoxic activity against a panel of human cancer cell lines investigeated. Thishas, however, give credibility to the ethnopharmacological approach as one of the best selection methods used for natural product bio-discovery from plant species.

In the present study, ten (10) compounds were isolated, out of which seven (7) compounds (CB1, CB2, CB3, CB5, CB6, CB7 and CR1) were characterised, while the other three compounds (CB4, CR2 and CR3) were not fully characterised yet due to insufficient spectroscopic data. Bioactivity-guidedfractionation of *C. benthamiana* leaf extract led to the isolation and characterisation of six compounds including a phenolic acid (methyl gallate), one tannin (benthamianoate), a dihydrofuran (benthamiacone), a monocarbonxylic acid (2-methoxyacrylic acid),an amine (1-aminohexane-1-ol) anda triterpenoid(benthamianol).In addition,a long chain fatty acid (racemonoate) was isolated and characterised from *C. racemosum* root bark through bioactive fractionation.

The crude methanol extract/ fractions and isolated compounds from the two plants (*C. benthamiana* and *C. racemosum*) possessed significant cytotoxic activity across the carcinoma cells investigated. However, a dihydrofuran characterised as benthamiaconeand a fatty acid, characterized as racemonoate displayed the highest cytotoxic activity amongst the isolated compounds across the panel of cancer cell lines investigated, compared with the cytotoxicity of the standard reference, methotrexate. It is worth mentioning that five of the seven compounds are new compounds previously unreported in the literature and all were isolated for the first time. This is the first report demonstrating their cytotoxic potentials on three human cancer cell lines. The results of this study validate the folkloric use of *C. benthamiana* and *C. racemosum* in the treatment of cancer.

5.2.1. Contributions to knowledge

1. Documentation of indigenous anticancer plants in three selected LGAs of Ekiti State using ethnobotanical/ethnopharmarcological approach,which has proven

to be one of the best selection methods for natural product bio-discovery from specific plant speciesin this study.

2. Four compounds have been isolated and charsacterised for the first time from *C.benthamiana* (Leguminosae) used in Nigerian enthnomedicine
3. One compound has been isolated and characterised from *C.racemosum* (Combretaceae) used in Nigerian ethnomedicine for the first time
4. Six compounds isolated and characterised from *C.benthamiana* and *C.racemosum* in this study have been reported for cytotoxic activity against three human carcinoma cells; breast (MCF-7), lung (A549), and prostate (PC3) for the first time
5. The rationale for the traditional application of *C.benthamiana* and *C.racemosum* in Nigerian ethnomedicine was validated in this study.
6. Documentation of medicinal knowledge of the use of indigenous plant for treatment of diseases, which is limited to the traditional healers and the elders have been archived in this study**.**

5.2.2. Recommendation

Following the outcome of this research, the following recommendations are presented:

1. Continuous documentation of indigenous knowledge should be encouraged to avoid loss of knowledge associated with respondents, most of which belong to the aging population.
2. Traditional healers, plant collectors, tertiary institutions as well as government agencies should encourage sustainable cultivation and conservation of medicinal plants, especially threatened species.
3. Further investigation on the selective ability and mechanism of action of the isolated cytotoxic compounds in this work needs to be carried out.
4. The unidentified isolated compounds in this work need to be fully characterised as they also displayed significant cytotoxic activity.
5. More medicinal plants used locally by traditional healers in cancer management should be investigated for their folklore claims in order to exploit their active secondary metabolites.
6. Clinical trial of isolated compounds needs to be carried out.

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APPENDICES

Appendix 1- Questionaire for Ethnobotanical Surveillance of Anticancer Plants

Department of Pharmacognosy
Faculty of Pharmacy
University of Ibadan, Ibadan.

Greetings,

Dear Respondent, I am Famojuro Tayo Ibukun, a PhD student of the Department of pharmacognosy, University of Ibadan. We are carrying out a study on the use of herbal preparations in the treatment of cancer disease. We are administeringthe questionaire to get information to be use for the research work. We are pleased to inform you that all your responses will be kept confidential and the outcome of this research will help us to develop drugs for the treatment of cancer. We also want to assure you that the outcome of our findings will be communicated to you. Your co-operation in giving us true and correct information will be highly appreciated.Thank You.

Place of interview……

Study site……

Date of interview…….

Section A: Demographic information of respondents

Name. .....

2.Village name .....

3. Local Government Area ……...

4. State of origin.....

5. Tribe: Yoruba Ha☐a Igbo☐ Others☐.....

6. Age:<20 ☐21-30 ☐31-40 ☐ 41-5051 a☐above ☐

7. Sex:Female ☐ Male ☐

8. Religion: Islam ☐ Christianity ☐ Traditionalist ☐ Others ☐

9. Educational level: Primary ☐ Secondary ☐ Tertiary ☐ None.....

10. Occupation: Traditionalmedicine practitioner ☐ Traditional birth attendant ☐

Herb sellerHer☐st others (p☐se specify) ☐

11. Marital status: Single ☐ Married ☐ Divorced ☐Widow☐Widower ☐

Section B: Cancer cultural characterisation

12. Did you know the disease called cancer? Yes ☐ No ☐

13. If yes, mention the different types of cancer you know

a) ……

b) ……

c) ……

d) ……....

e) ……

14. What do you think causes cancer disease?

a) ……

b) ……

c) ……

d) ……....

e) ……

15. What are the symptoms of cancer disease?

a) ……

b) ……

c) ……

d) ……....

e) ……

16. How do you diagnose cancer disease?

a) ……

b) ……

c) ……

d) ……....

e) ……

17. How do you think cancer disease can be prevented?

a) ……

b) ……

c) ……

d) ……....

e) ……

18. How do you treat the disease?

a) Herbal preparations only ☐ b) Divination/oracle only ☐ c)Incantation only ☐

d) Herbal preparation + incantation ☐ e) Herbal preparation + other things ☐

19 Please rank in ascending order the most common cancer among those mentioned

a) ……

b) ……

c) ……

d) ……...

e) ……

Section C: Medicinal plants used for the treatment of cancer

20. Do you know any medicinal plant(s) used in the treatment of cancer disease?

Yes ☐ No ☐

21. Give local names of the plant(s) and plant parts used in the treatment of cancer disease.

Plant(s) used	Plant(s) part used
(a)	.....
(b)	.....
(c)	.....
(d)	.....
(e)	

22. Which of the plant(s) listed above is the most used in the treatment of cancer disease?.....

23. What are the other medicinal uses of the plant(s) mentioned above?

(a) .....

(b) .....

(c) .....

24. Where do you collect your medicinalplant(s)?.....

25. What form of plant material did you prefer to use when preparing the cancer recipe? a) Fresh ☐ b) dried ☐

26. State the method of preparation of the medicinal plant(s):.....

.....

27. State the method of administration of the medicinal plant(s): .....

28. What are the side effects of the recipe you have noticed? .....

29. Do you have any dosage for it? (How do you apply it?)

(a) Once daily ☐

(b) Two times daily ☐

(c) Three times daily ☐

(d) Others .....

30. Do you add any other material to the medicinal plant(s) during preparation?

Yes☐ No ☐

31.If yes, what are they? .....

32. How are the medicinal recipes preserved?.....

33. How did you learn about the medicinal properties of plant(s)?

a) Ancestral (grandparent/parent) ☐ b) Training☐ c) Ancestral and Training☐

d) Dream ☐

34.Your years of experience in the trade: ….....

35. Have you registered your trade? Yes☐ No ☐

36. Do you have any suggestion(s) on how to improve local drugs for cancer treatment

…….
….

37. Are you aware of conservation of indigenous knowledge and plant diversity?

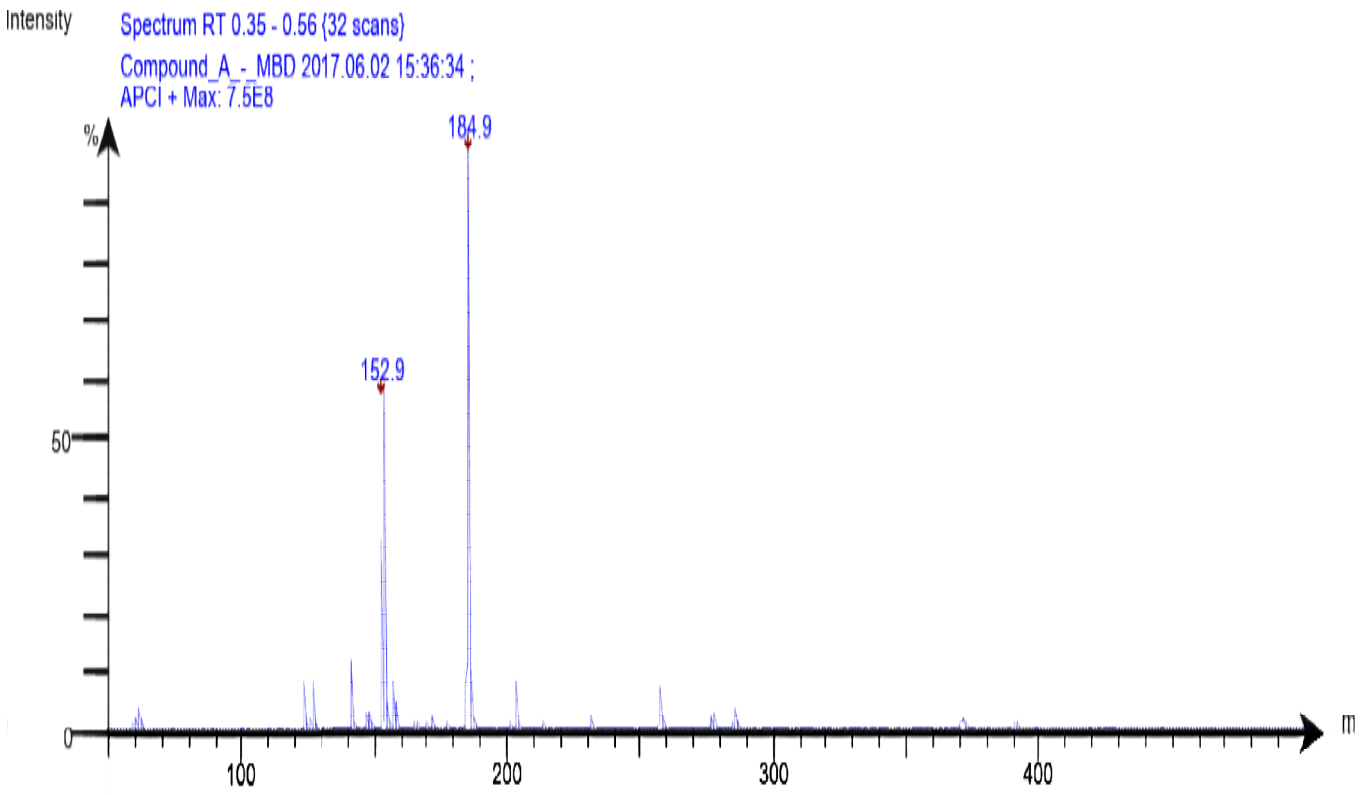
Yes☐ No ☐

If yes, give details

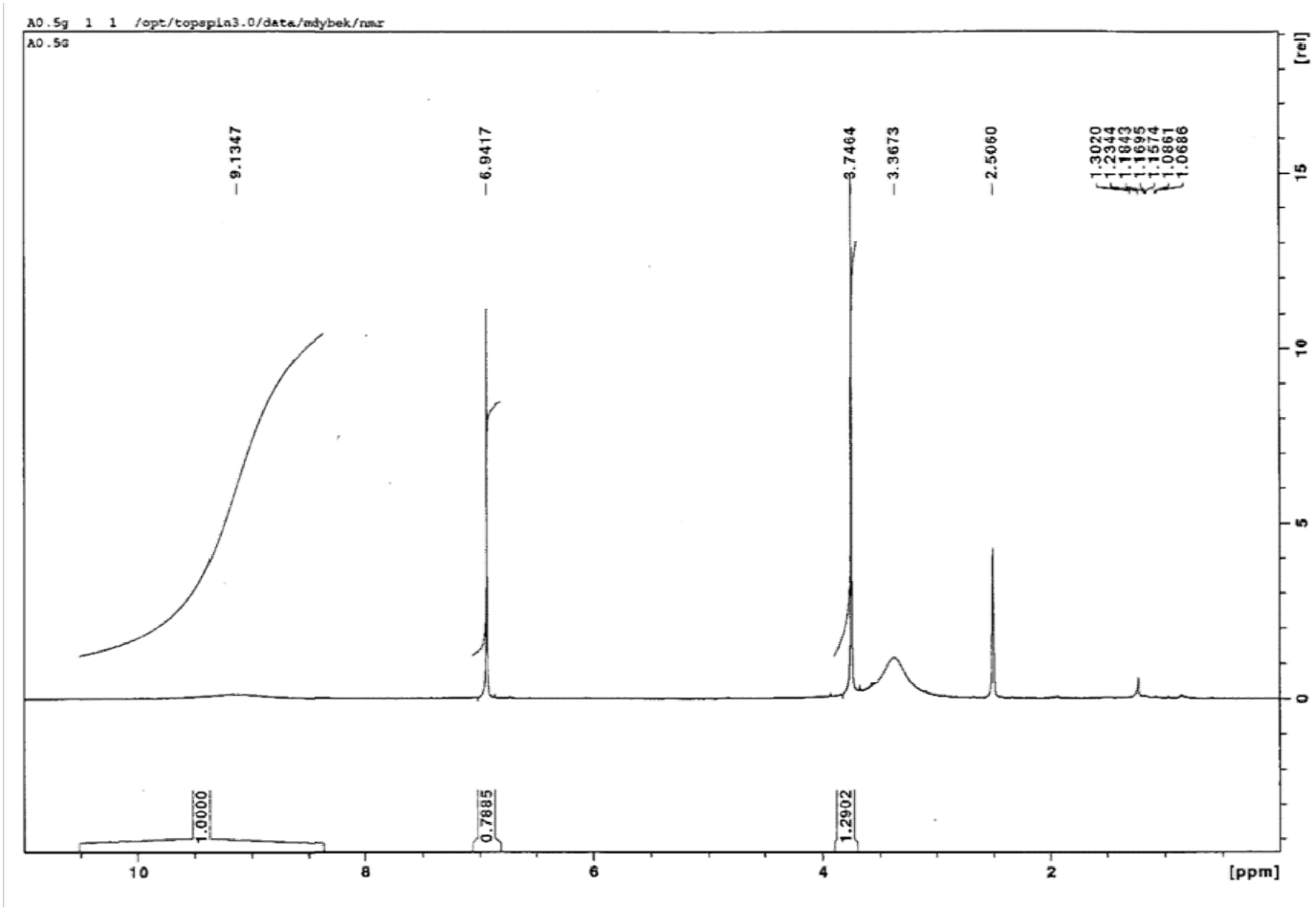
……
……

Thanks so much for your time to respond to this questionnaire.

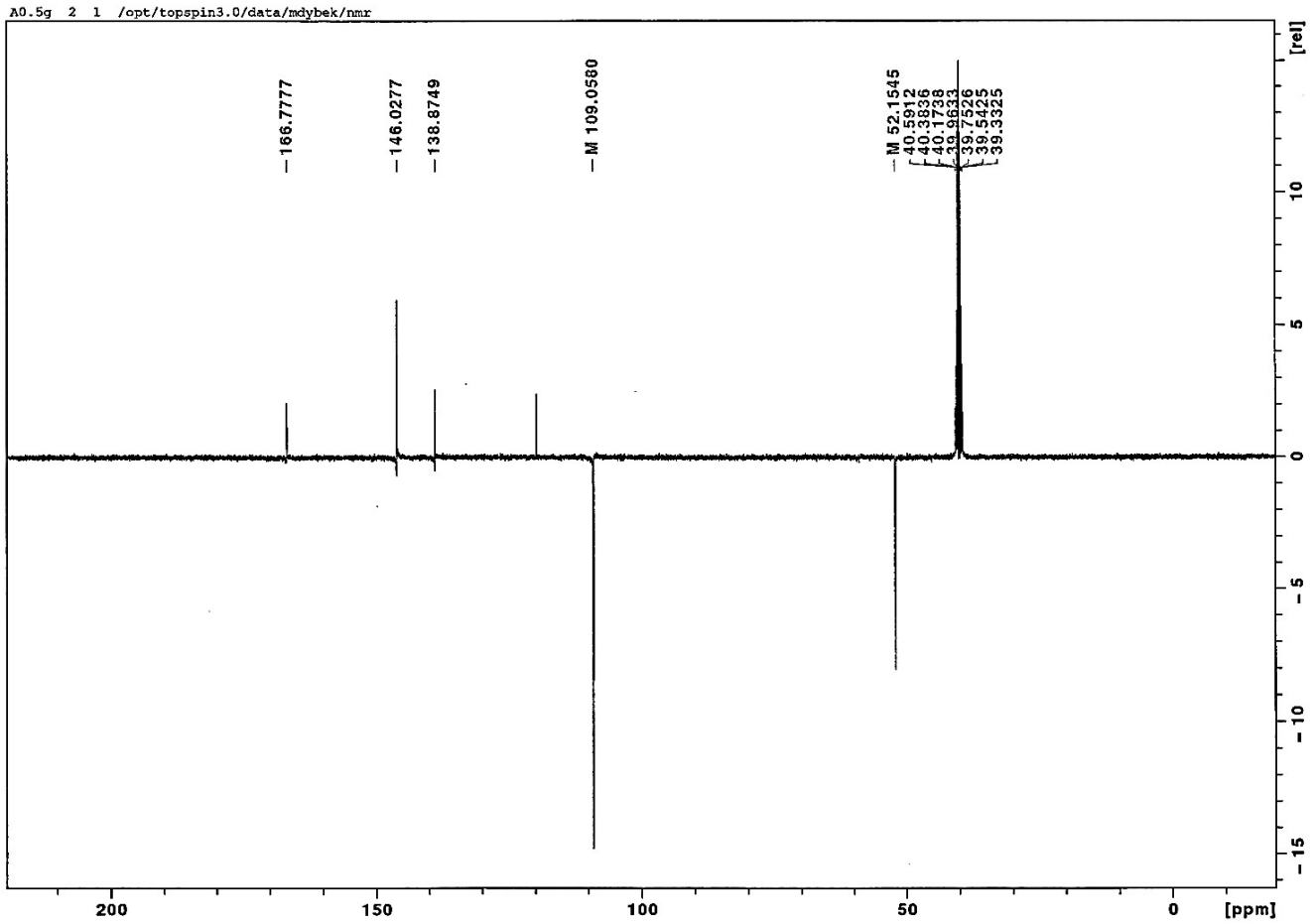
Appendix 2a– Mass spectra of compound CB1



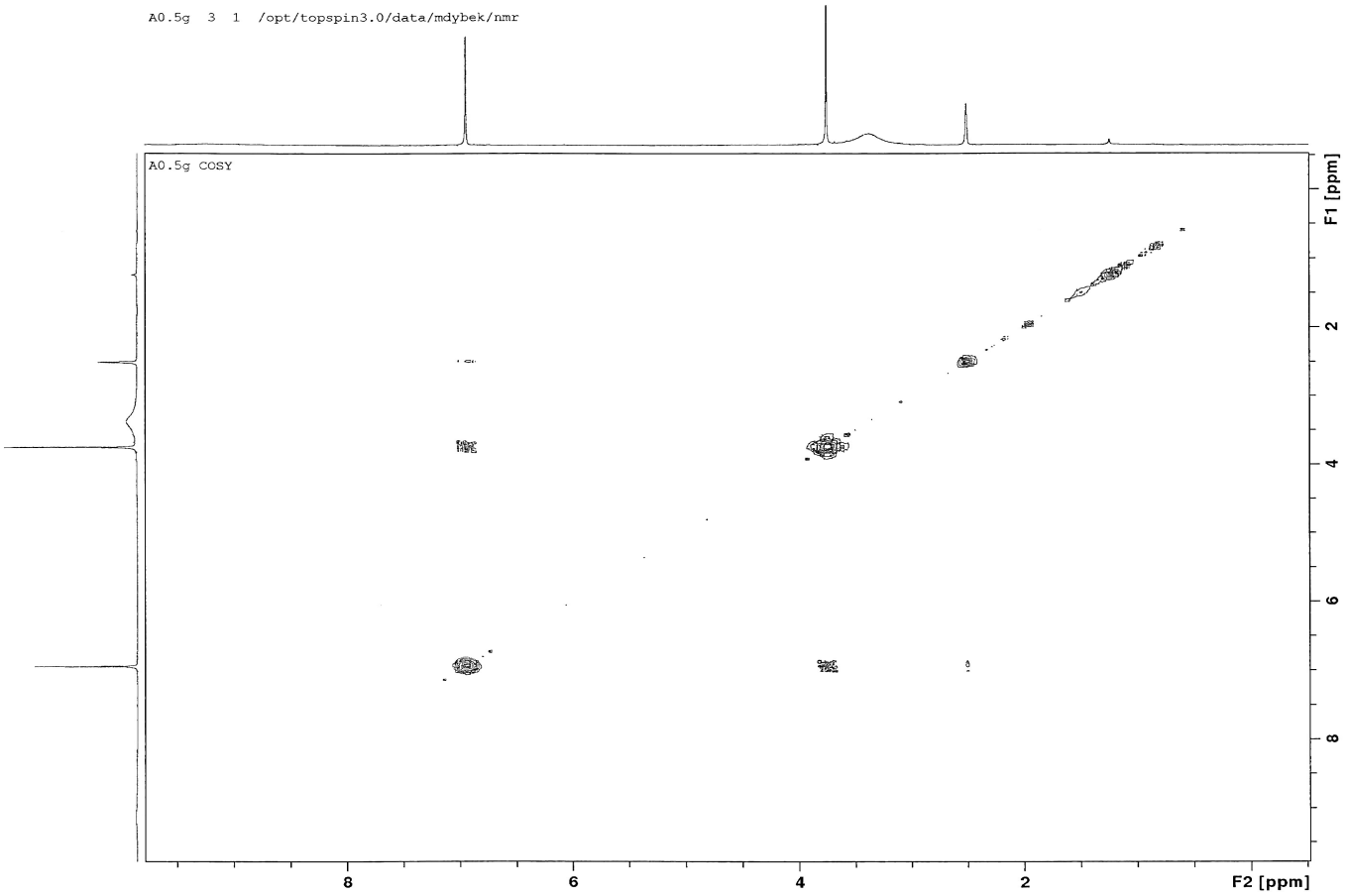
Appendix 2b– ^{1}H spectra of compound CB1



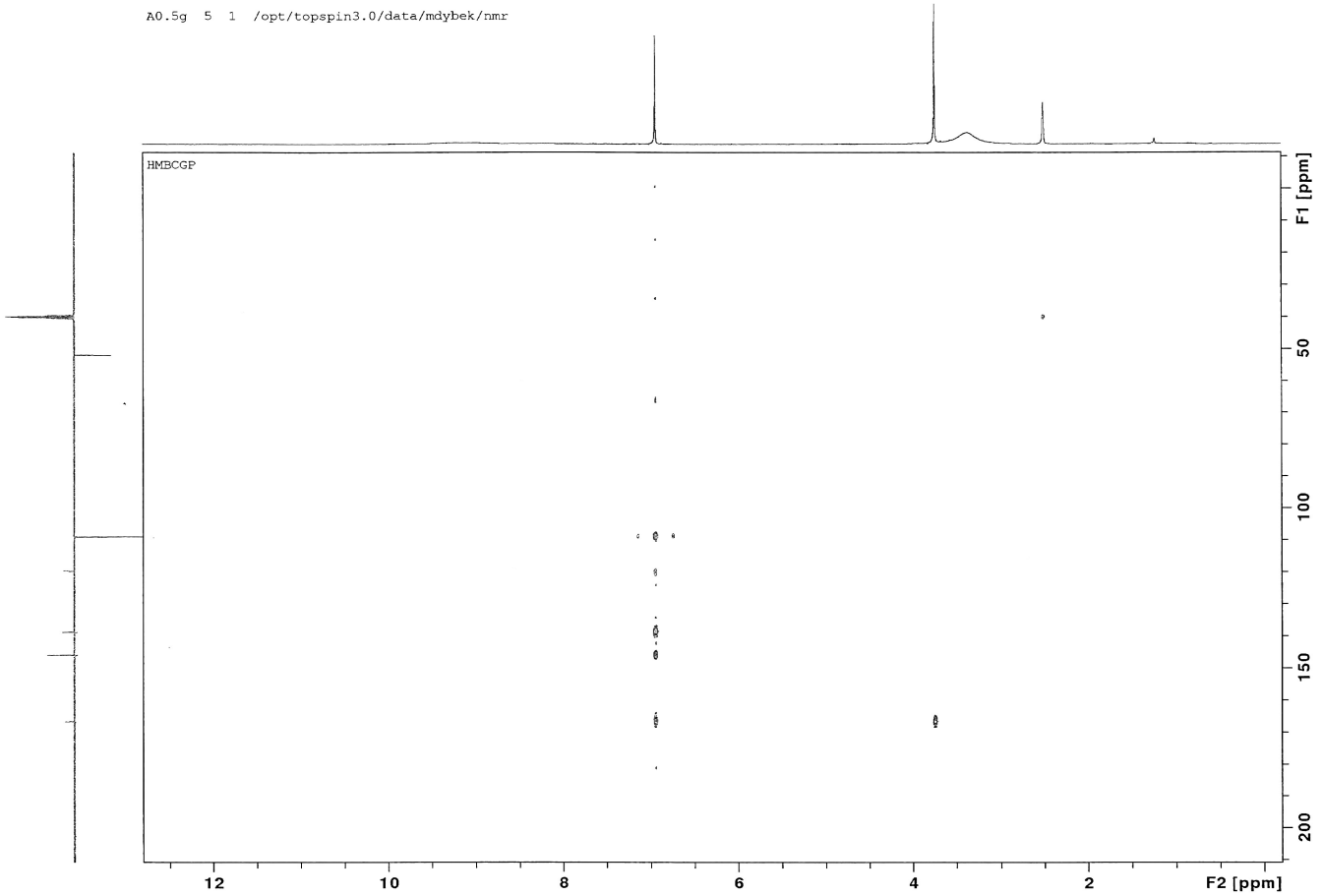
Appendix 2c– DEPT spectra of compound CB1



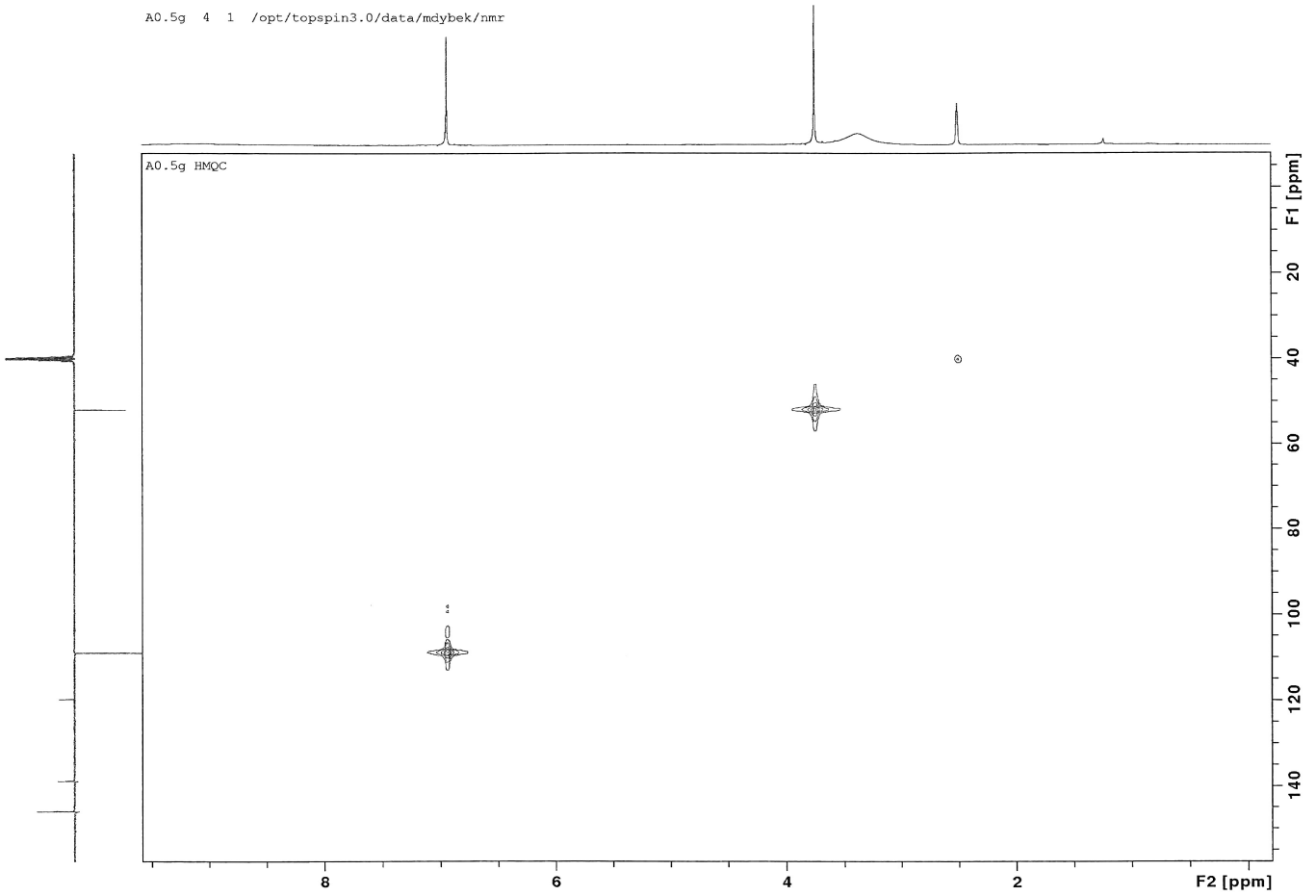
Appendix 2d– COSY spectra of Compound CB1



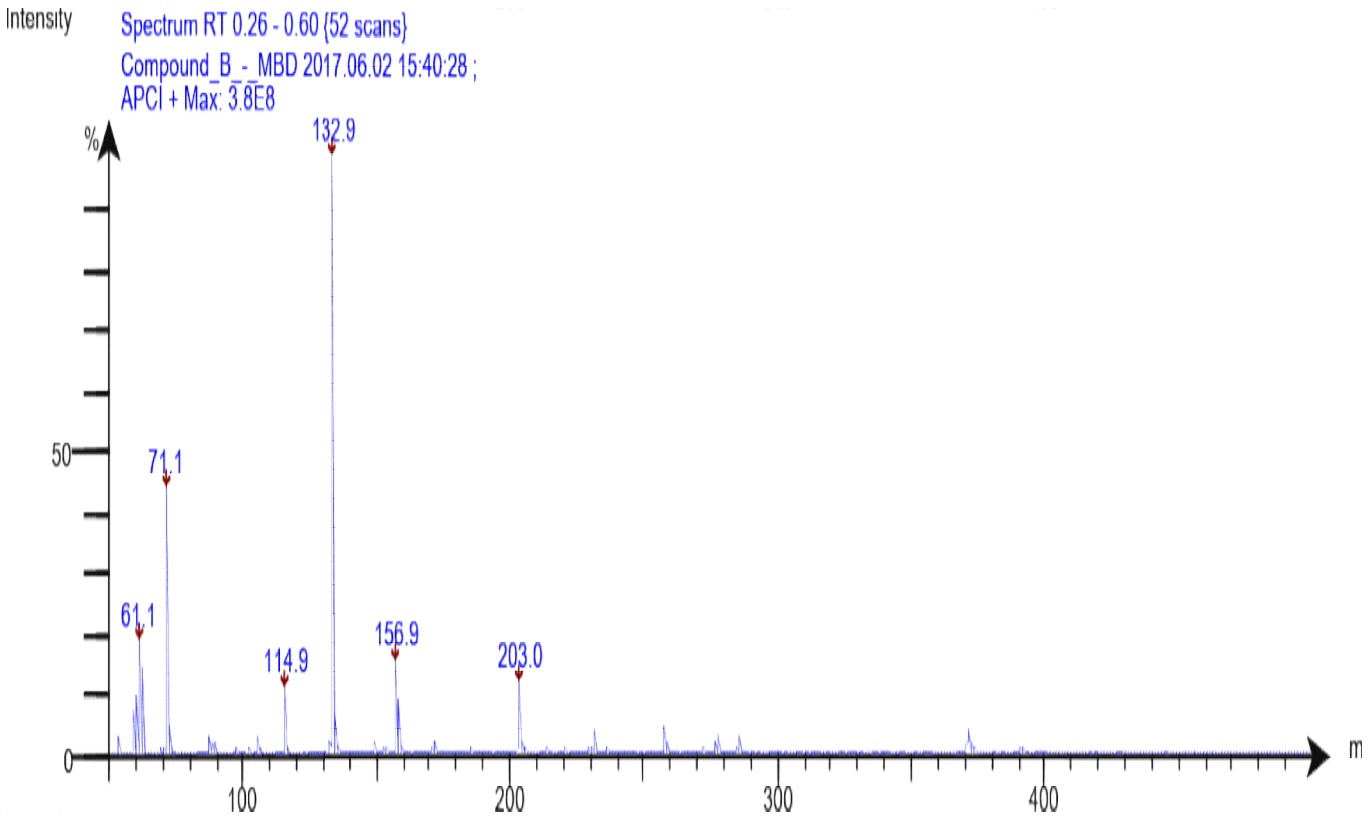
Appendix 2e– HMBC spectra of compound CB1



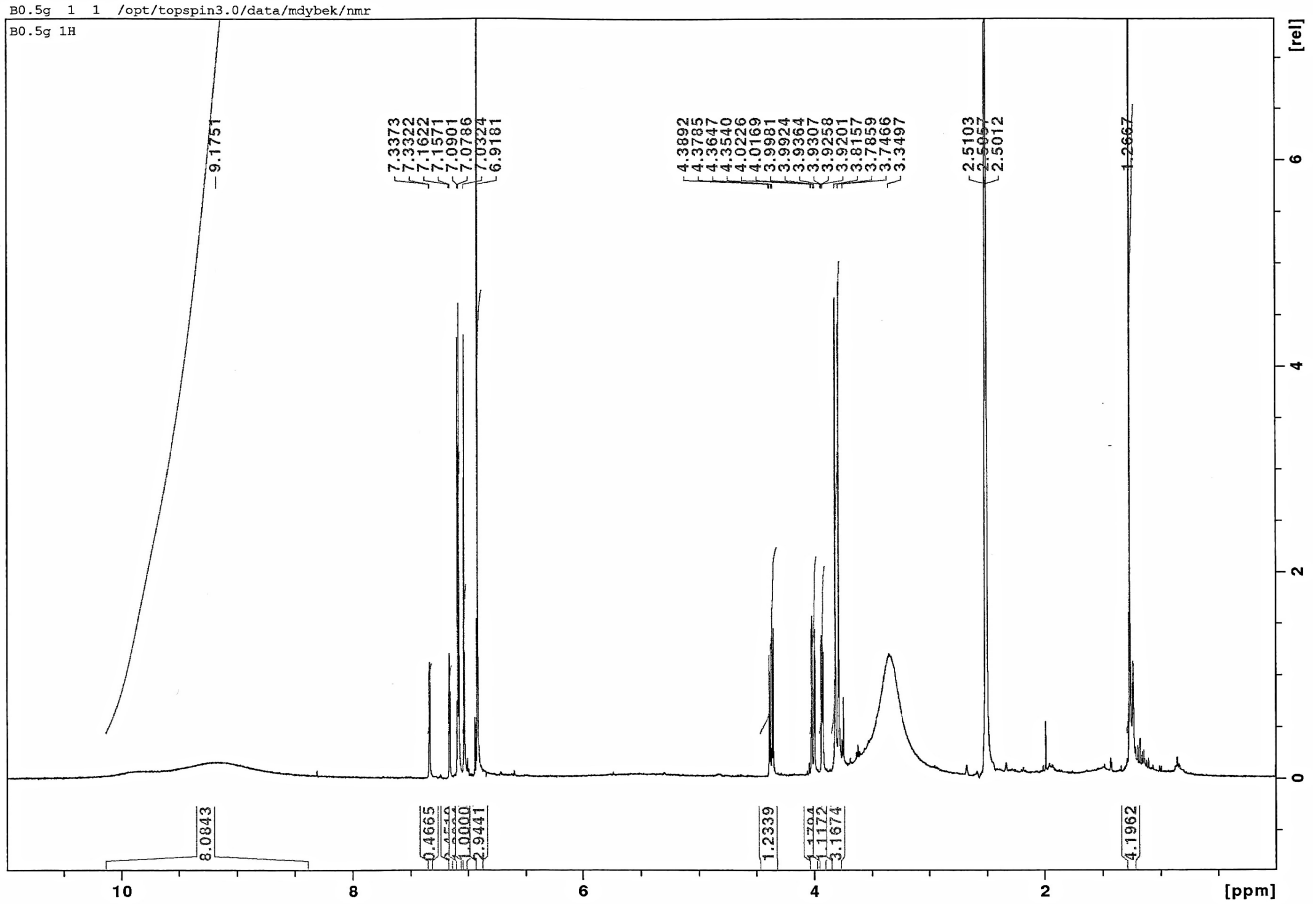
Appendix 2f– HSQC spectra of compound CB1



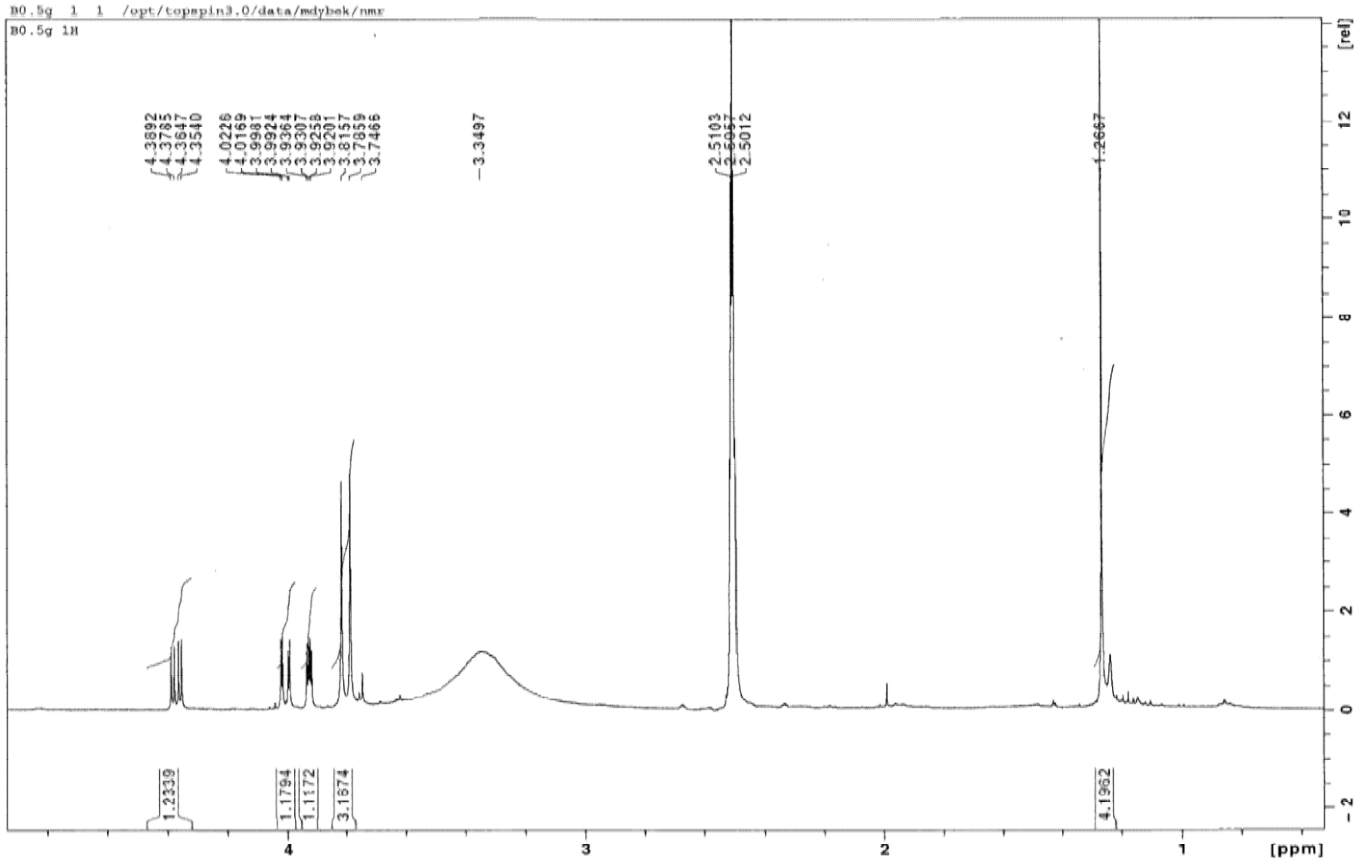
Appendix 3a– Mass spectra of compound CB2



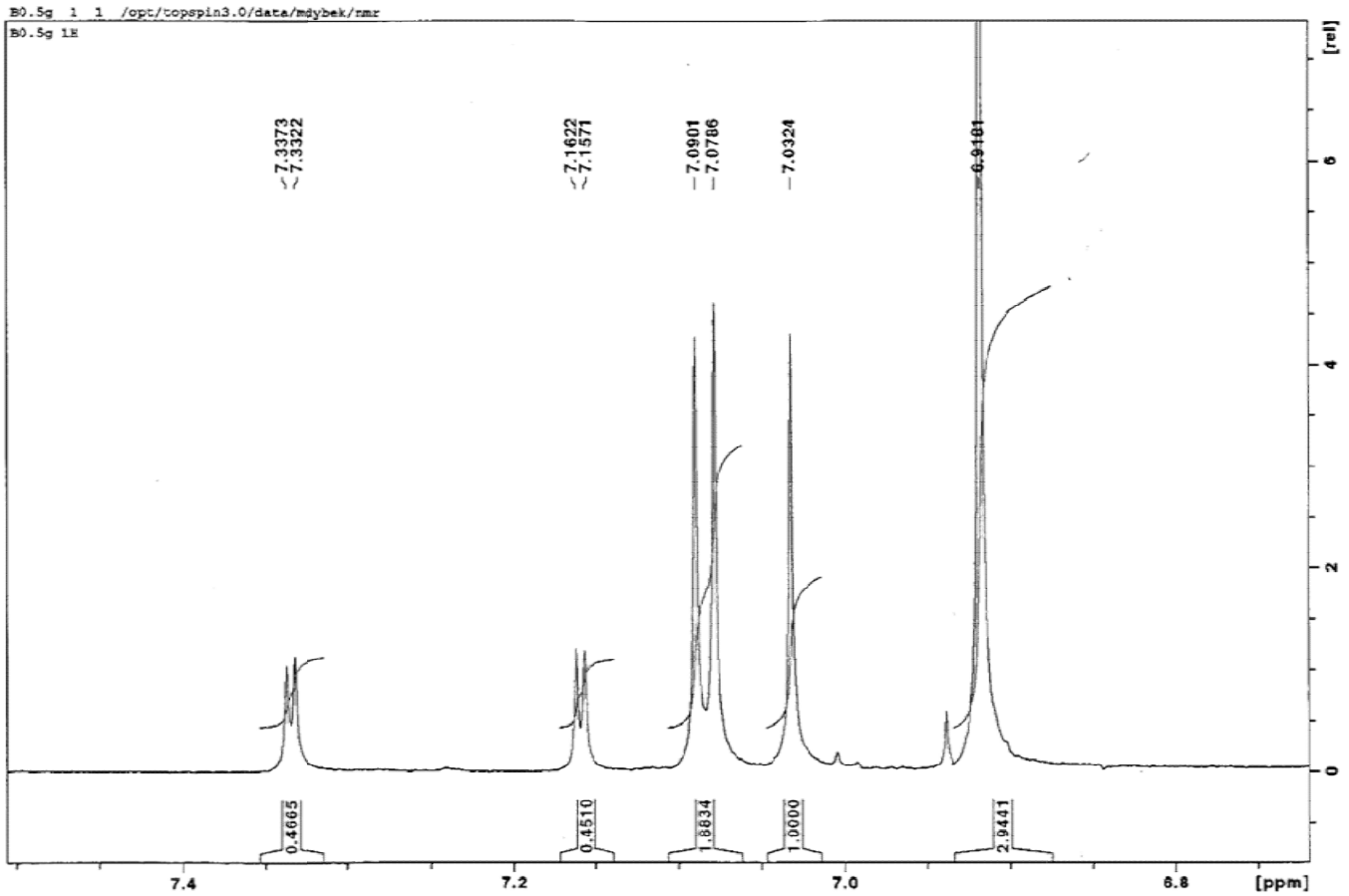
Appendix 3b i– ^{1}H spectra of compound CB2



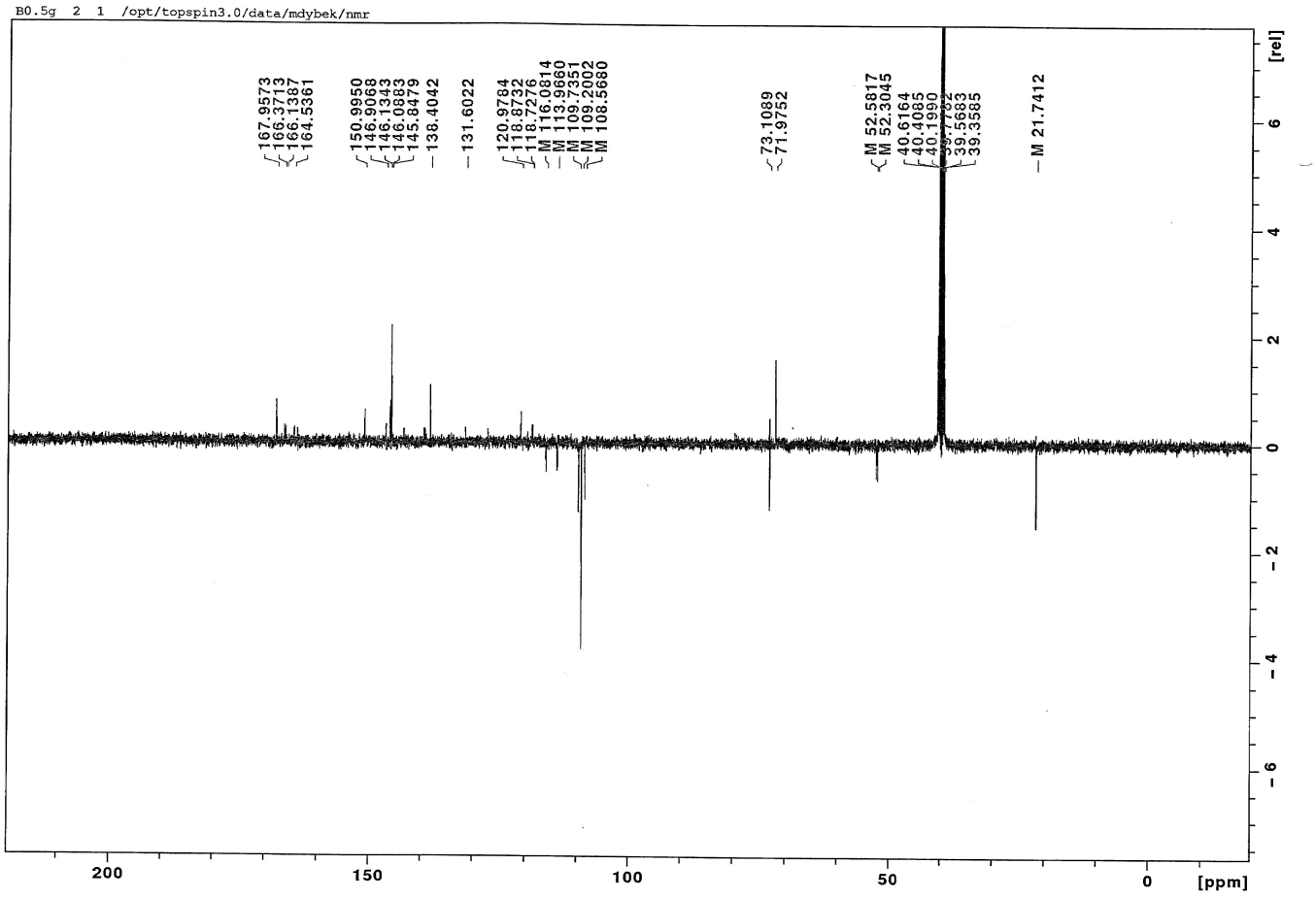
Appendix 3b ii– ^{1}H spectra of compound CB2



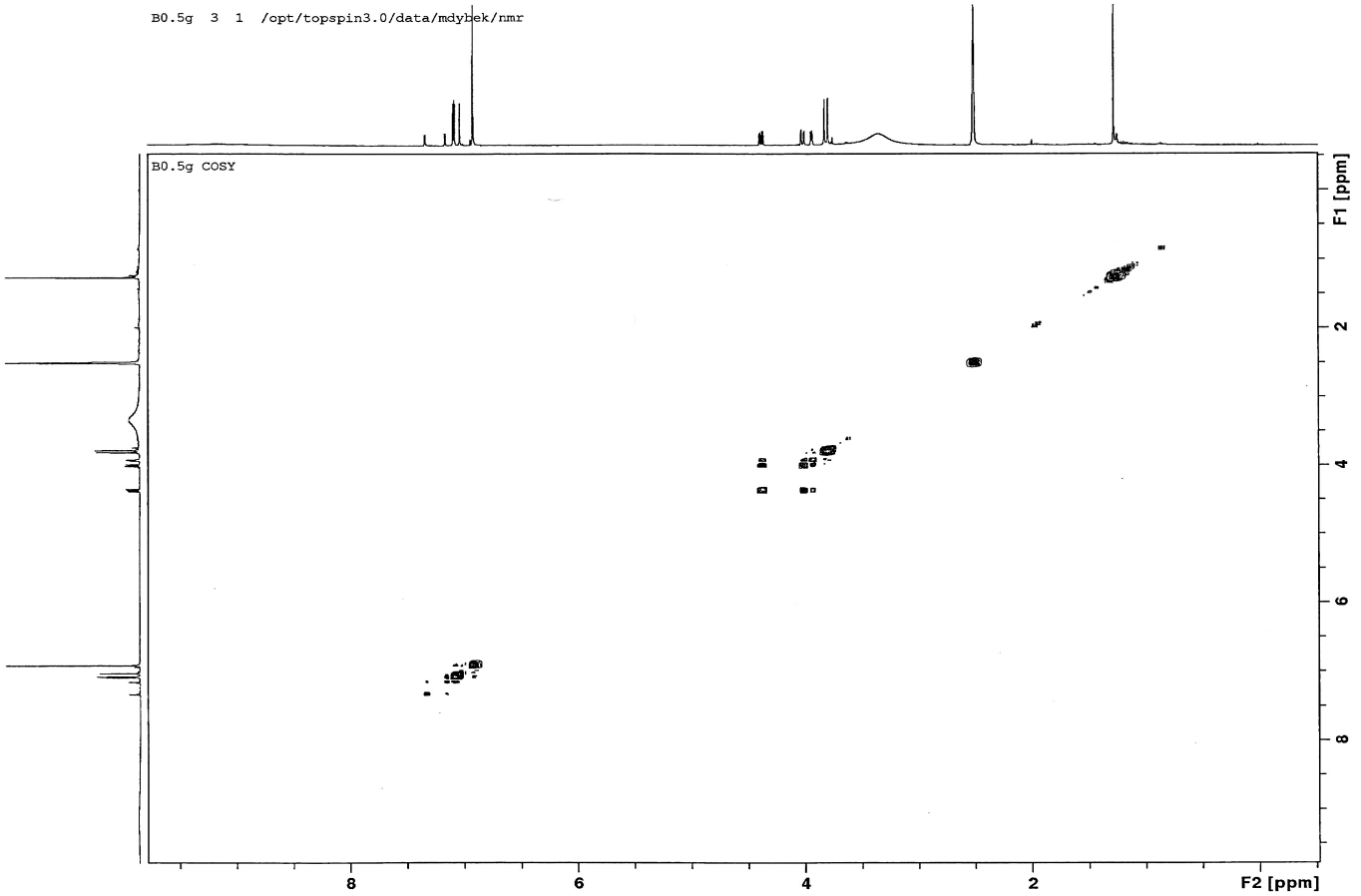
Appendix 3b iii– ^{1}H spectra of compound CB2



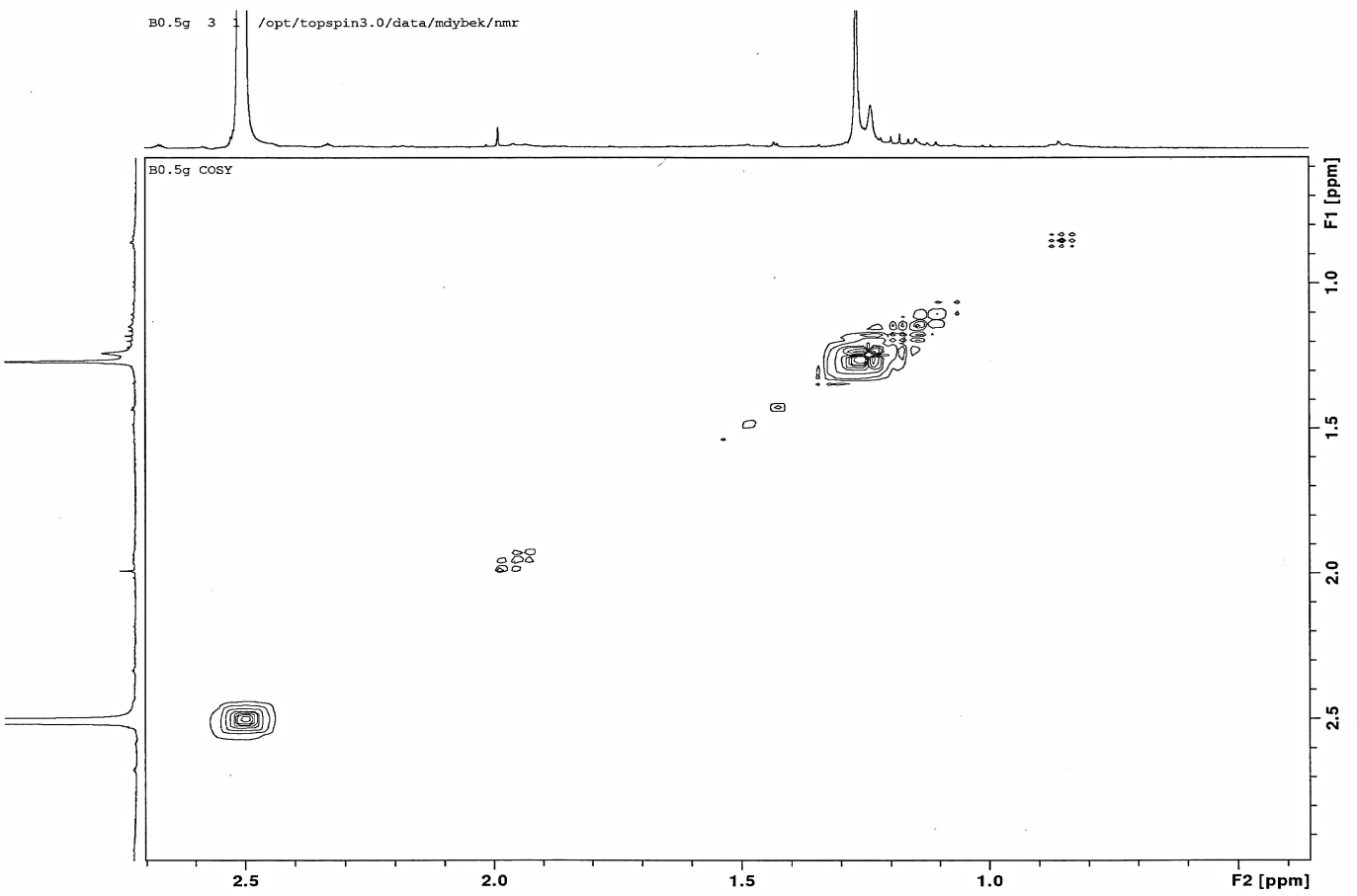
Appendix 3c– DEPT spectra of compound CB2



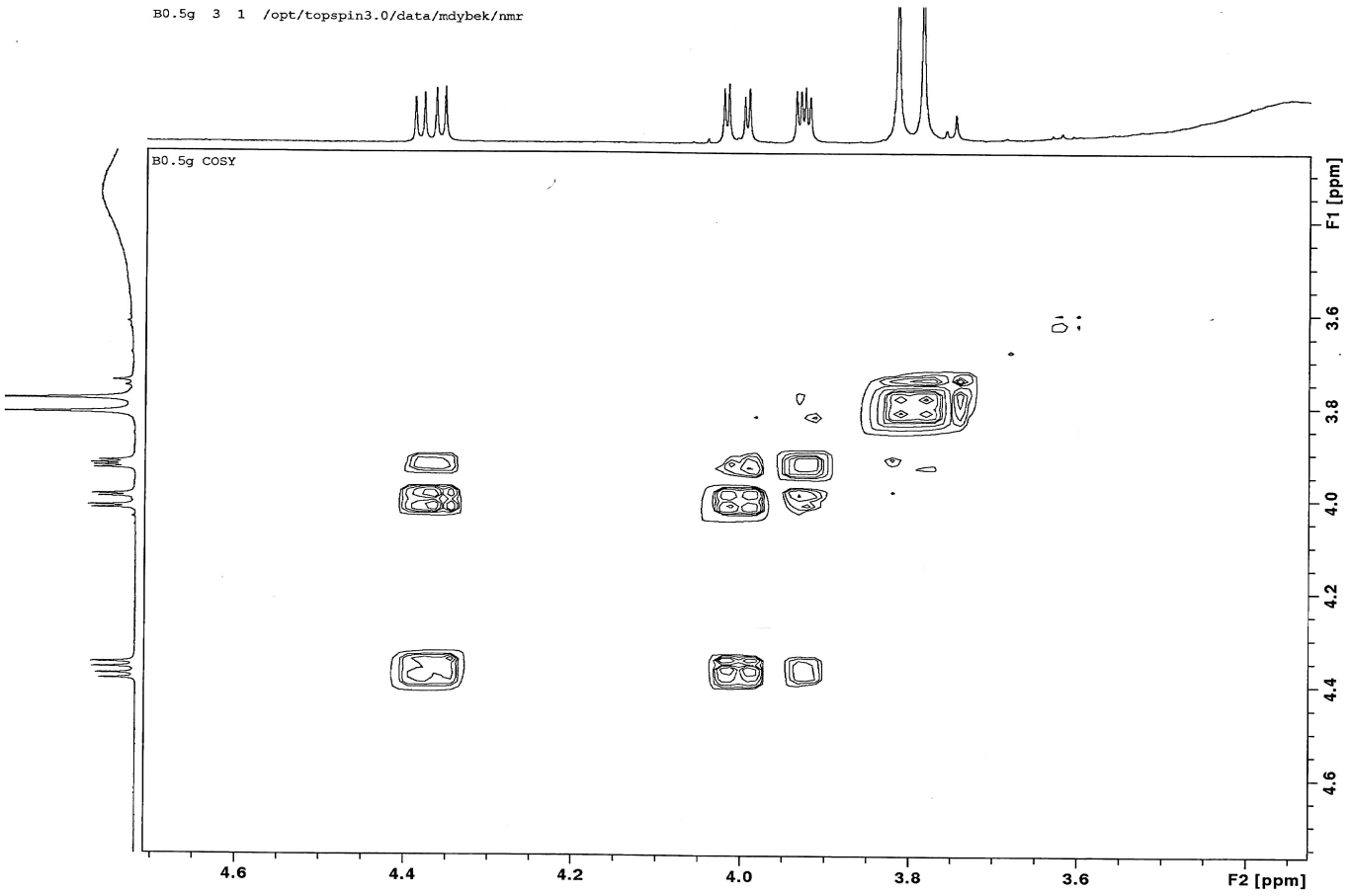
Appendix 3d i– COSY spectra of compound CB2



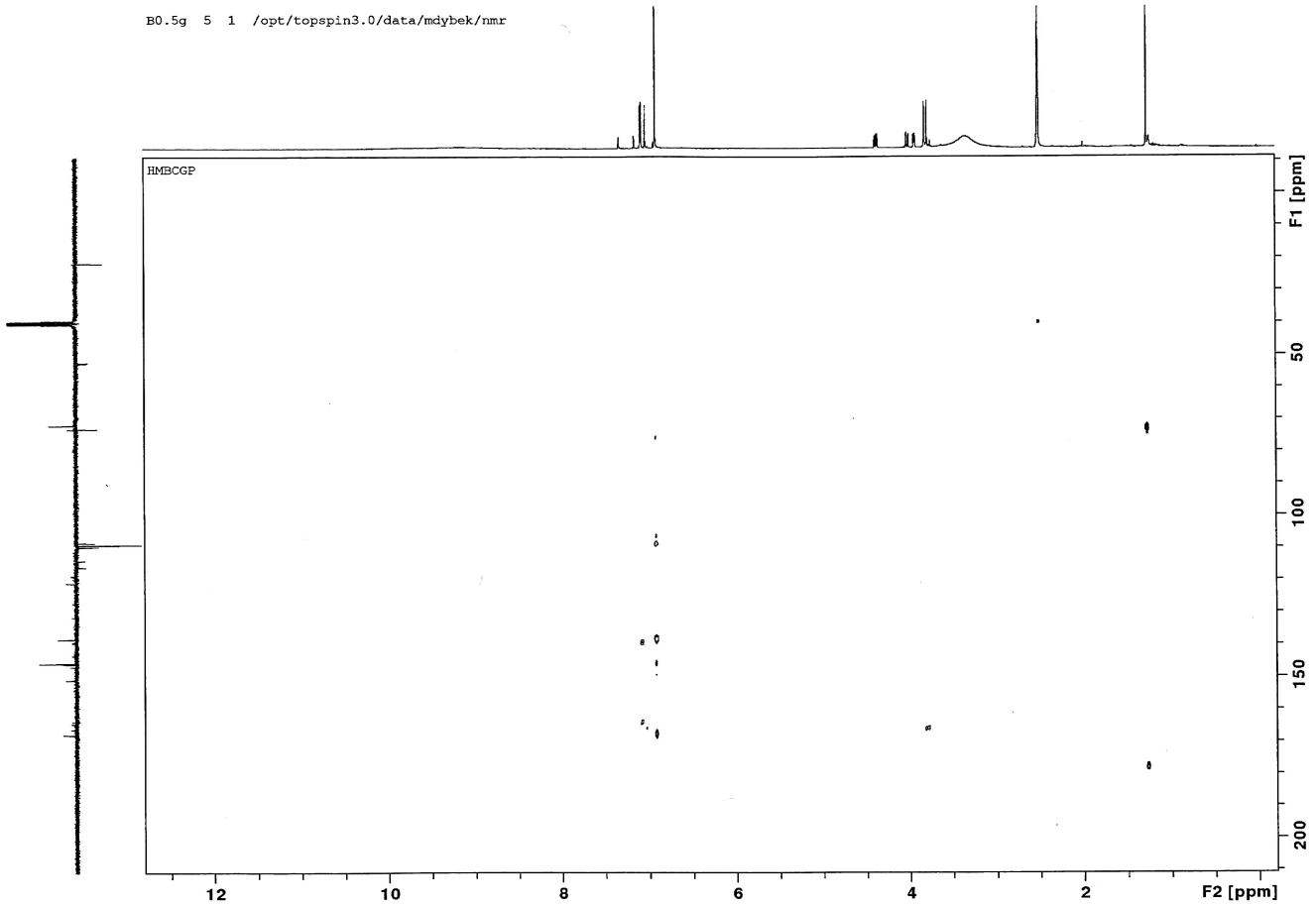
Appendix 3d ii– COSY spectra of compound CB2



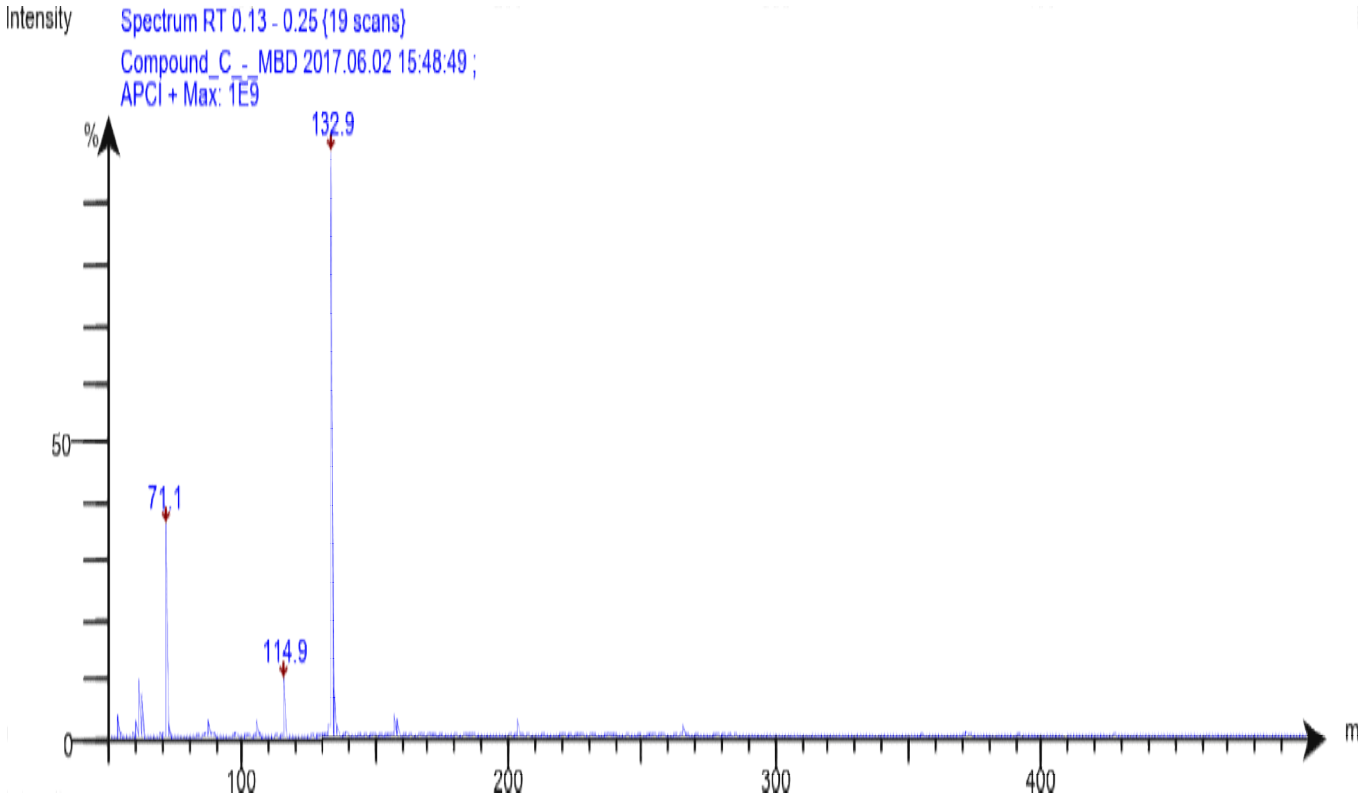
Appendix 3d iii– COSY spectra of compound CB2



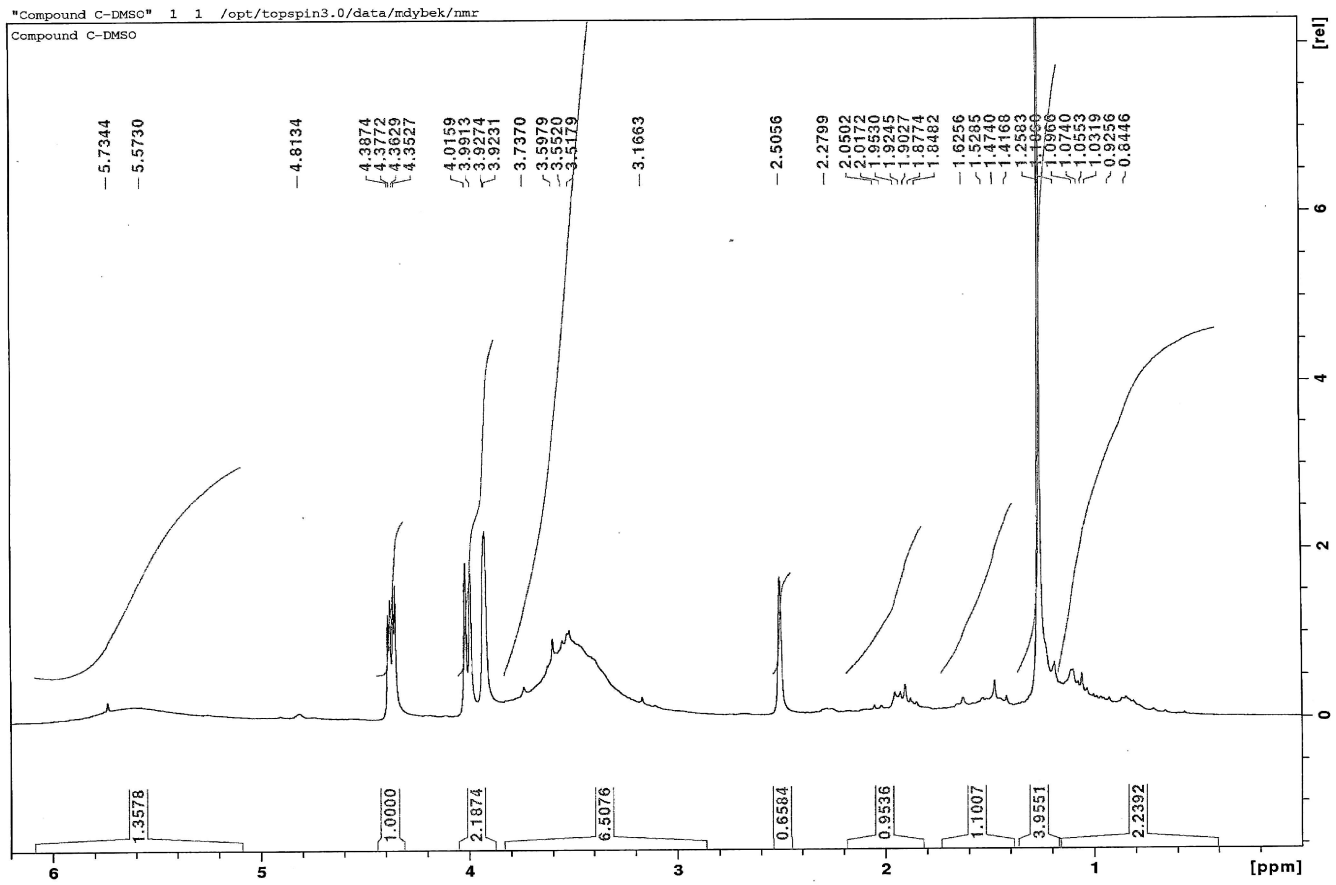
Appendix 3e– HMBC spectra of compound CB2



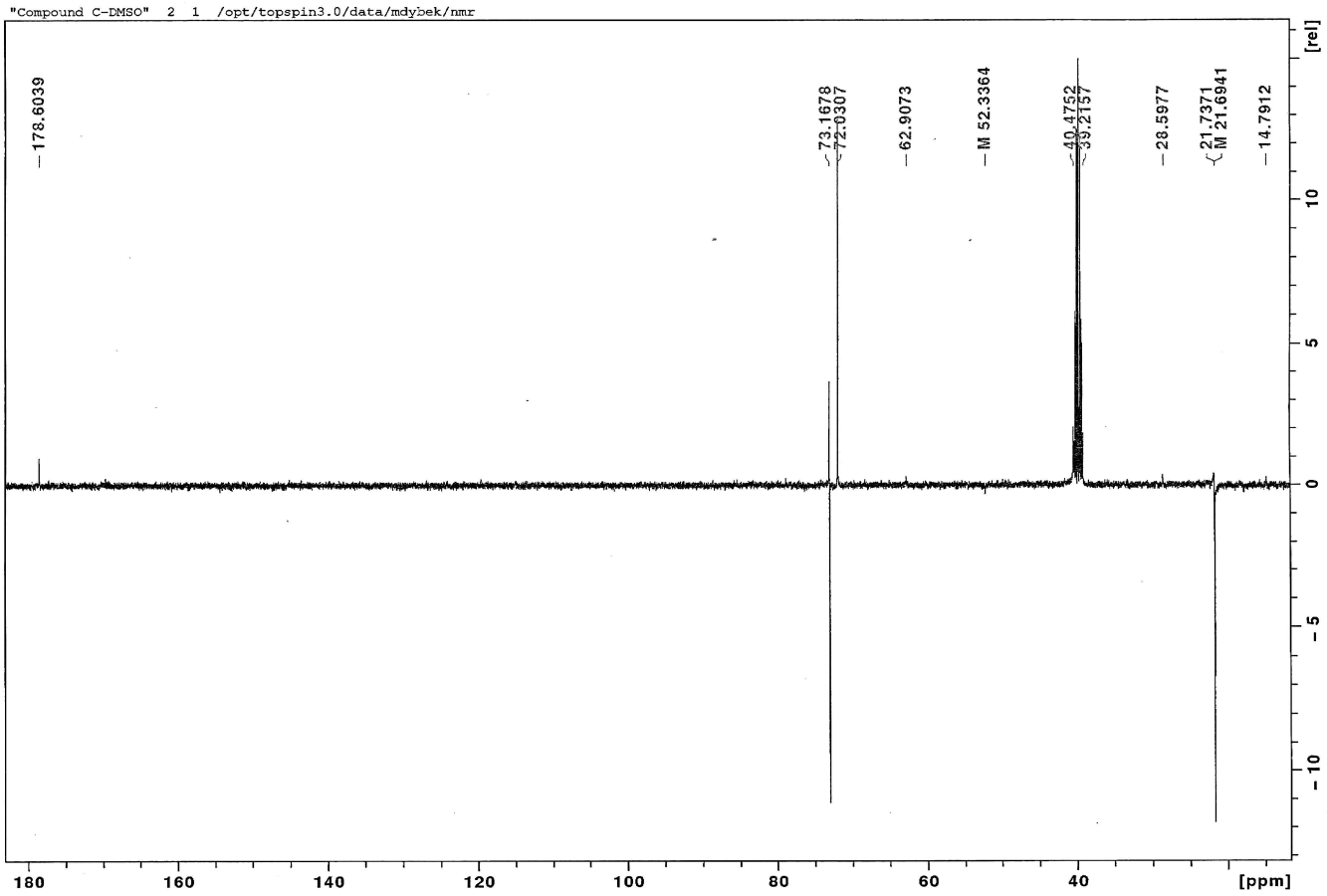
Appendix 4a– Mass spectra of compound CB3



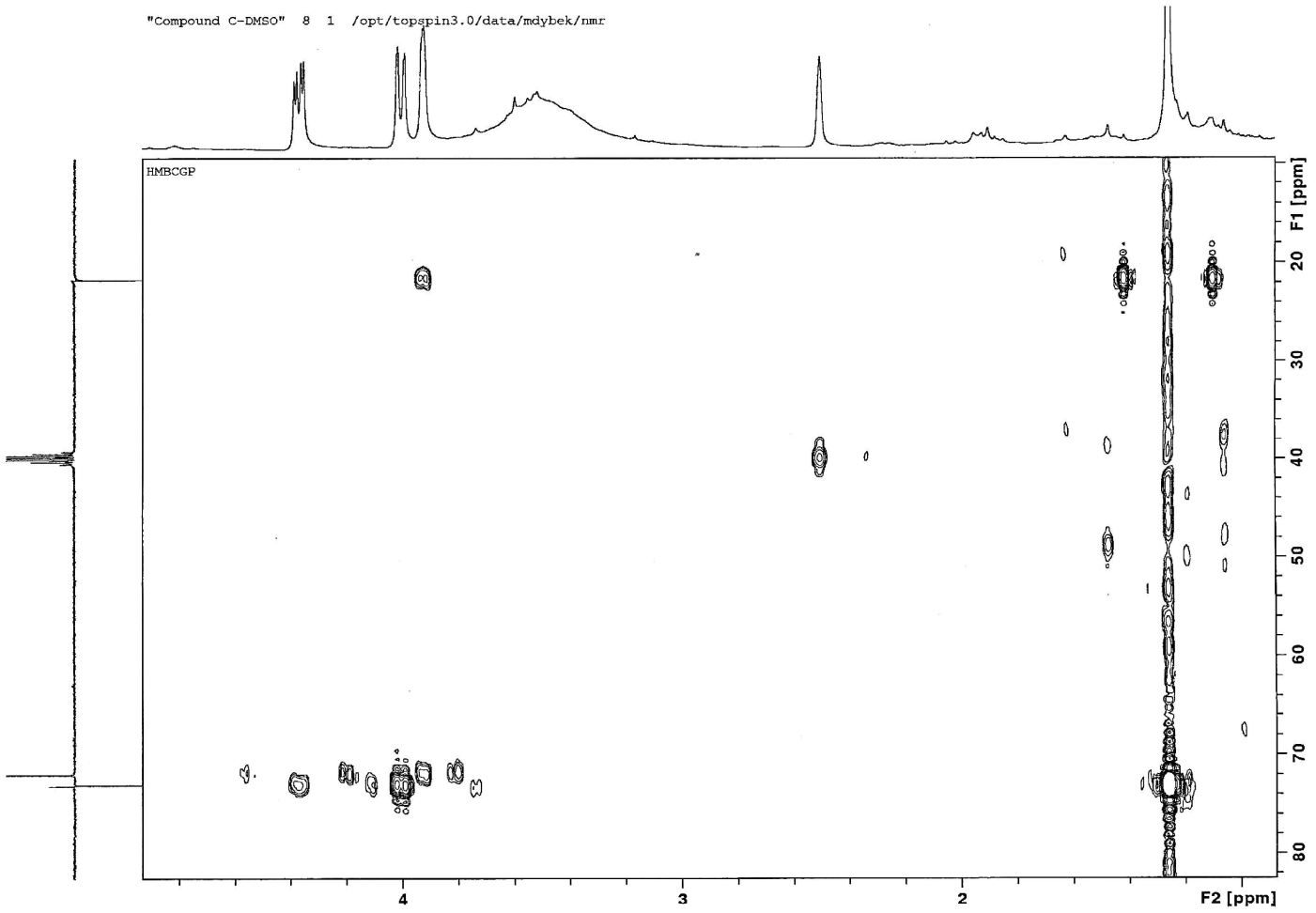
Appendix 4b– ^{1}H spectra of compound CB3


"Compound C-DMSO" 1 1 /opt/topspin3.0/data/mdybek/nmr
Compound C-DMSO
5.7344
5.5730
4.8134
4.3874
4.3772
4.3629
4.3527
4.0159
3.9913
3.9274
3.9231
3.7370
3.5979
3.5520
3.5179
3.1663
2.5056
2.2799
2.0502
2.0172
1.9530
1.9245
1.9027
1.8774
1.8482
1.6256
1.5285
1.4740
1.4168
1.2583
1.1060
1.0966
1.0740
1.0553
1.0319
0.9256
0.8446
1.3578
1.0000
2.1874
6.5076
0.6584
0.9536
1.1007
3.9551
2.2392
[rel]
[ppm]

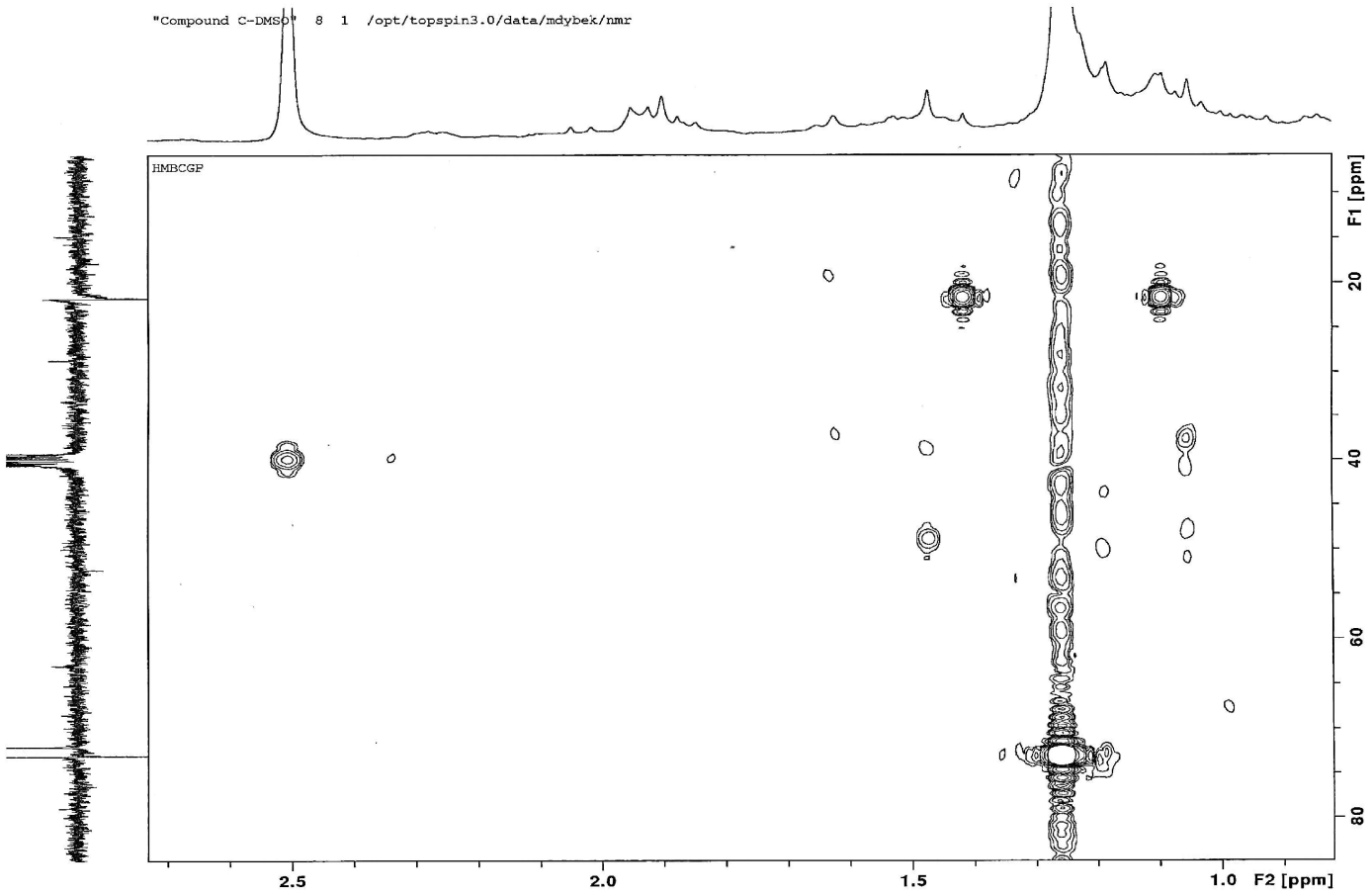
Appendix 4c– DEPT spectra of compound CB3


"Compound C-DMSO" 2 1 /opt/topspin3.0/data/mdybek/nmr
178.6039
73.1678
72.0307
62.9073
M 52.3364
40.4752
39.2157
28.5977
21.7371
M 21.6941
14.7912
[rel]
10
5
0
- 5
- 10
180
160
140
120
100
80
60
40
[ppm]

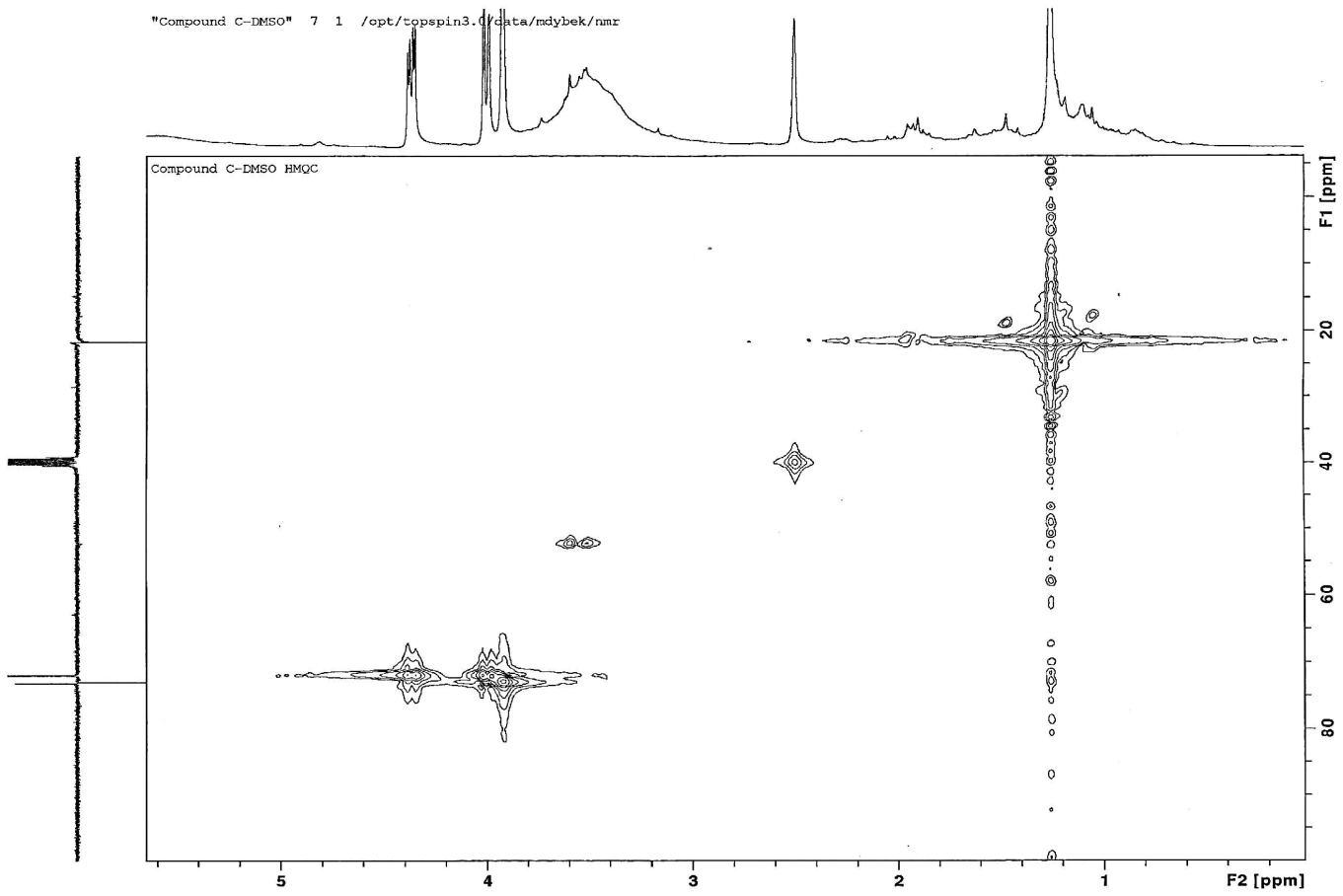
Appendix 4d i– HMBC spectra of compound CB3


"Compound C-DMSO" 8 1 /opt/topspin3.0/data/mdybek/nmr
HMBCGP
F1 [ppm]
20
30
40
50
60
70
80
4
3
2
F2 [ppm]

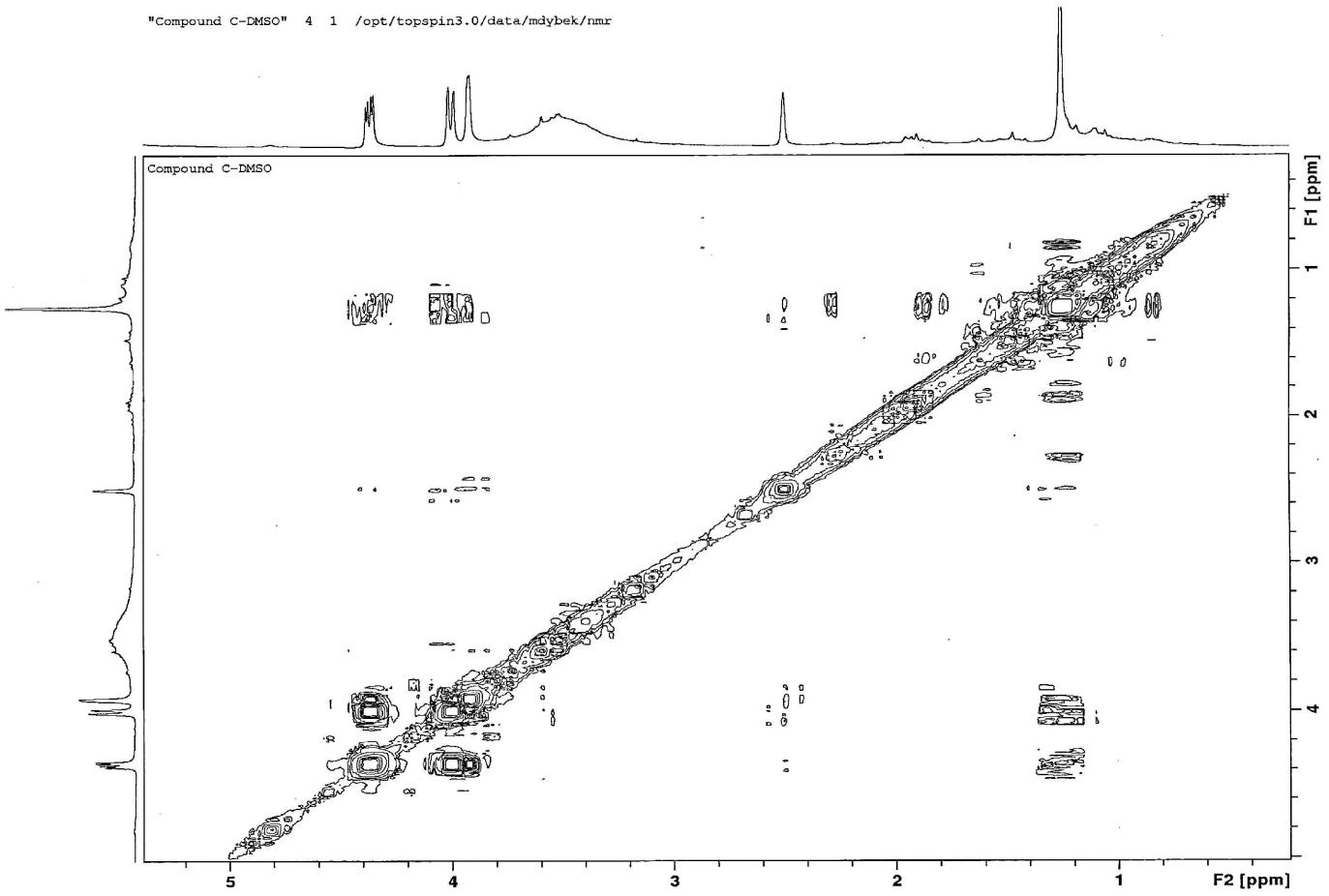
Appendix 4d ii– HMBC spectra of compound CB3


"Compound C-DMSO" 8 1 /opt/topspin3.0/data/mdybek/nmr
HMBCGP
F1 [ppm]
20
40
60
80
2.5
2.0
1.5
1.0
F2 [ppm]

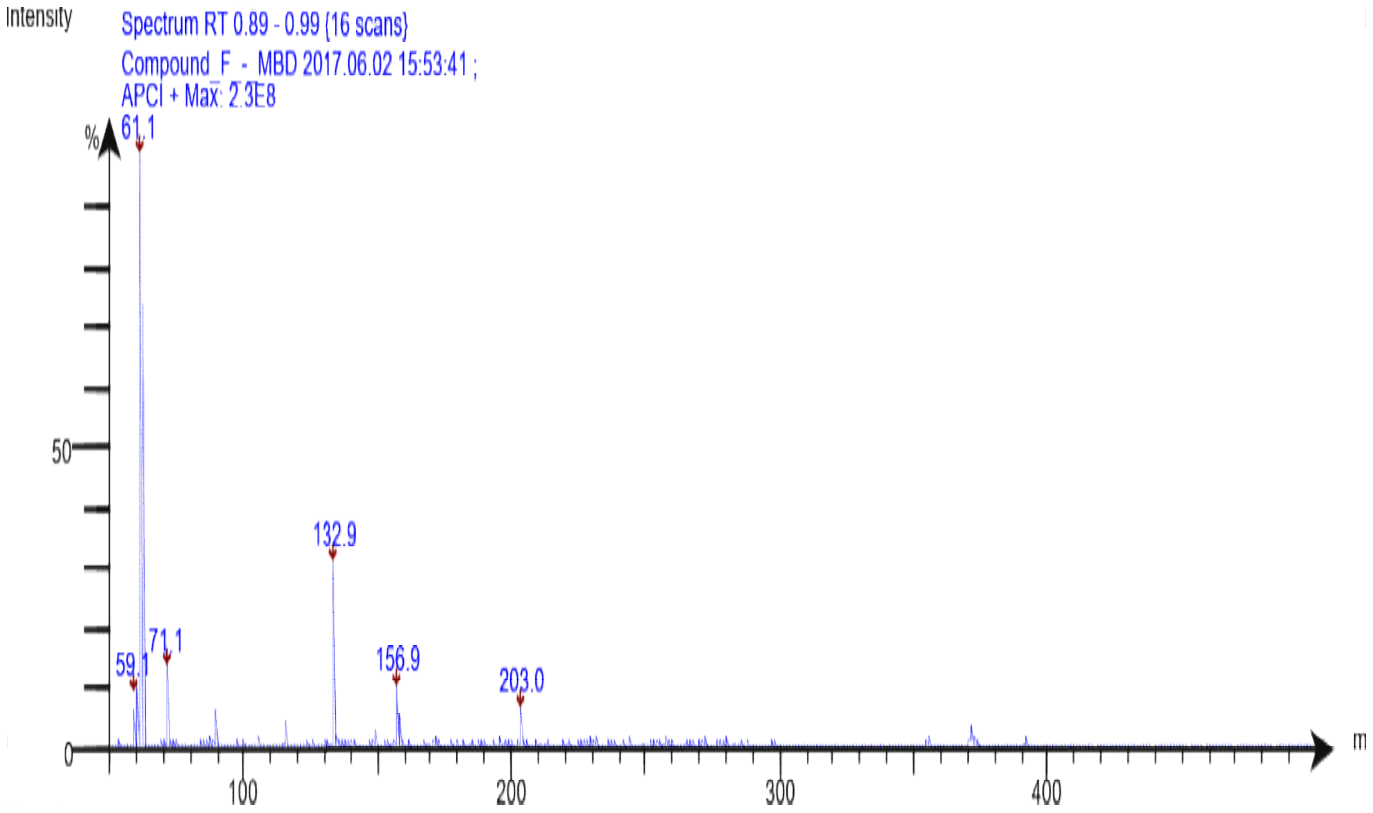
Appendix 4e– HSQC spectra of compound CB3


"Compound C-DMSO" 7 1 /opt/topspin3.0/data/mdybek/nmr
Compound C-DMSO HMQC
F1 [ppm]
20
40
60
80
5
4
3
2
1
F2 [ppm]

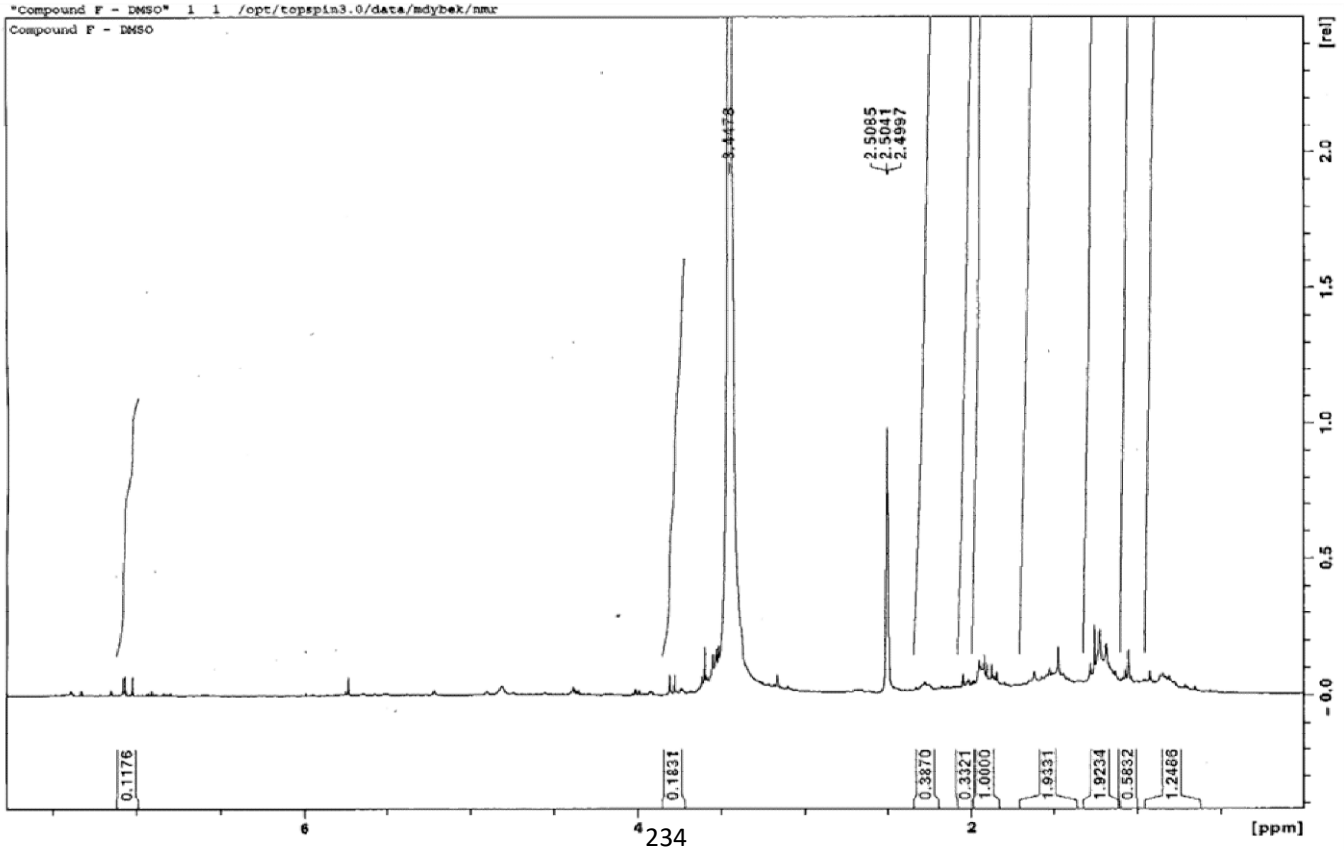
Appendix 4f– NOESY spectra of compound CB3


"Compound C-DMSO" 4 1 /opt/topspin3.0/data/mdybek/nmr
Compound C-DMSO
F1 [ppm]
F2 [ppm]
5
4
3
2
1

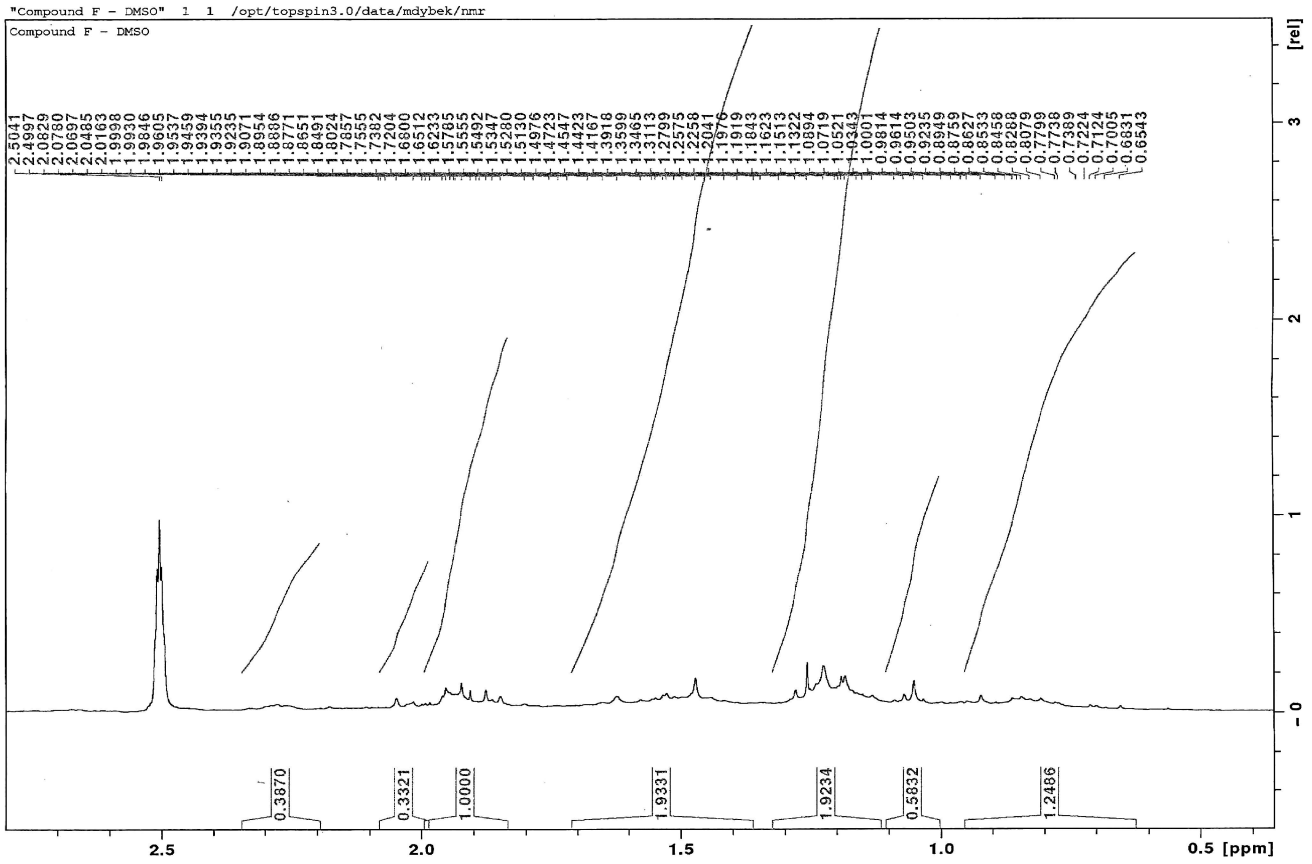
Appendix 5a– Mass spectra of compound CB4



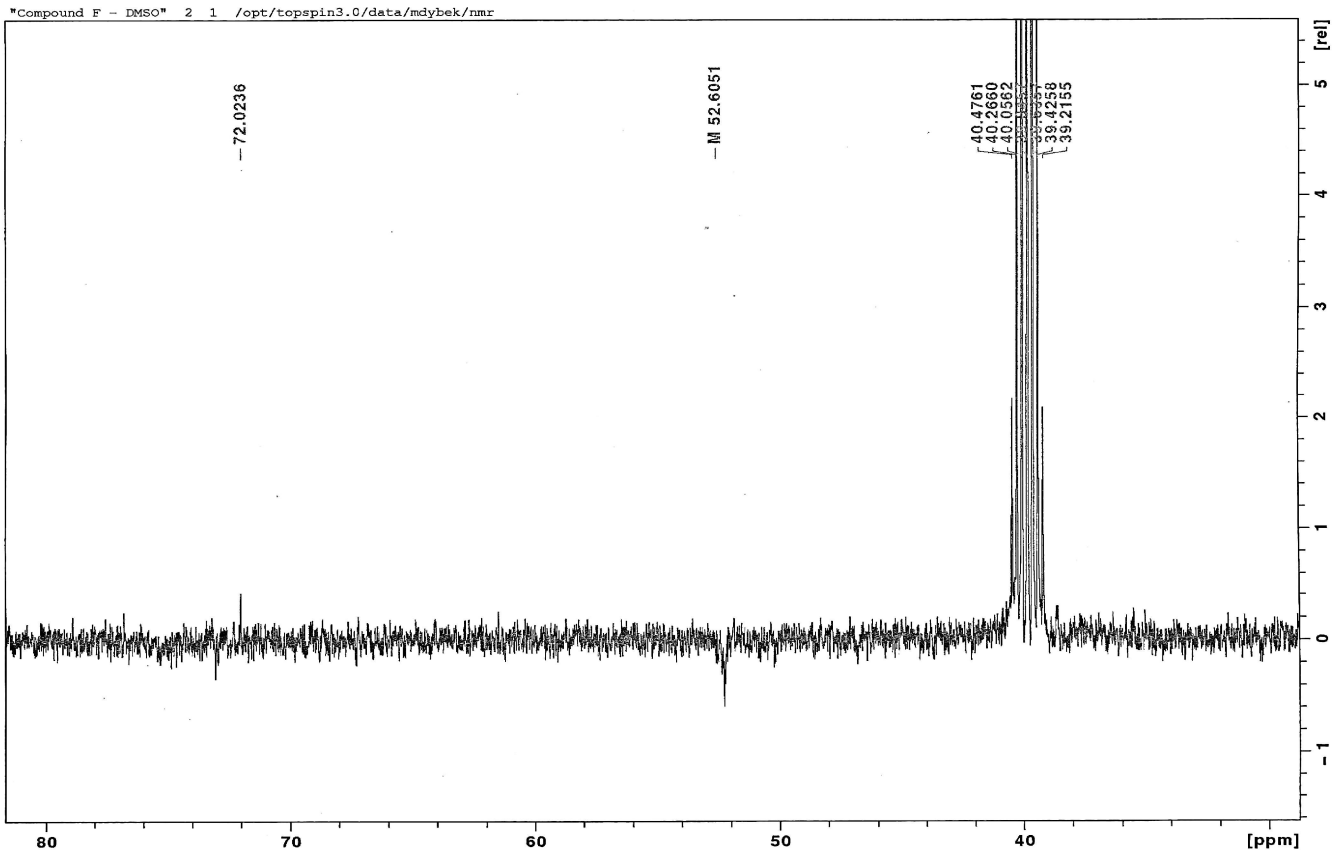
Appendix 5b i– ^{1}H spectra of compound CB4


"Compound F - DMSO" 1 1 /opt/topspin3.0/data/mdybek/nmr
Compound F - DMSO
3.4478
2.5085
2.5041
2.4997
0.1176
0.1831
0.3870
0.3321
1.0000
1.9331
1.9234
0.5832
1.2466
6
4
2
[ppm]
[rel]
2.0
1.5
1.0
0.5
-0.0

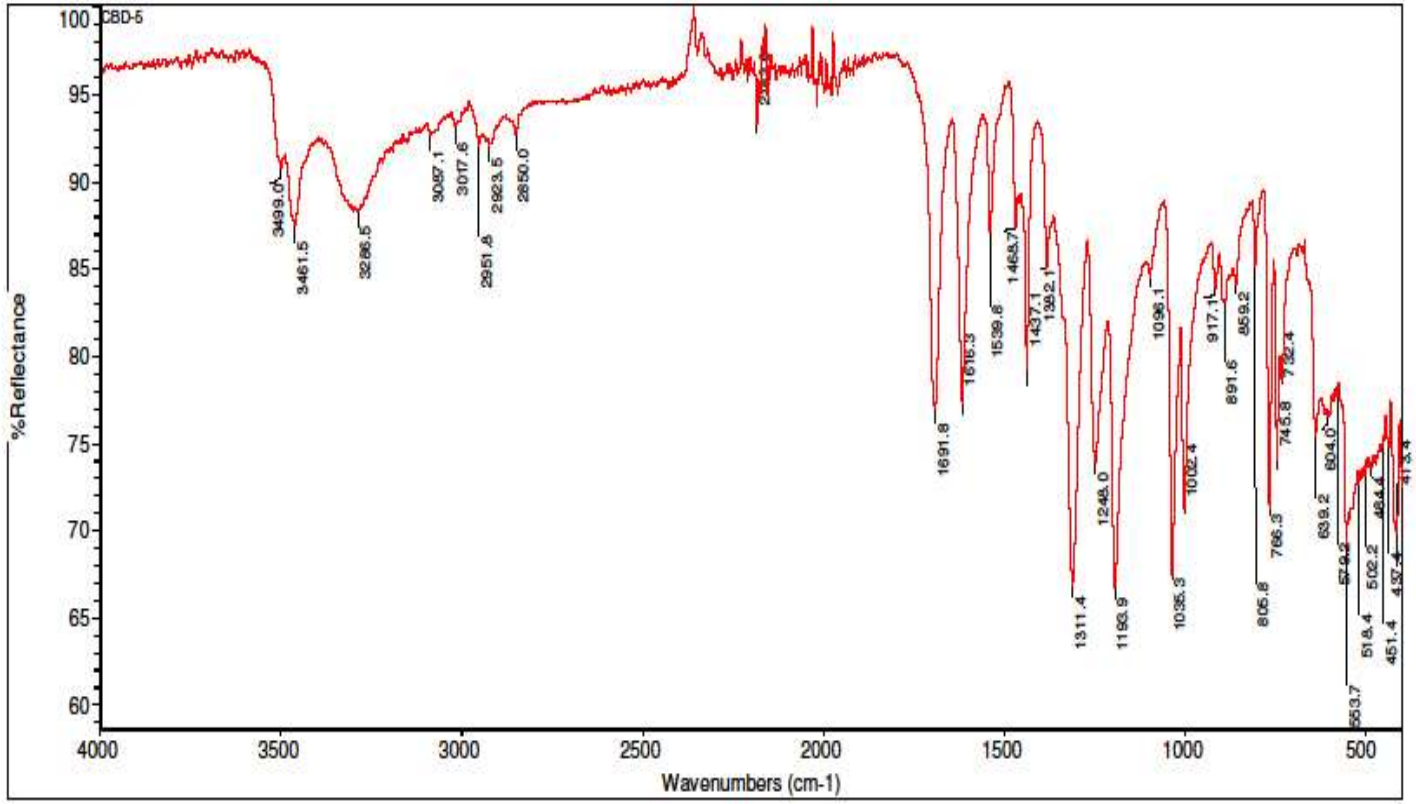
Appendix 5b ii– ^{1}H spectra of compound CB4


"Compound F - DMSO" 1 1 /opt/topspin3.0/data/mdybek/nmr
Compound F - DMSO
2.5041
2.4997
2.0829
2.0780
2.0697
2.0485
2.0163
1.9998
1.9930
1.9846
1.9605
1.9537
1.9459
1.9394
1.9355
1.9235
1.9071
1.8954
1.8836
1.8696
1.8771
1.8651
1.8491
1.8024
1.7857
1.7555
1.7382
1.7204
1.6800
1.6512
1.6233
1.5785
1.5555
1.5492
1.5347
1.5280
1.5130
1.4976
1.4723
1.4547
1.4423
1.4167
1.3918
1.3599
1.3465
1.3113
1.2799
1.2575
1.2256
1.2041
1.1976
1.1919
1.1843
1.1623
1.1513
1.1322
1.0894
1.0719
1.0521
1.0343
1.0001
0.9814
0.9614
0.9503
0.9235
0.8949
0.8759
0.8627
0.8533
0.8458
0.8288
0.8079
0.7799
0.7738
0.7389
0.7224
0.7124
0.7065
0.6831
0.6543
0.3870
0.3321
1.0000
1.9331
1.9234
0.5832
1.2486
2.5
2.0
1.5
1.0
0.5 [ppm]
[rel]
3
2
1
0

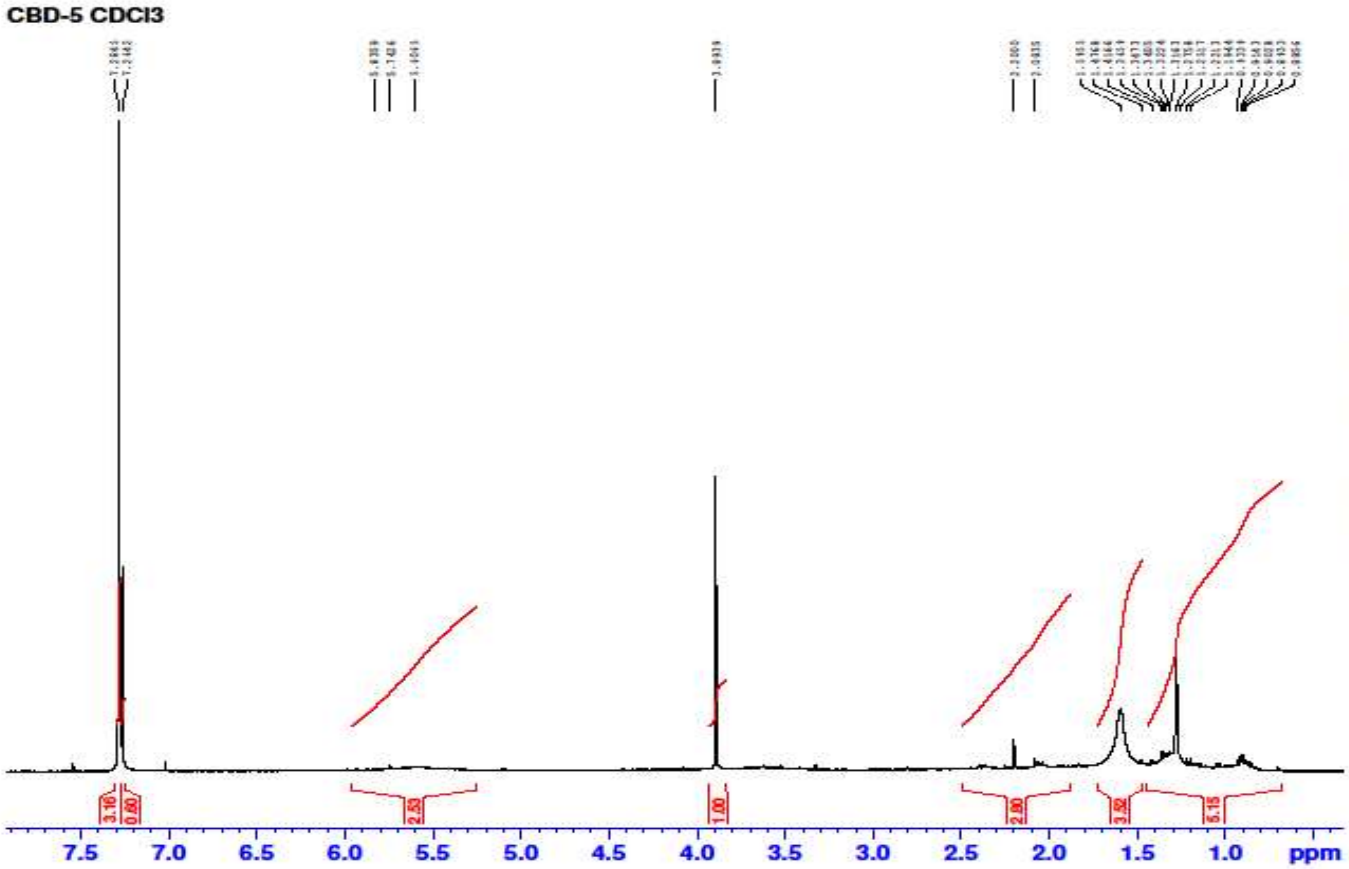
Appendix 5c– DEPT spectra of compound CB4


"Compound F - DMSO" 2 1 /opt/topspin3.0/data/mdybek/nmr
72.0236
M 52.6051
40.4761
40.2660
40.0582
39.4258
39.2155
[rel]
5
4
3
2
1
0
-1
80
70
60
50
40
[ppm]

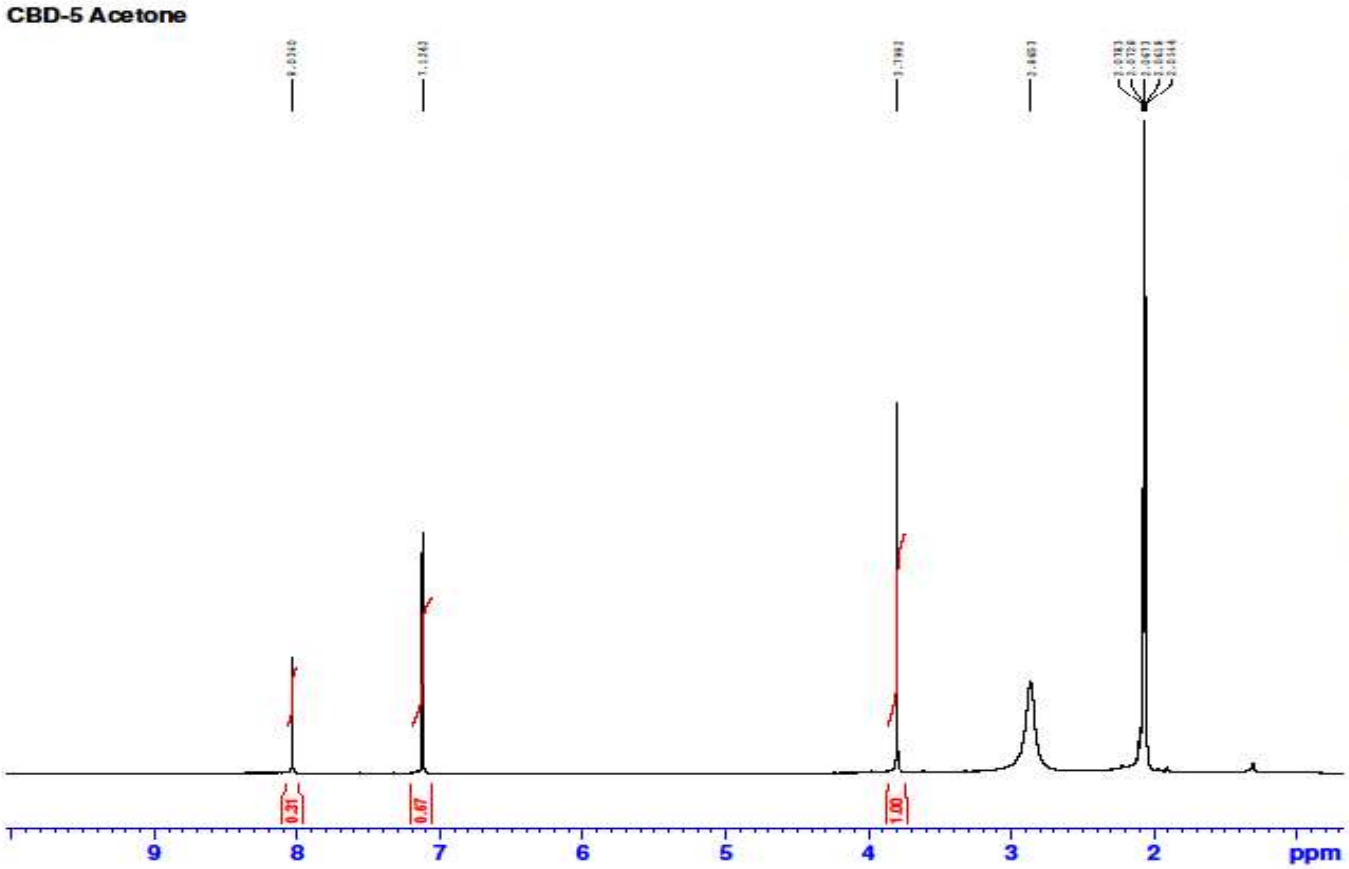
Appendix 6a– IF spectra of compound CB5



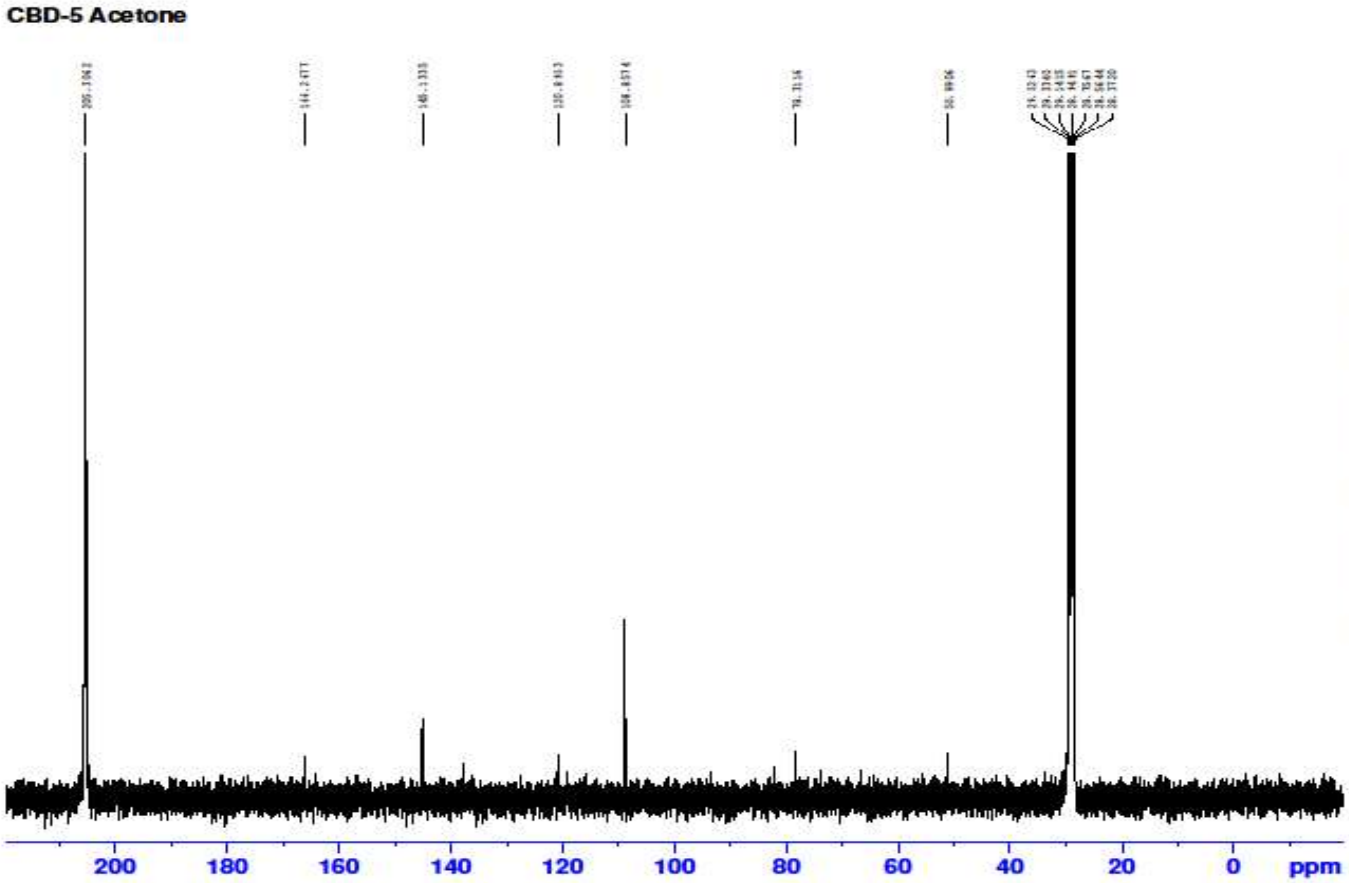
Appendix 6b i– ^{1}H spectra of compound CB5



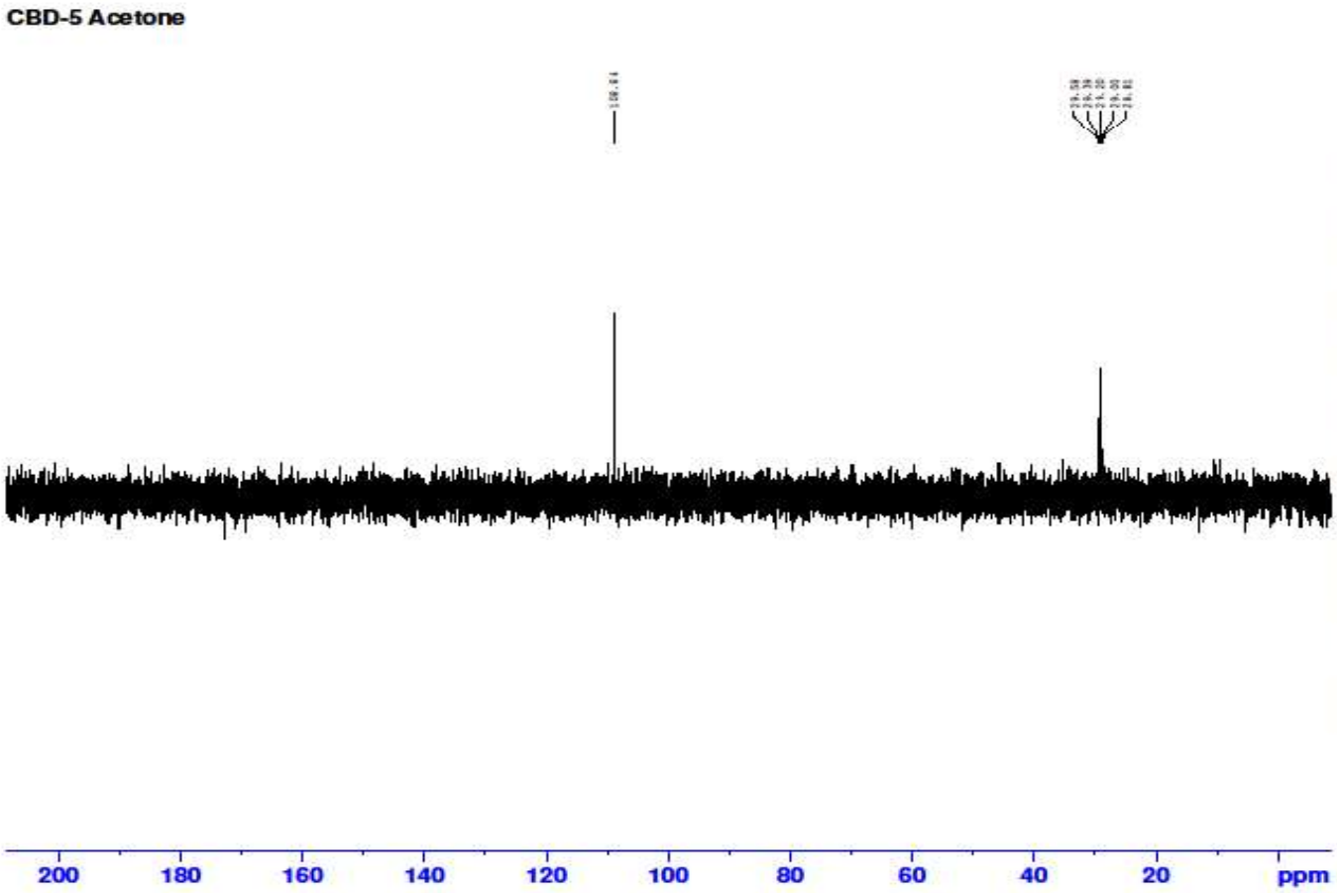
Appendix 6bii– ^{1}H spectra of compound CB5



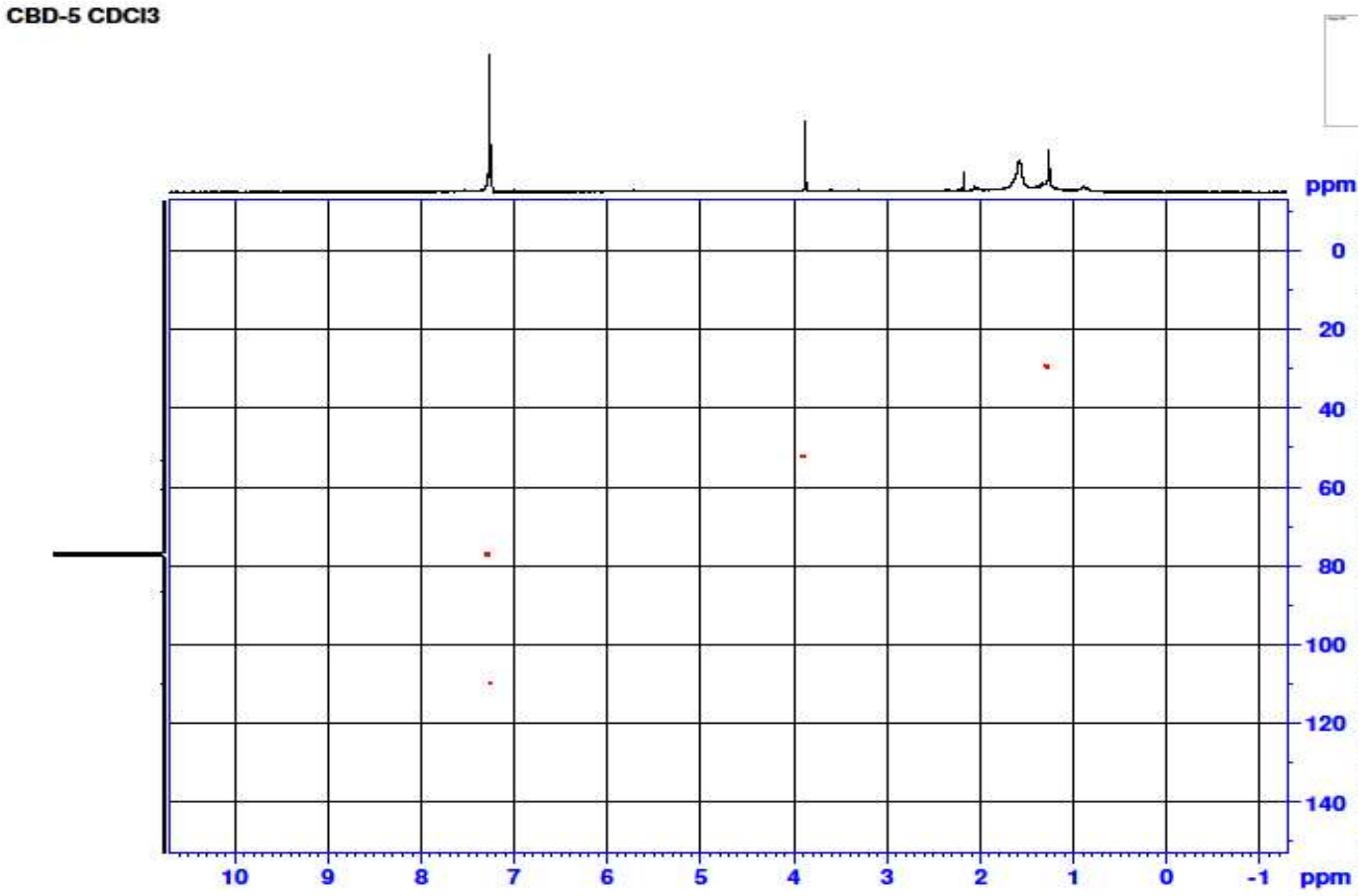
Appendix 6c– ^{13}C spectra of compound CB5



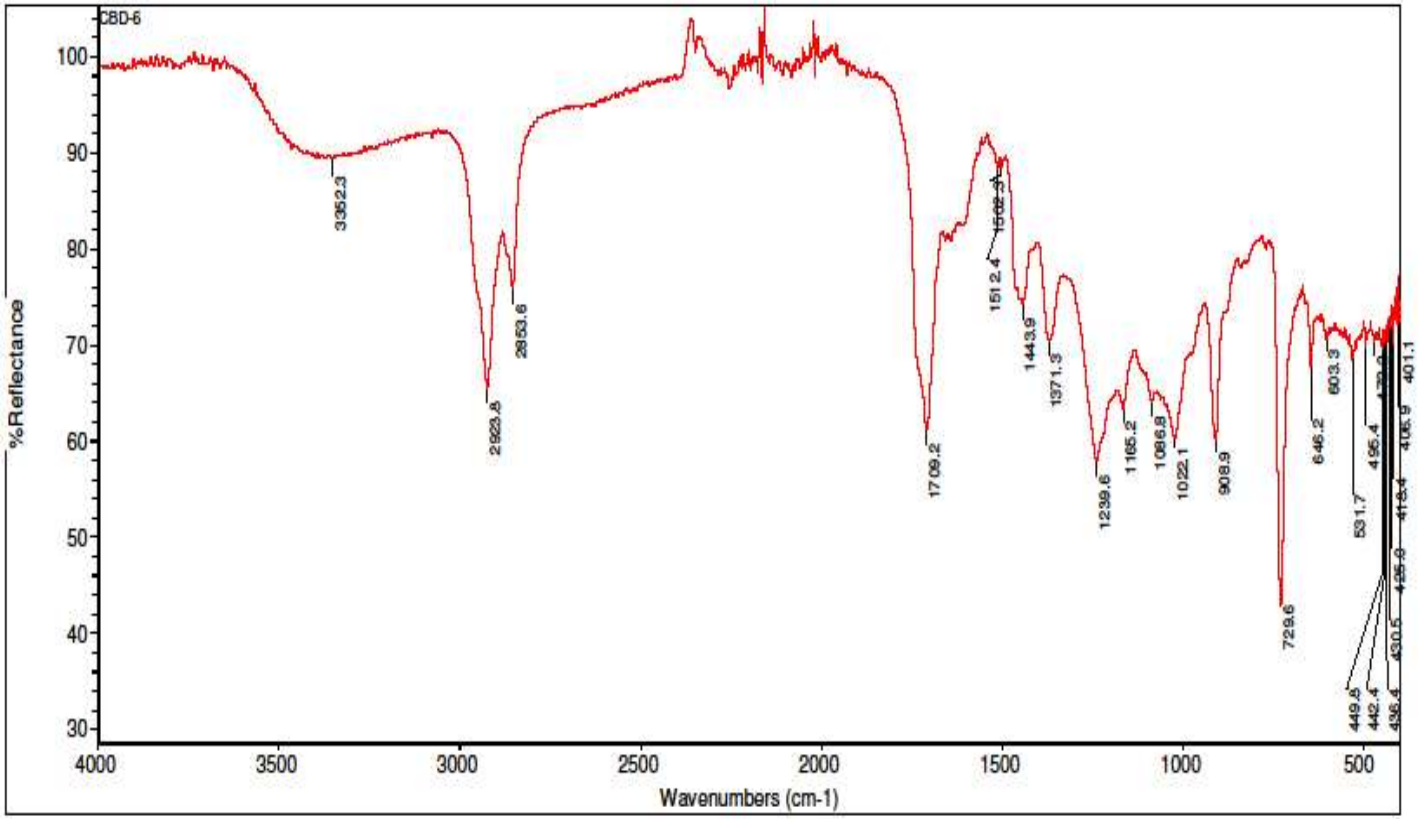
Appendix 6d– DEPT spectra of compound CB5



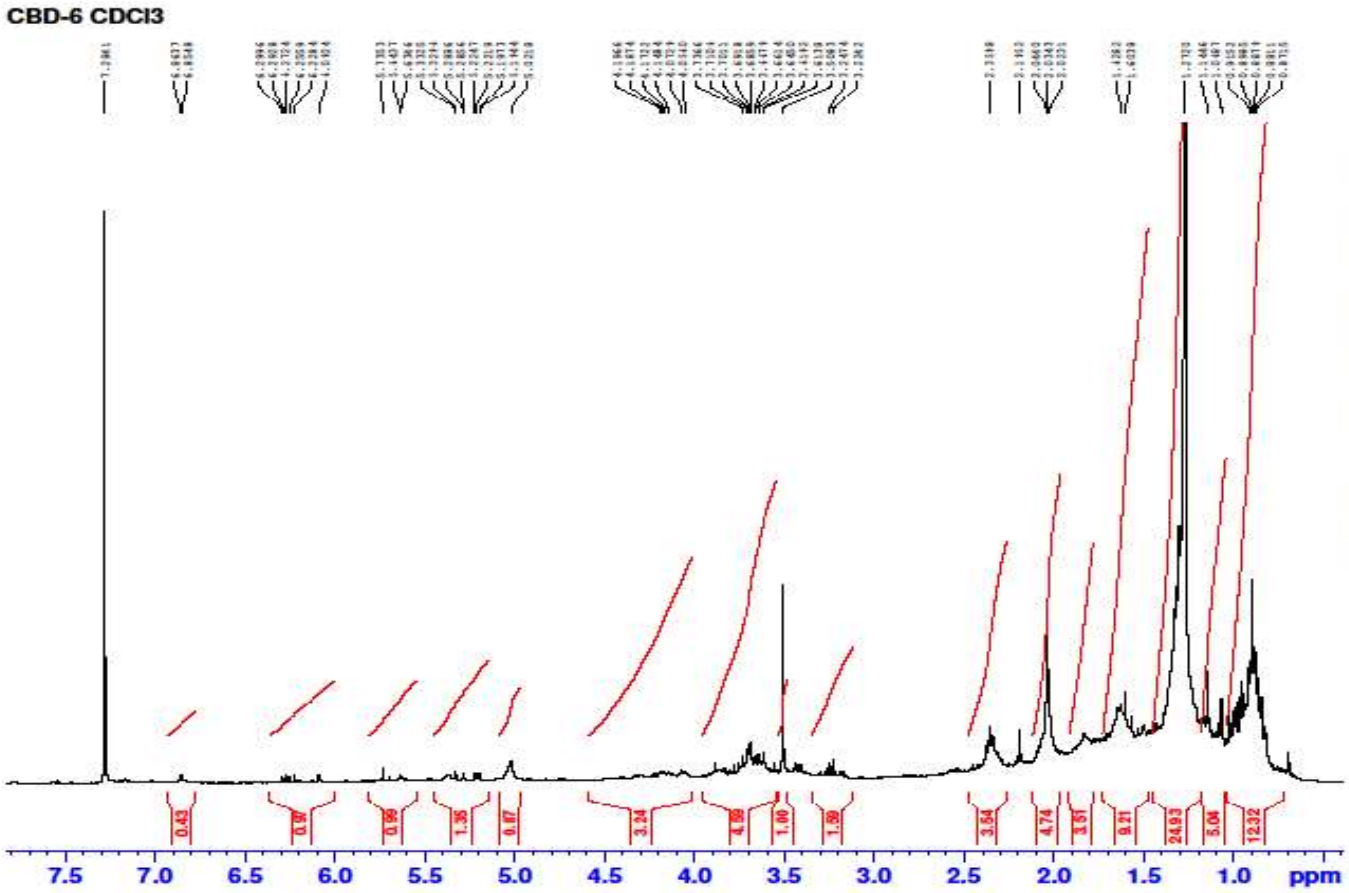
Appendix 6e– COSY spectra of compound CB5



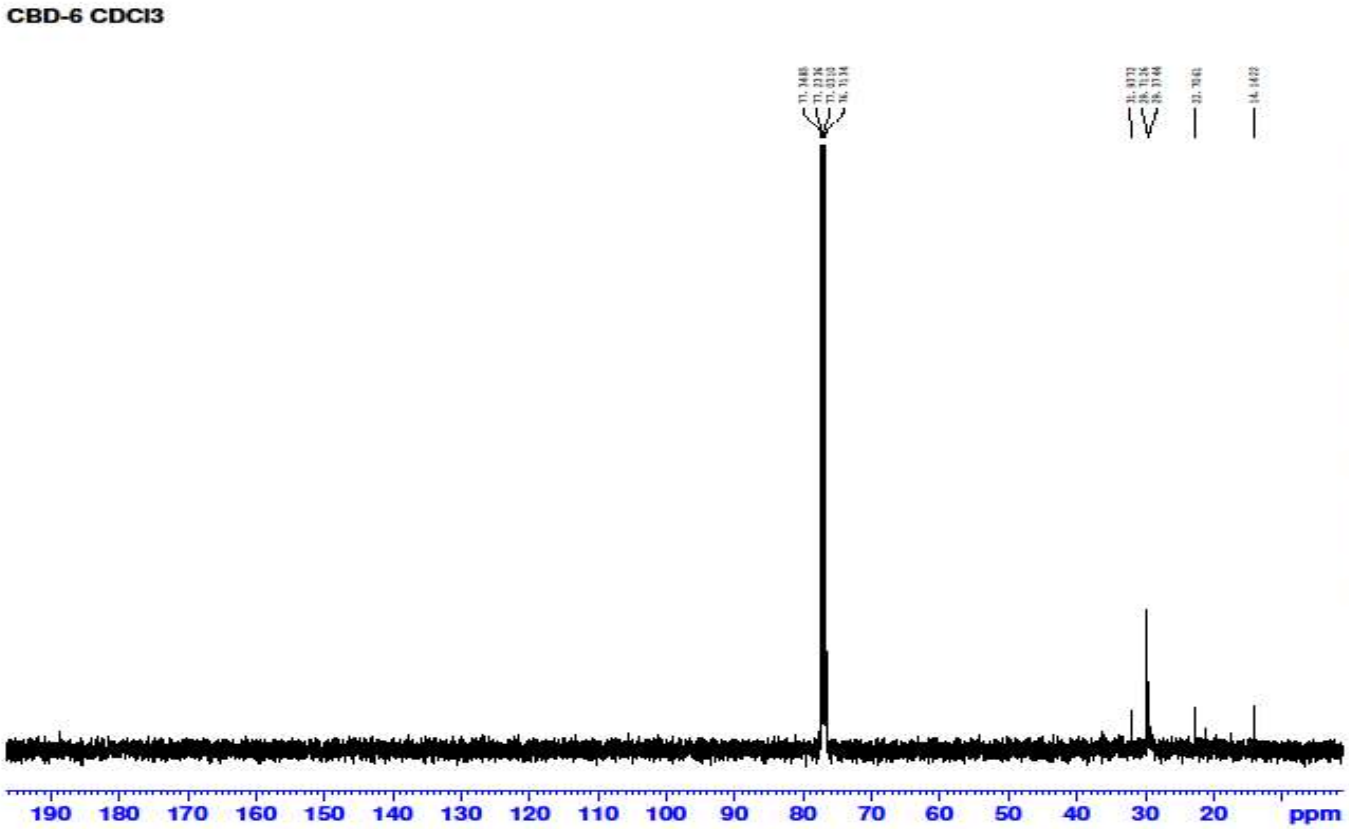
Appendix 7a– IF spectra of compound CB6



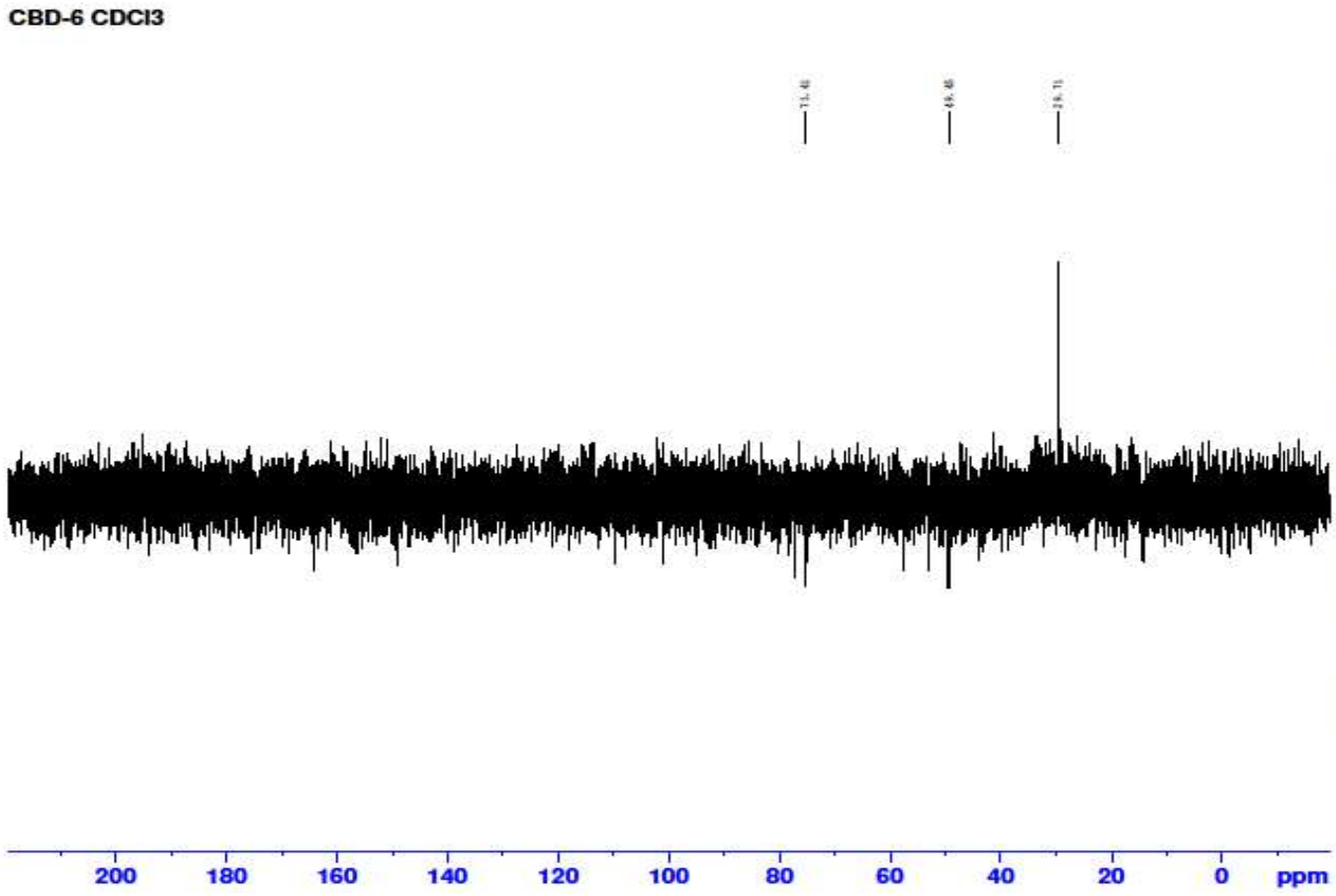
Appendix 7b– ^{1}H spectra of compound CB6



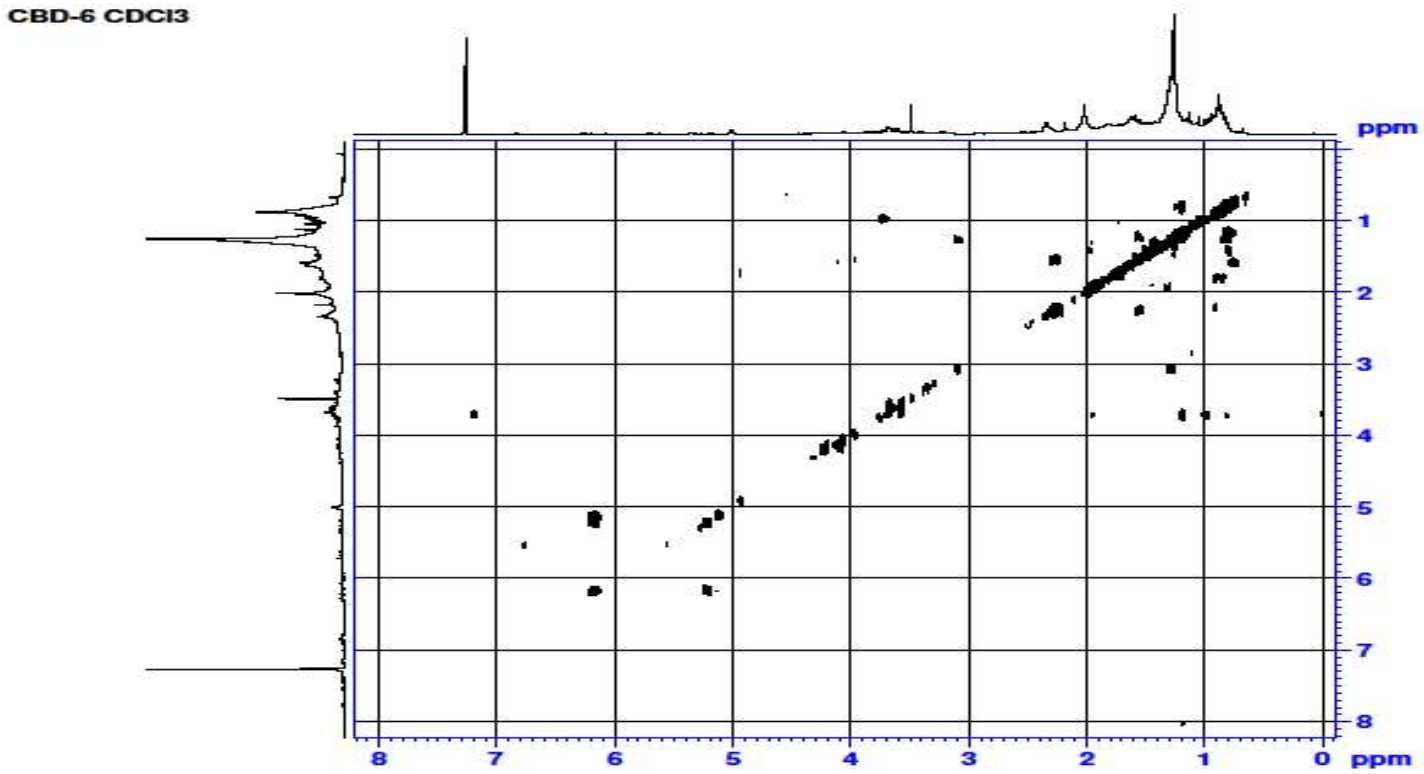
Appendix 7c– ^{13}C spectra of compound CB6



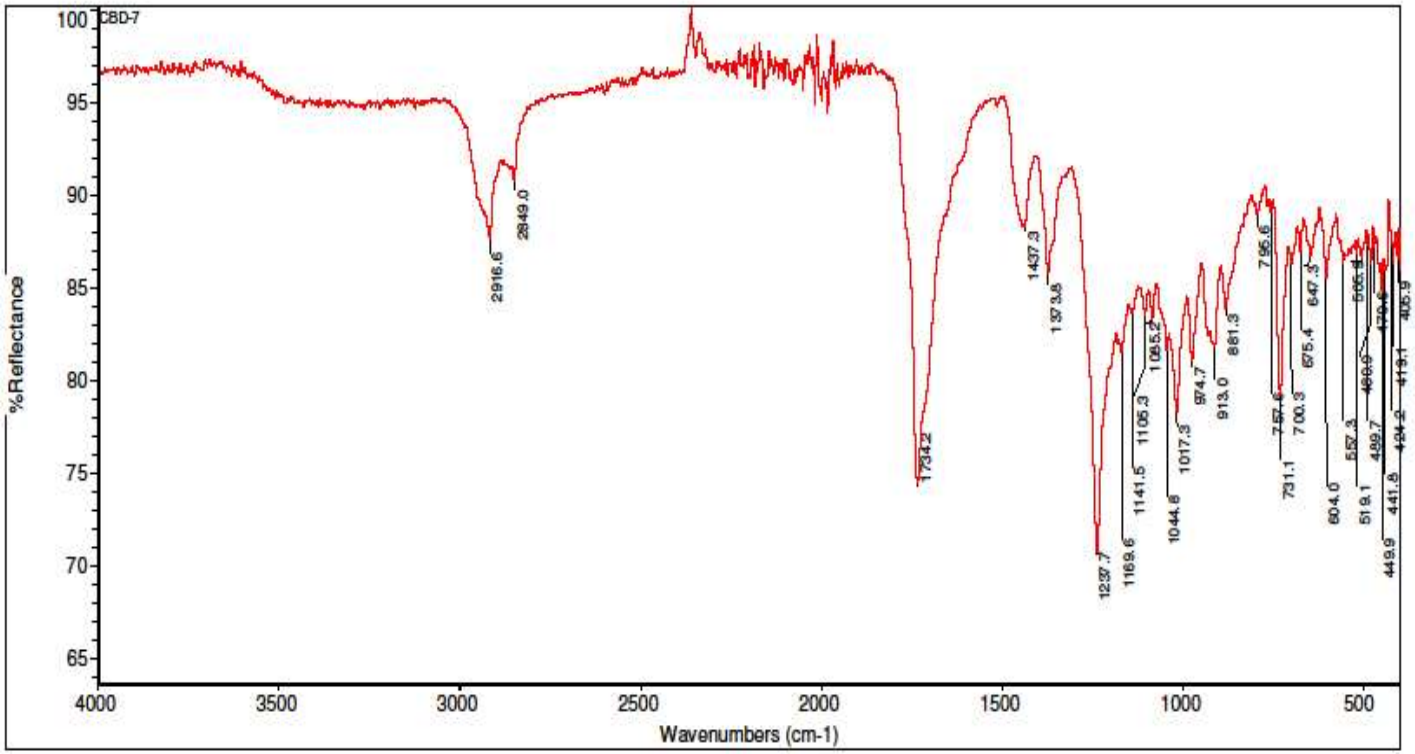
Appendix 7d– DEPT spectra of compound CB6



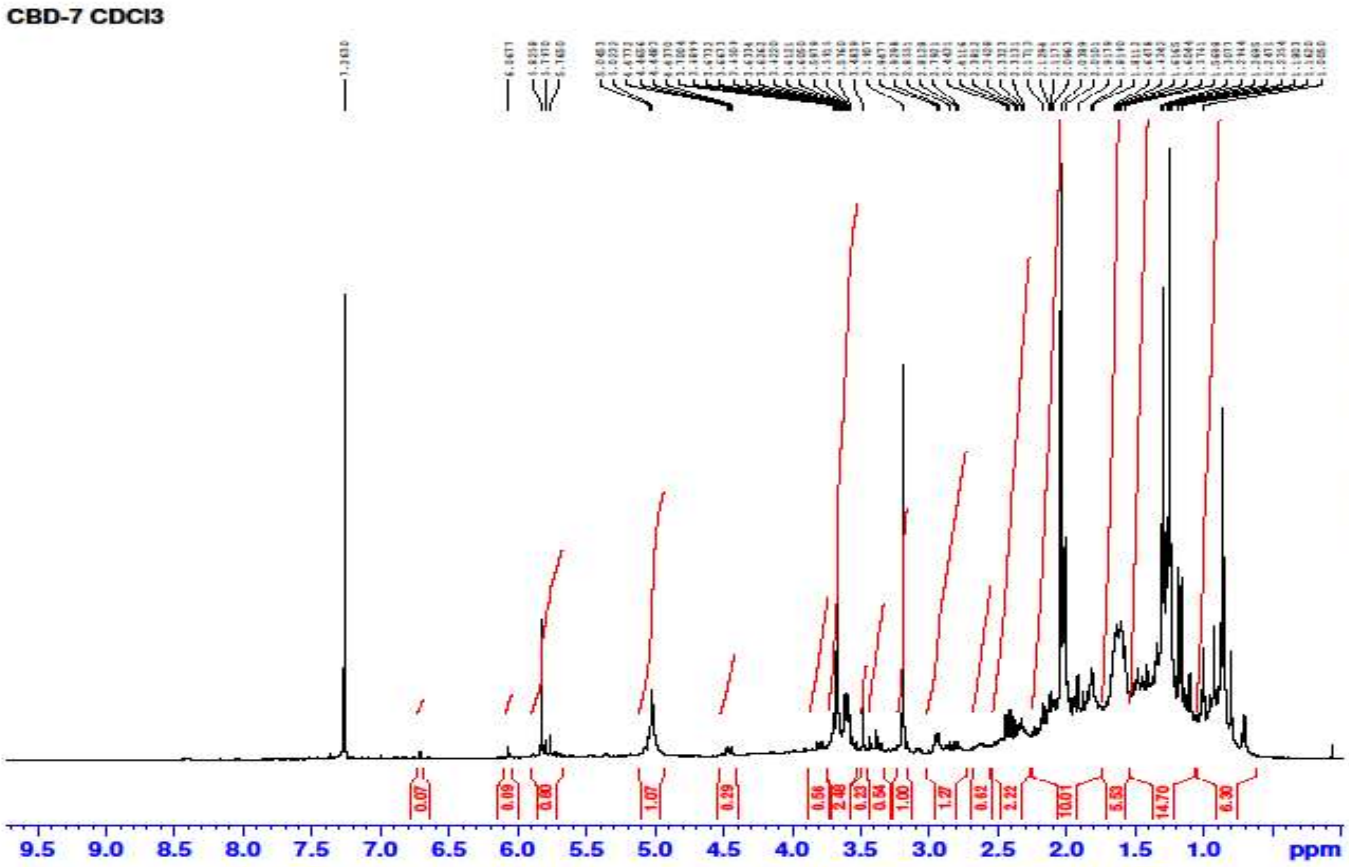
Appendix 7e– COSY spectra of compound CB6



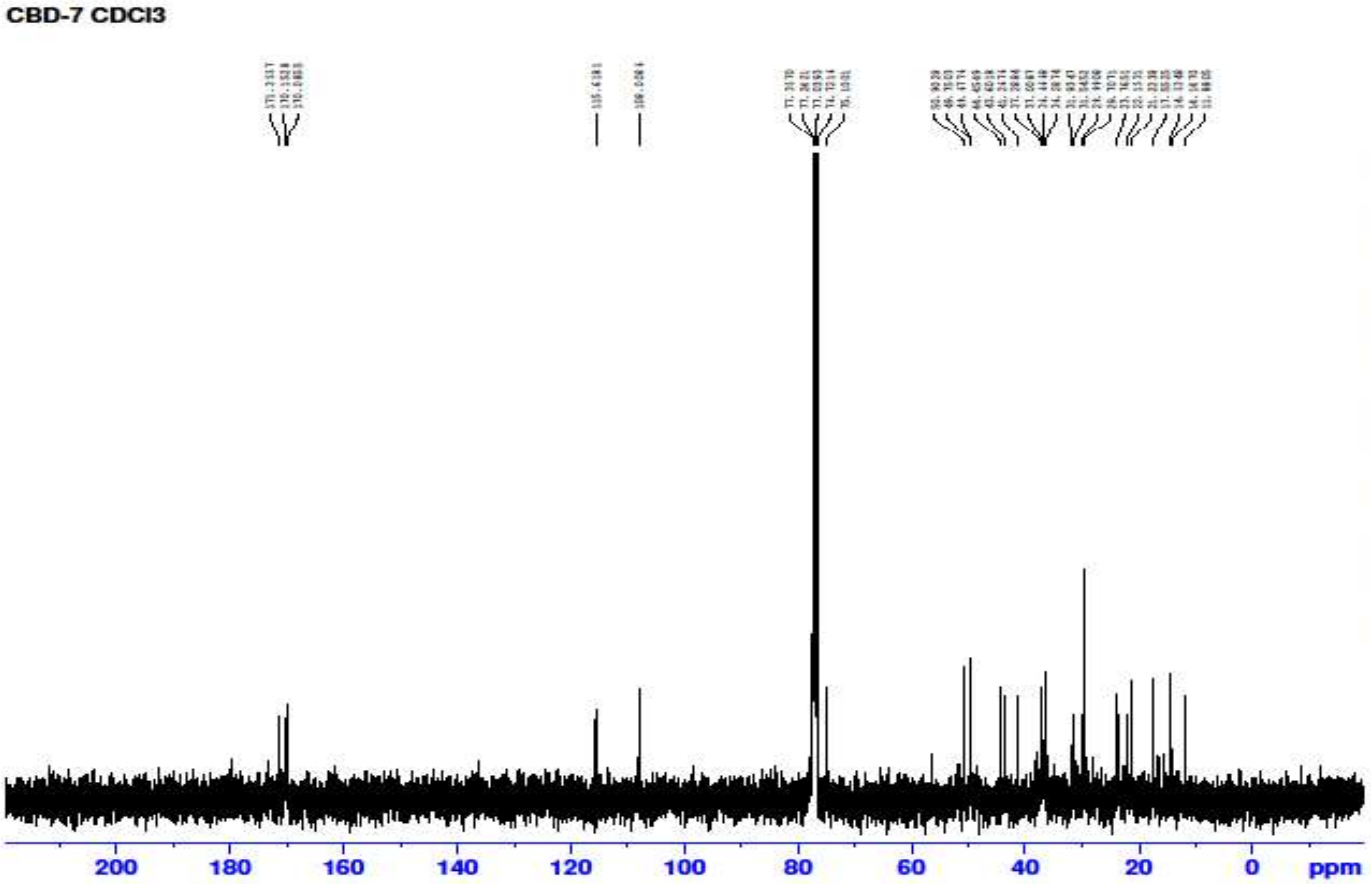
Appendix 8a– IF spectra of compound CB7



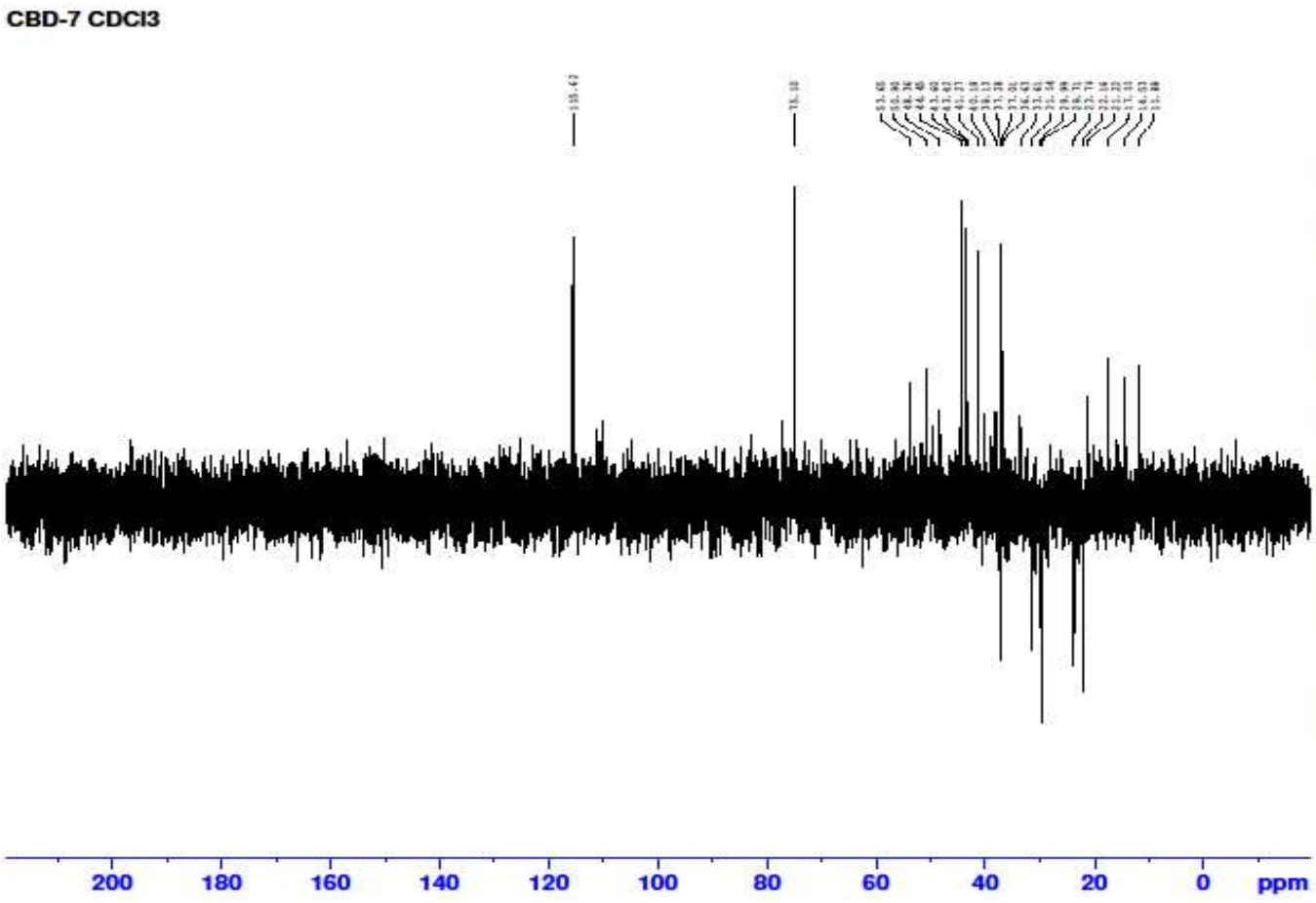
Appendix 8b– ^{1}H spectra of compound CB7



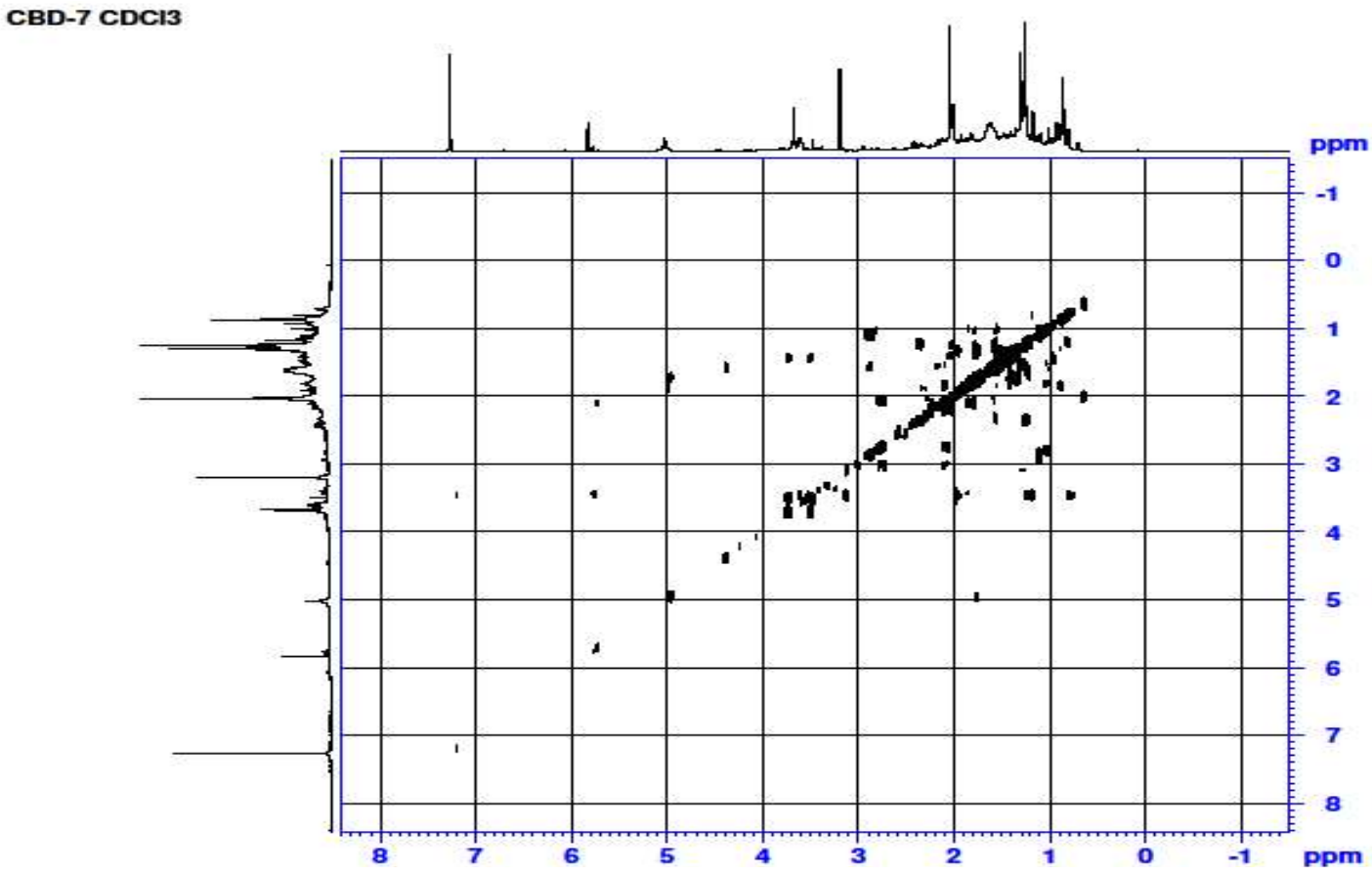
Appendix 8c– ^{13}C spectra of compound CB7



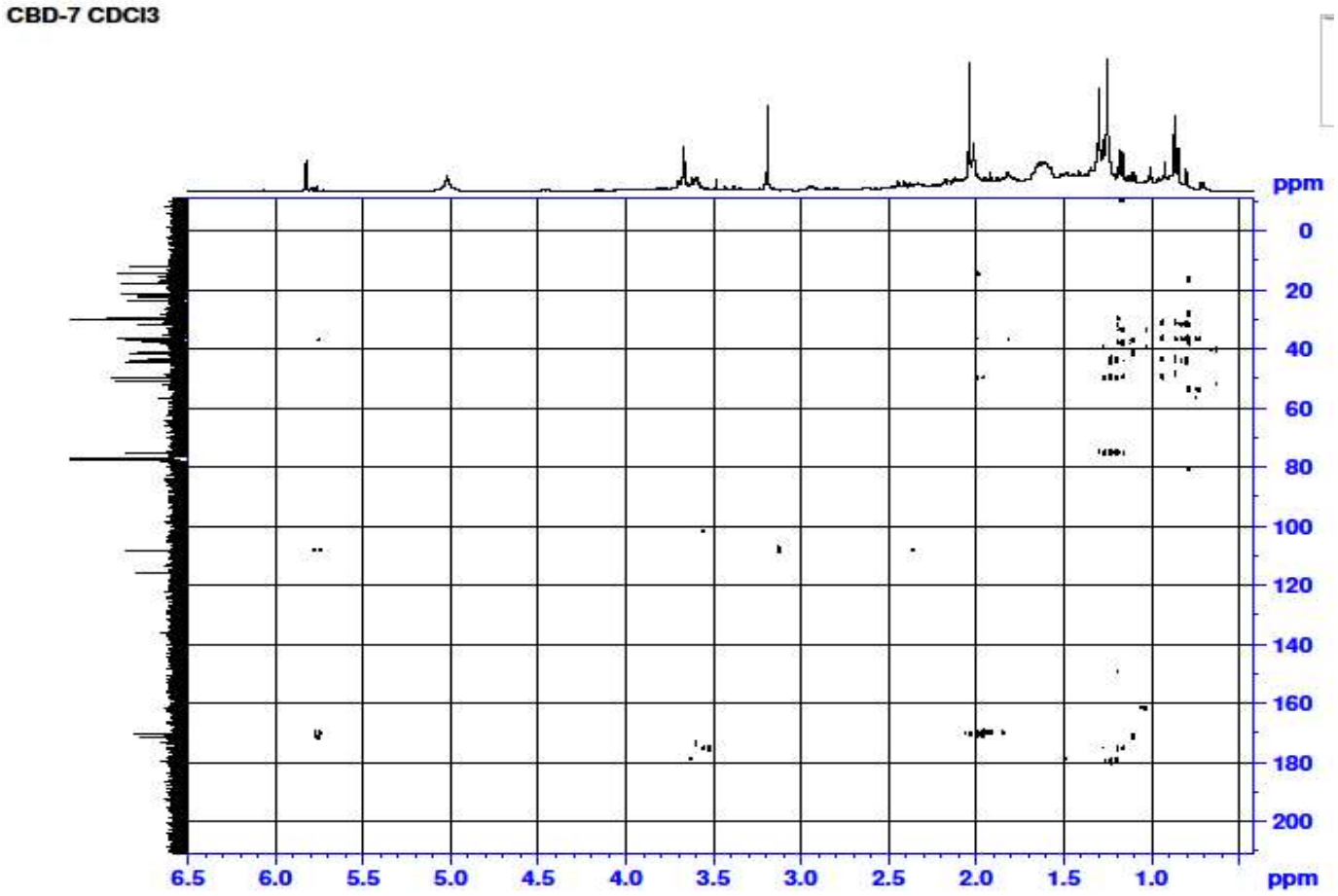
Appendix 8d– DEPT spectra of compound CB7



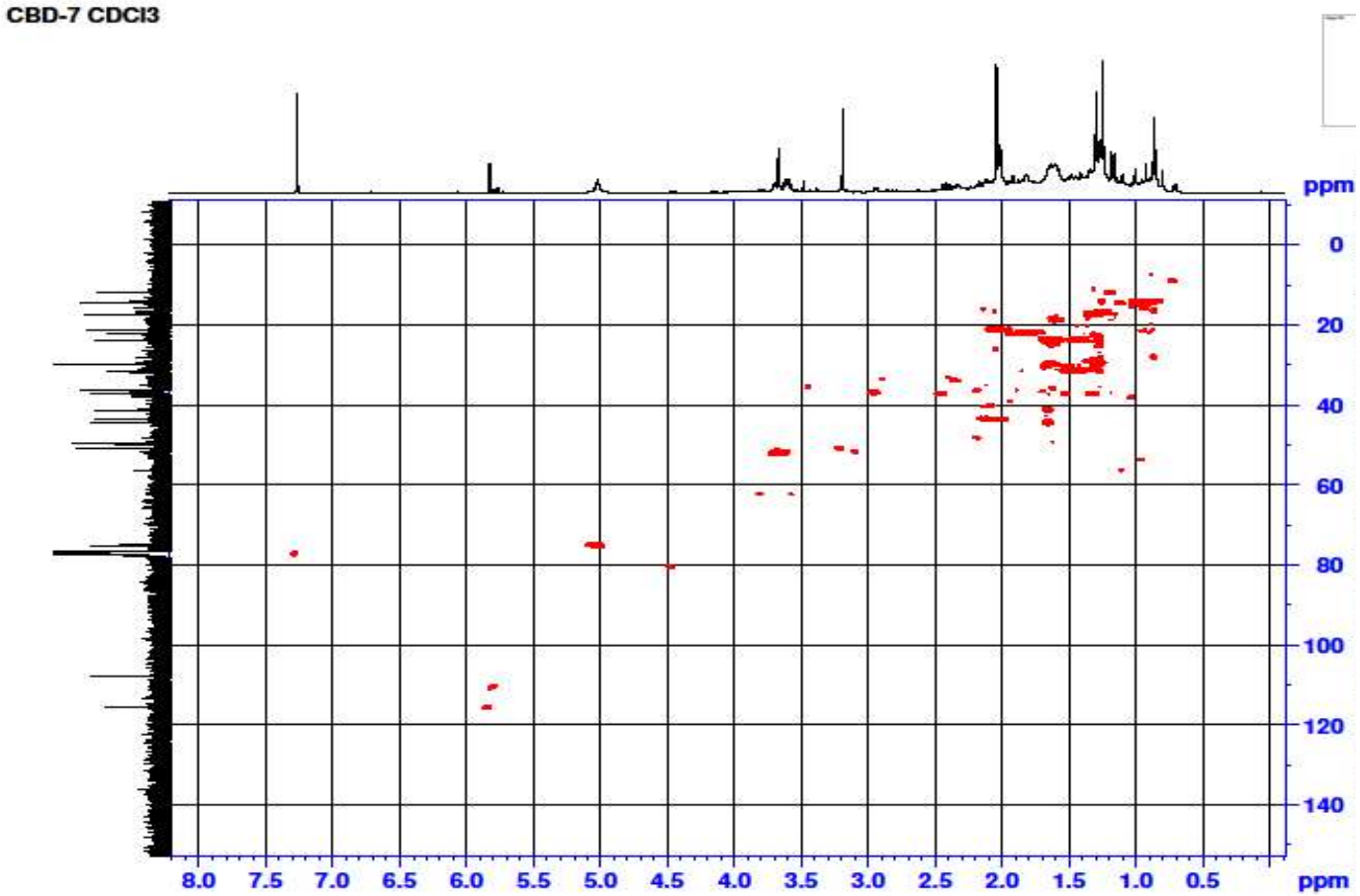
Appendix 8e– COSY spectra of compound CB7



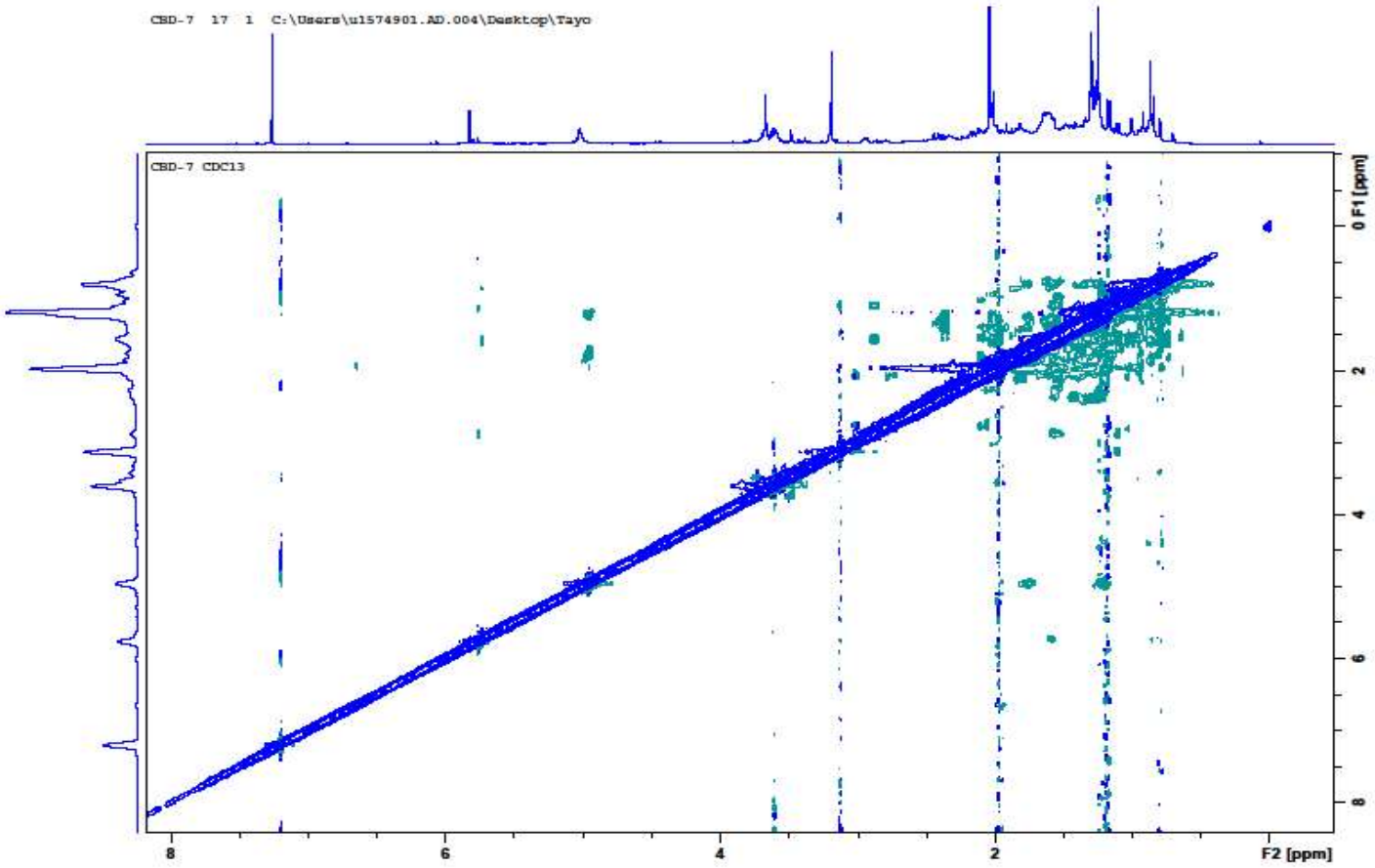
Appendix 8f– HMBC spectra of compound CB7



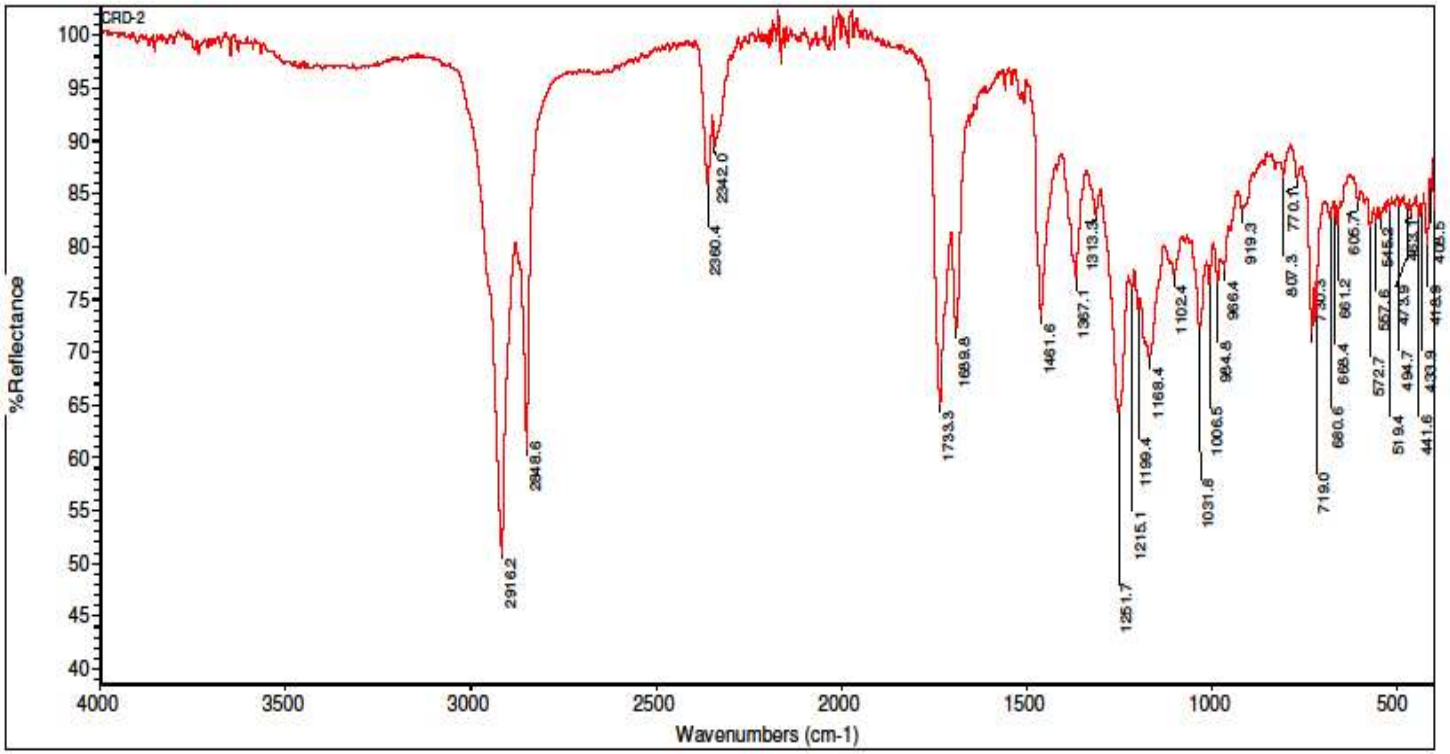
Appendix 8g– HSQC spectra of compound CB7



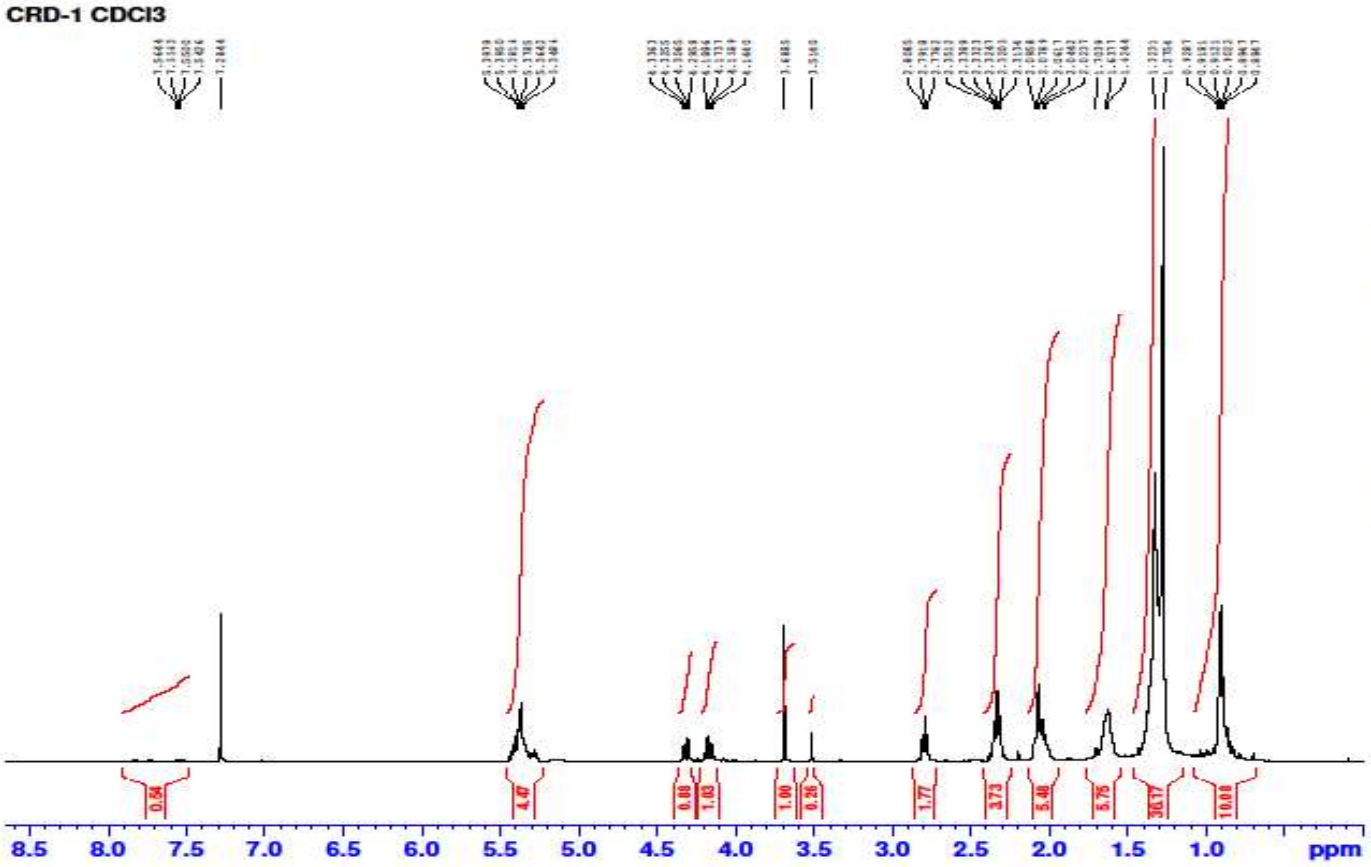
Appendix 8h– NOESY spectra of compound CB7



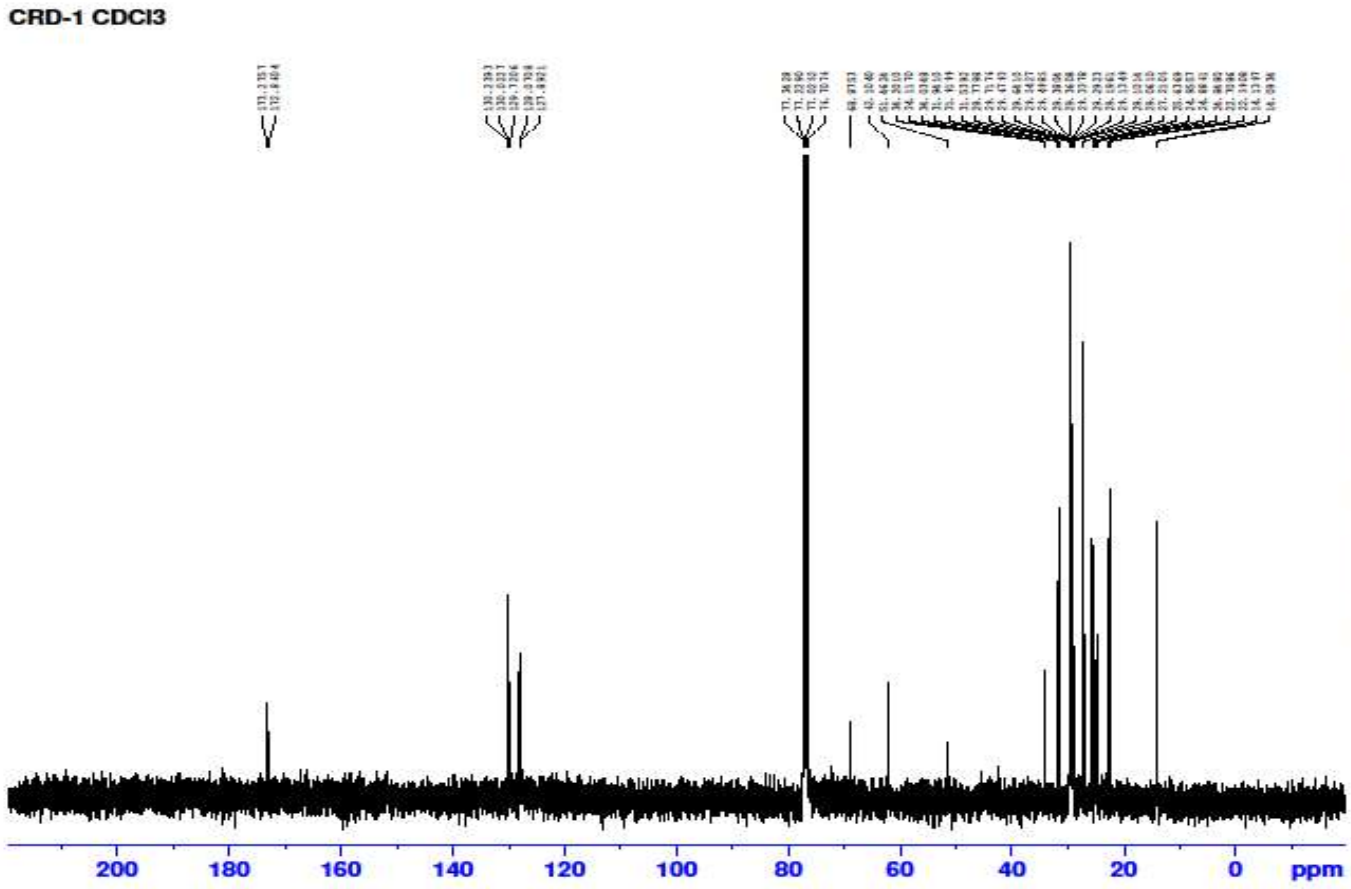
Appendix 9a– IR spectra of compound CR1



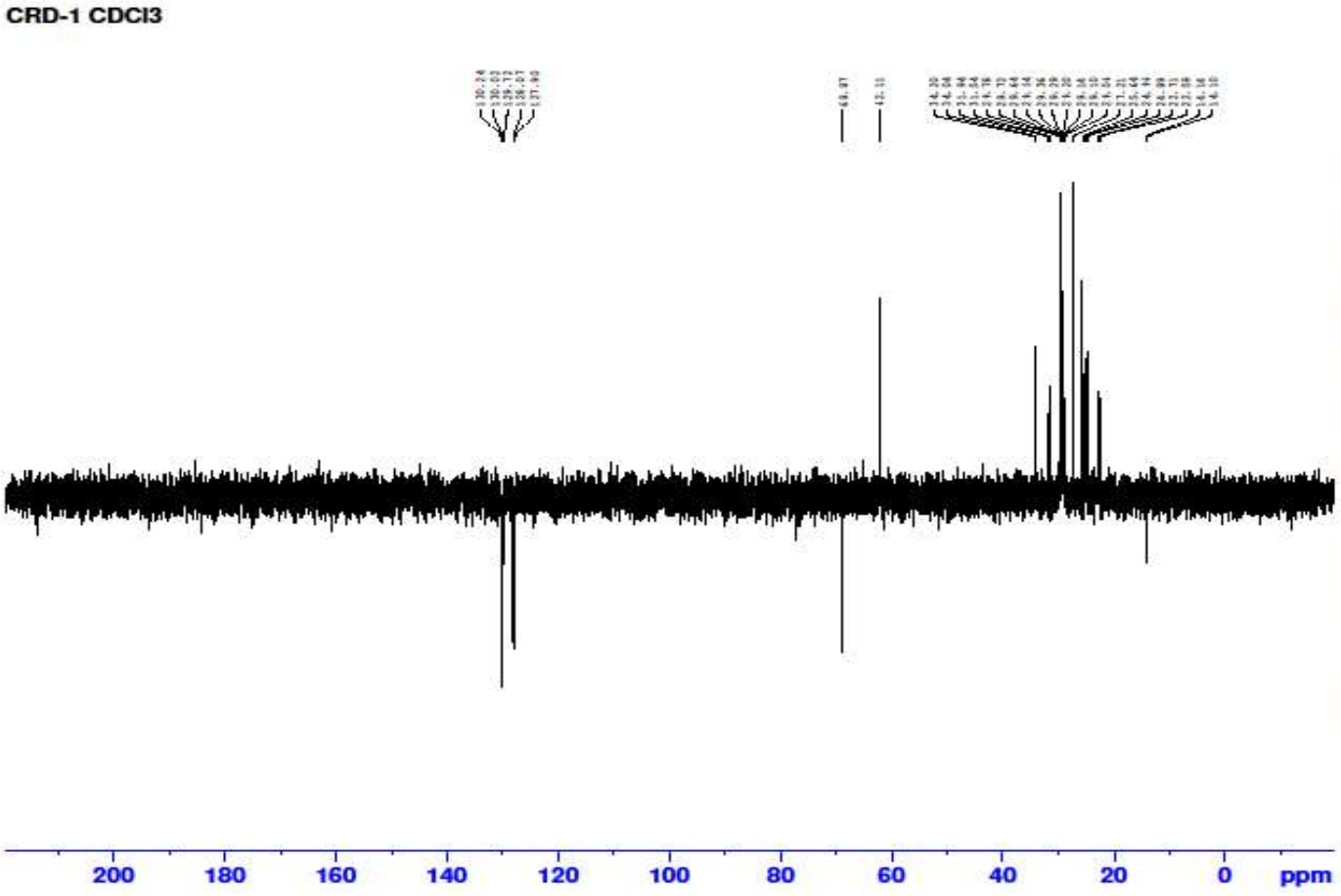
Appendix 9b– ^{1}H spectra of compound CR1



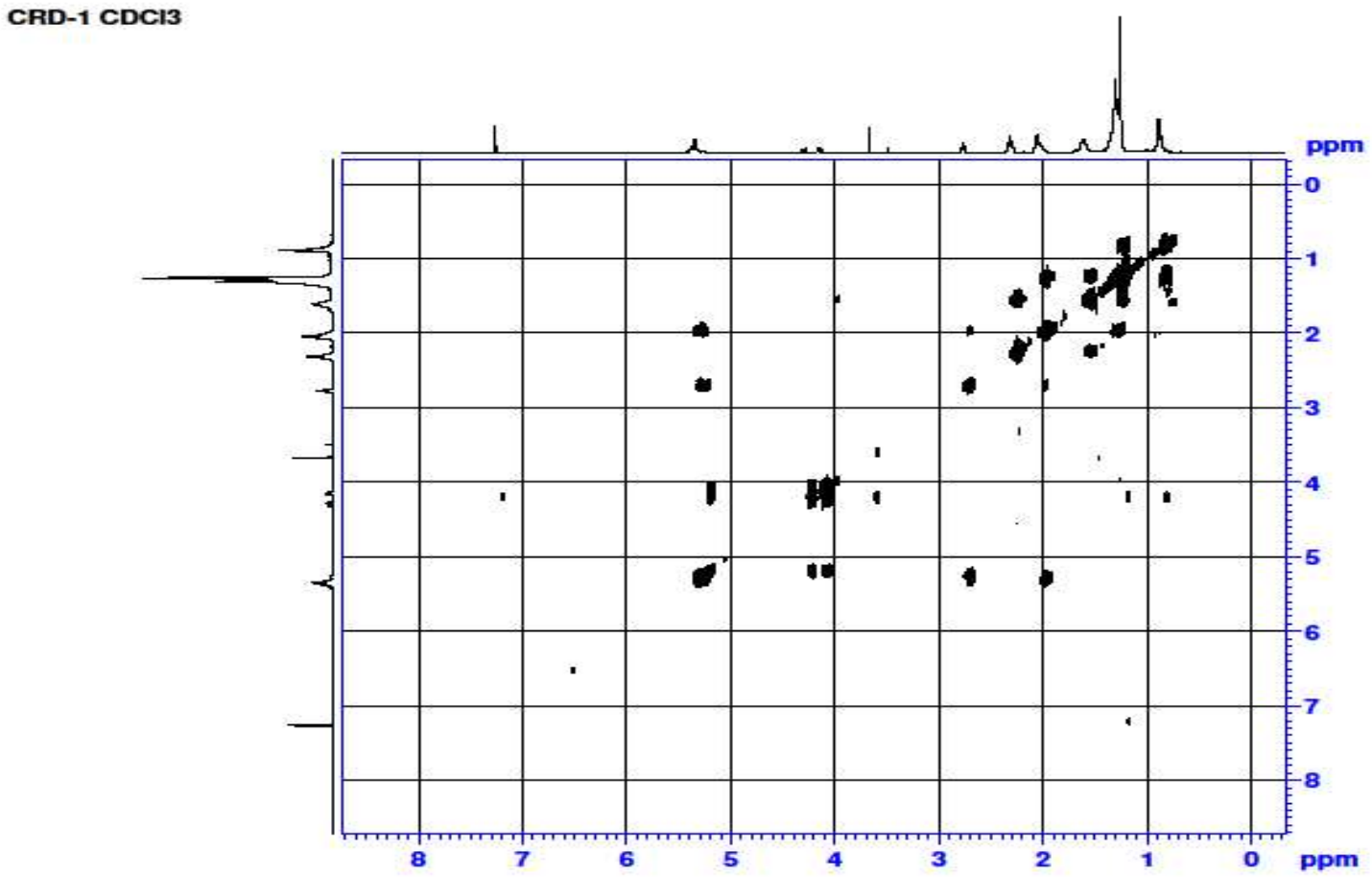
Appendix 9c– ^{13}C spectra of compound CR1



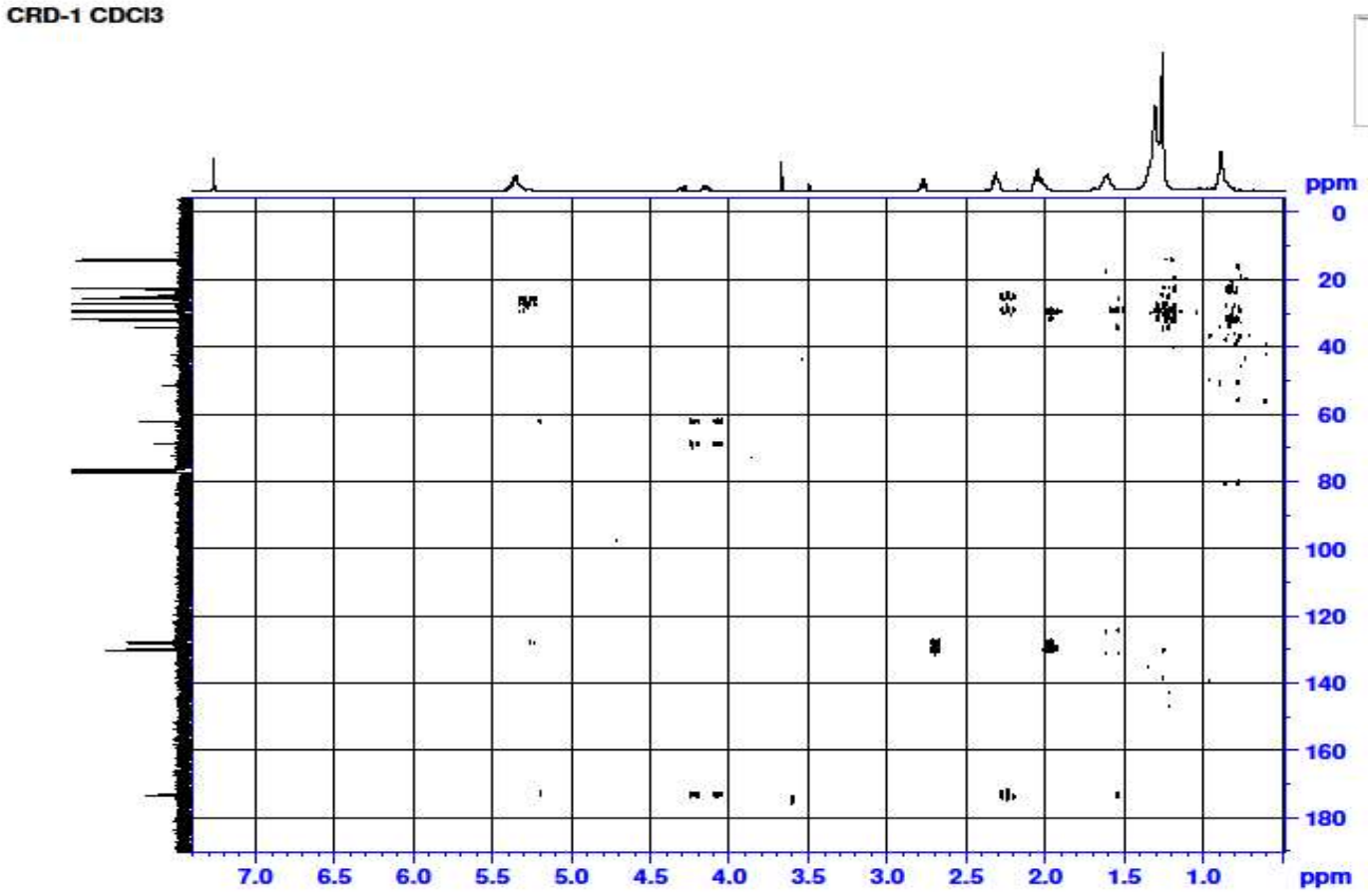
Appendix 9d– DEPT spectra of compound CR1



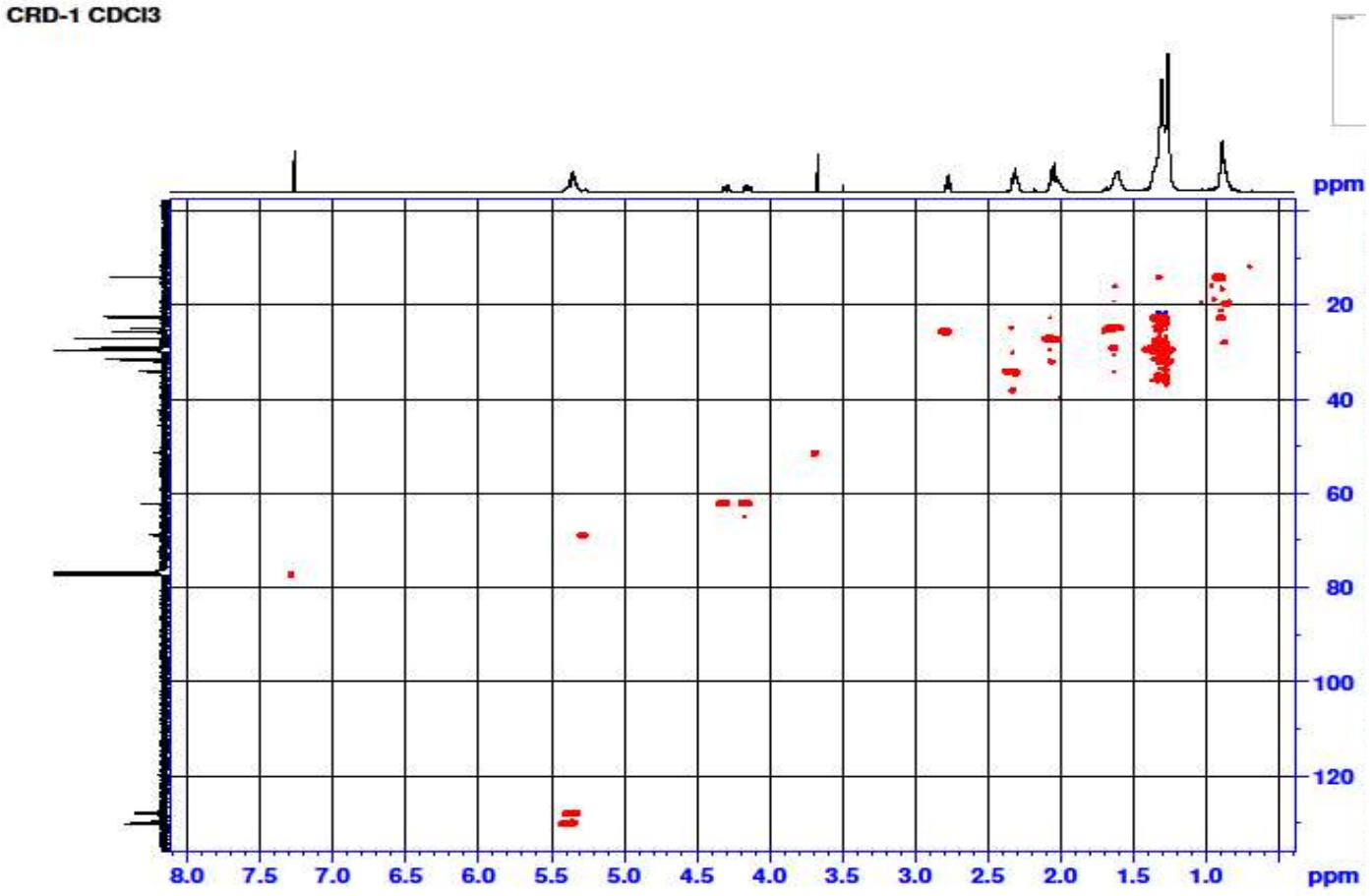
Appendix 9e– COSY spectra of compound CR1



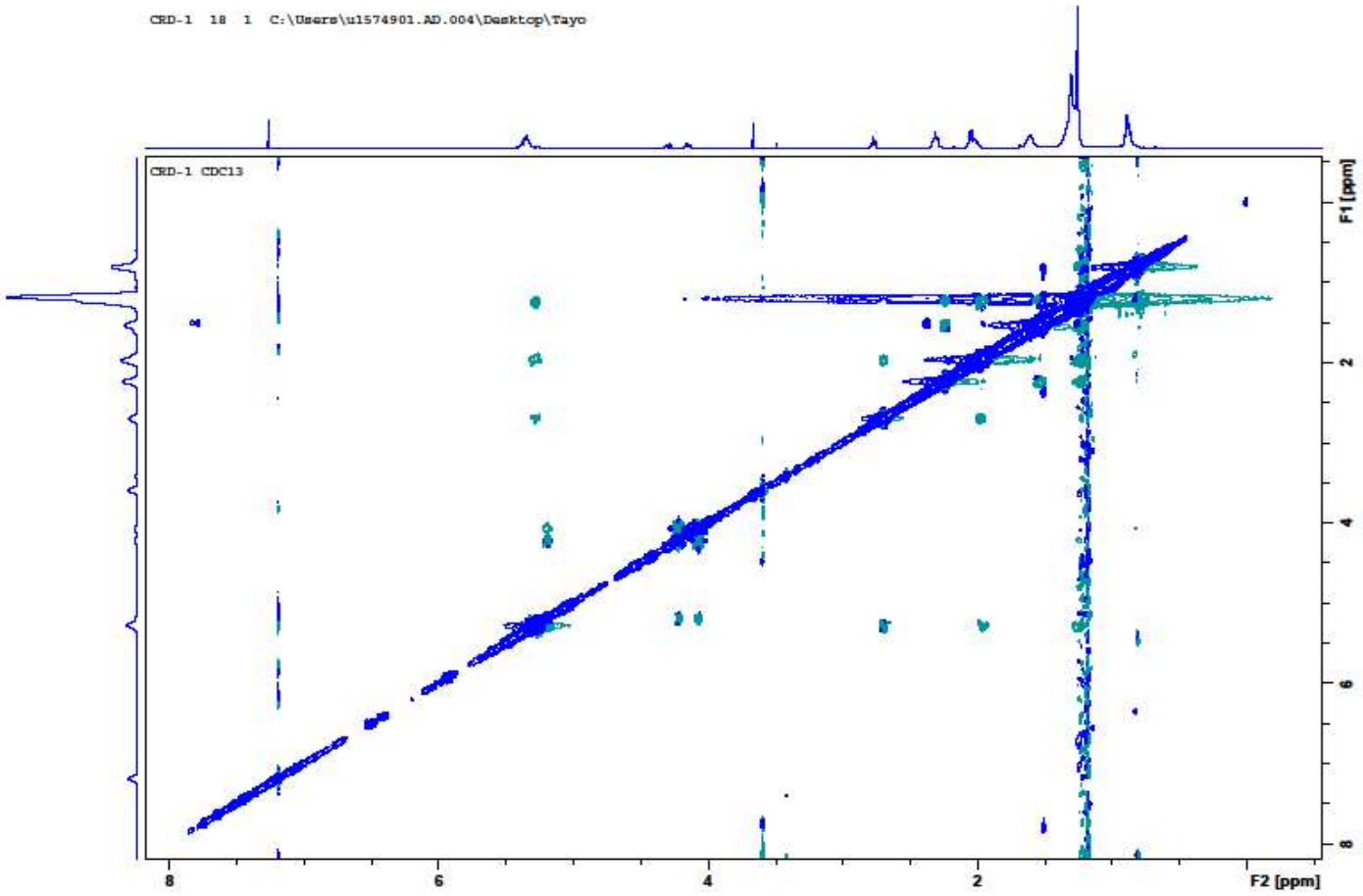
Appendix 9f– HMBC spectra of compound CR1



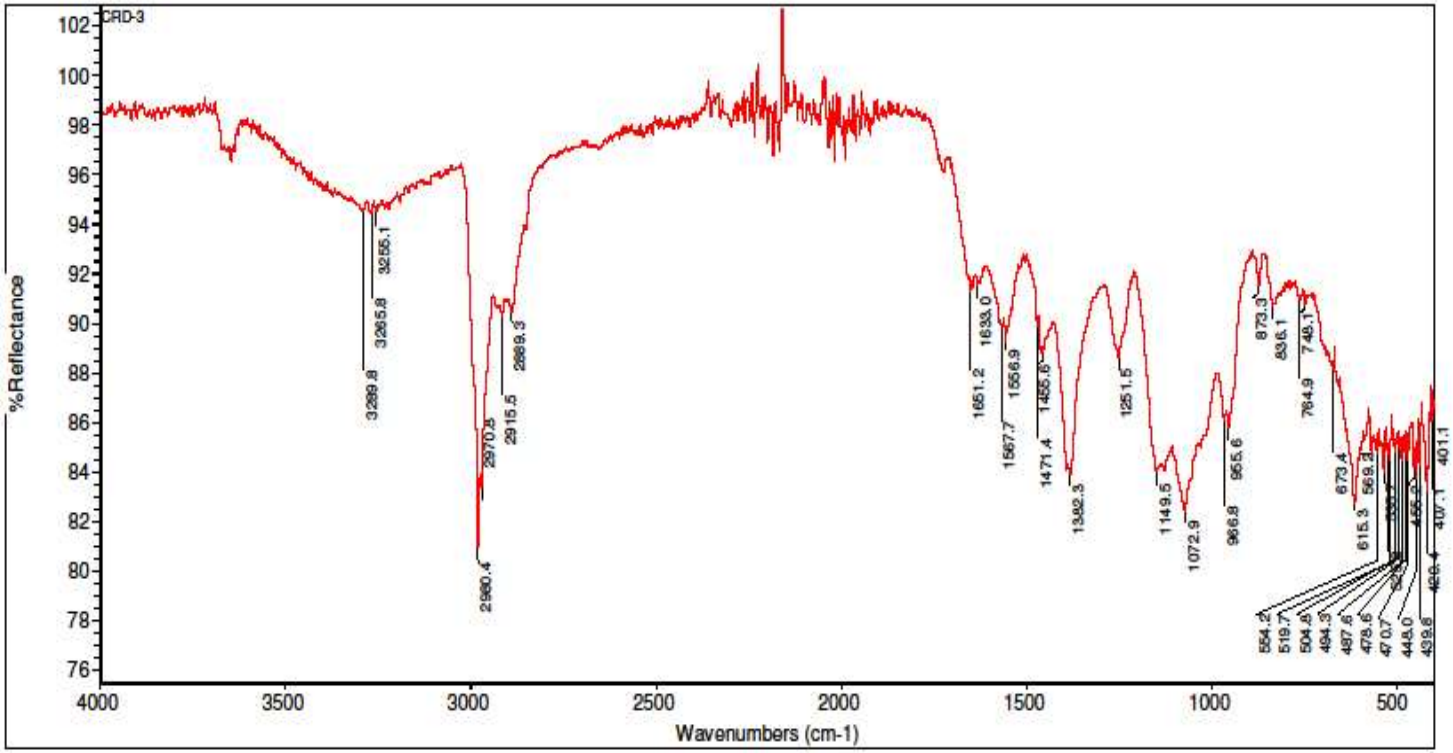
Appendix 9g– HSQC spectra of compound CR1



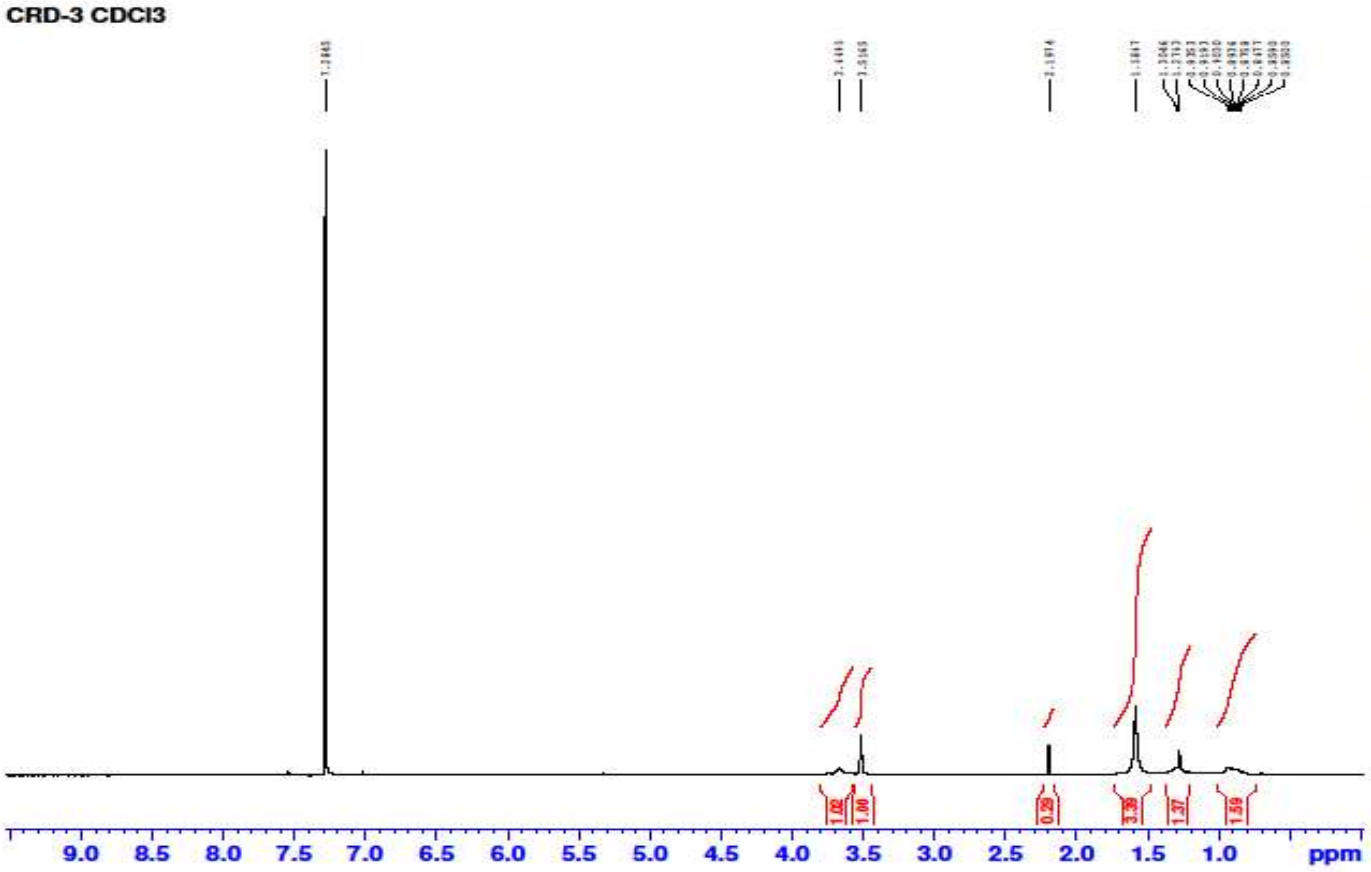
Appendix 9h – NOESY spectra of compound CR1



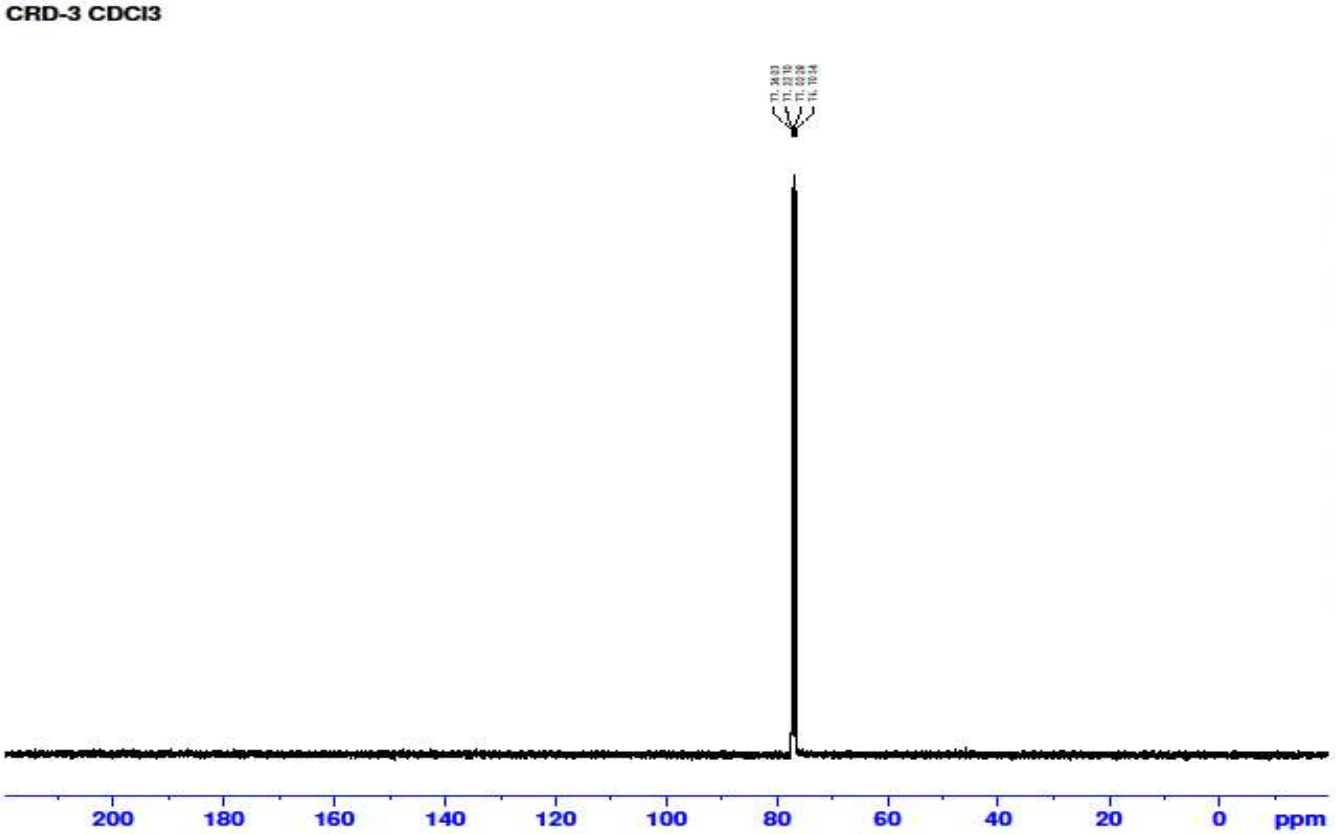
Appendix 10a– IR spectra of compound CR3



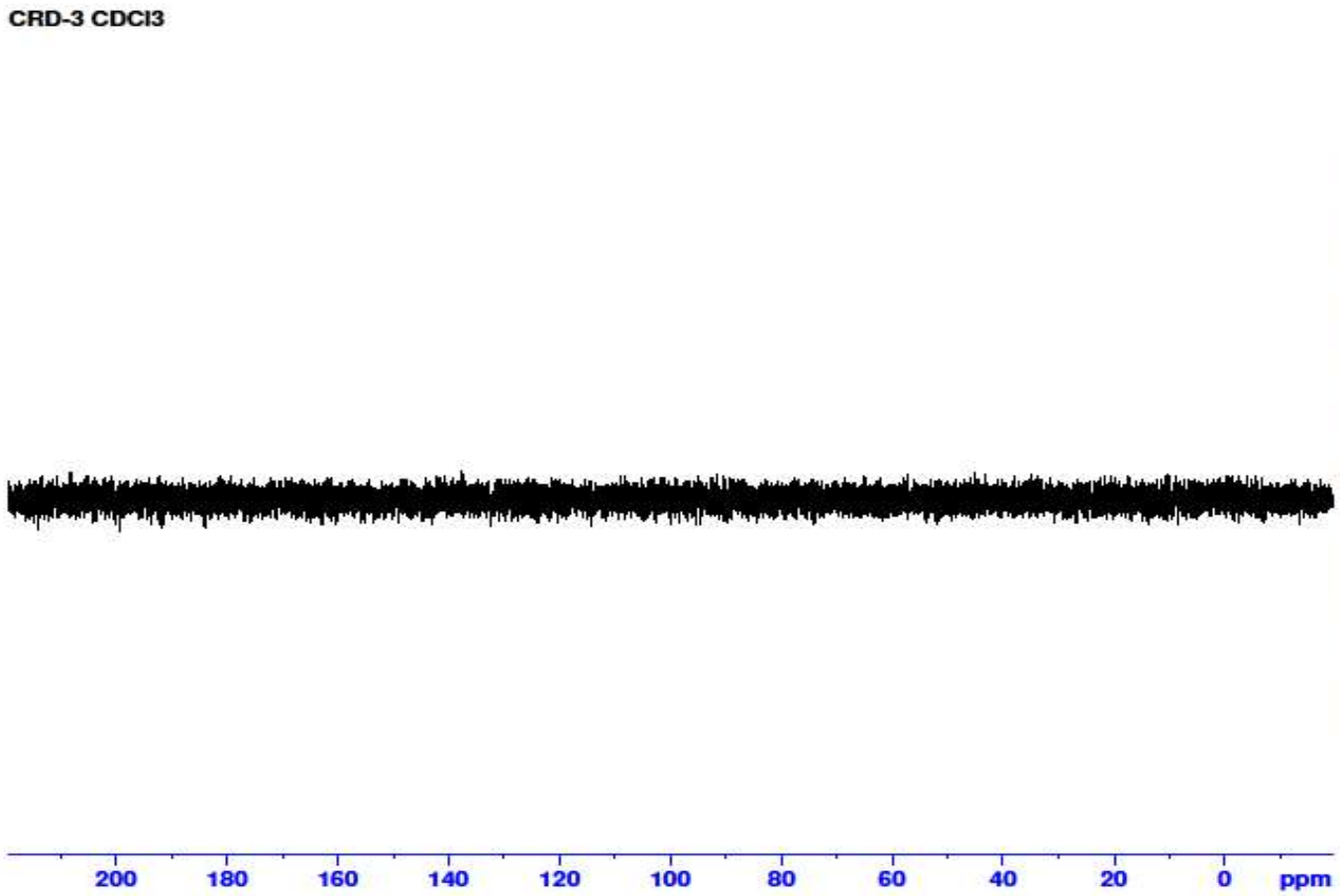
Appendix 10b– ^{1}H spectra of compound CR3



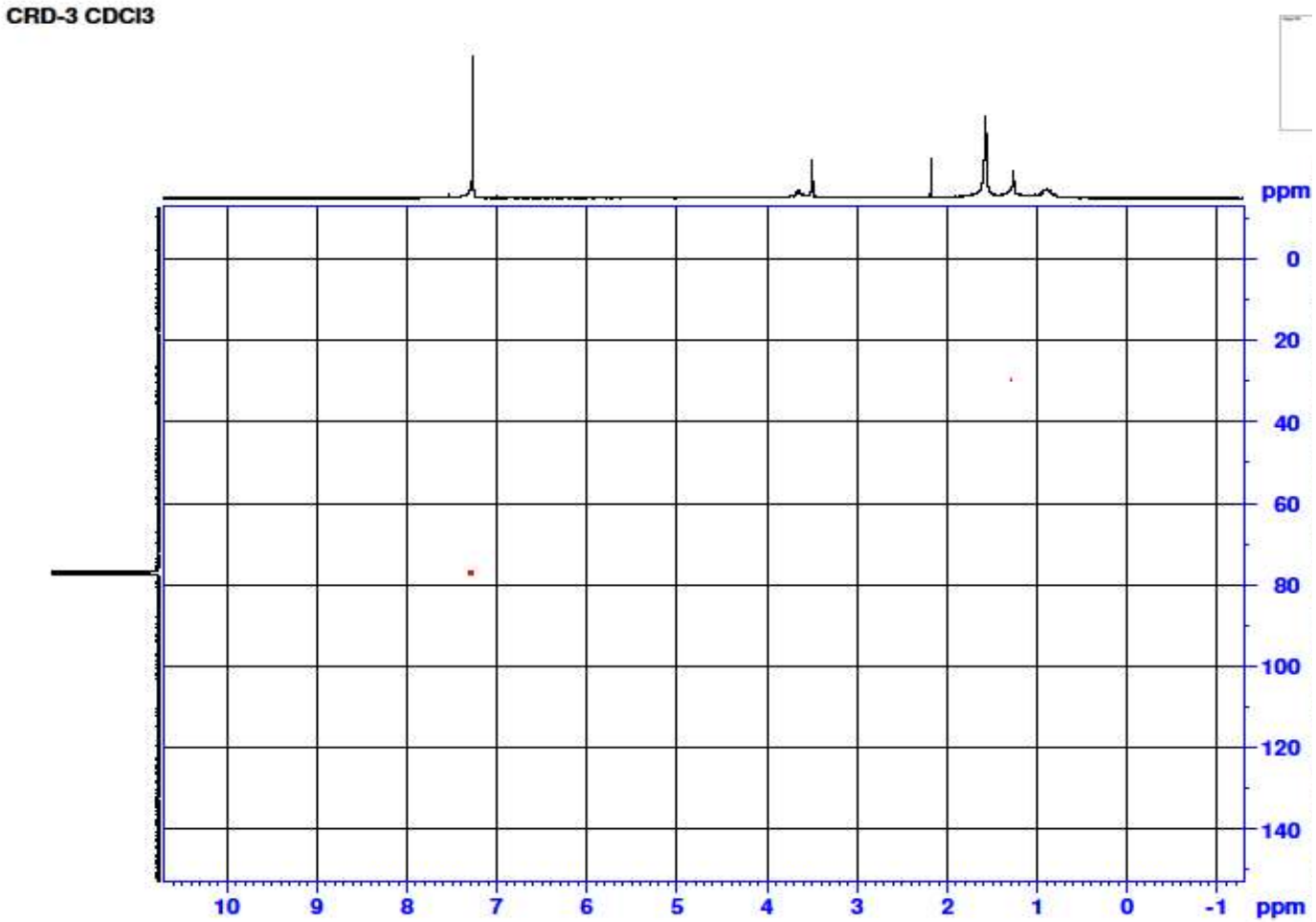
Appendix 10c– ^{13}C spectra of compound CR3



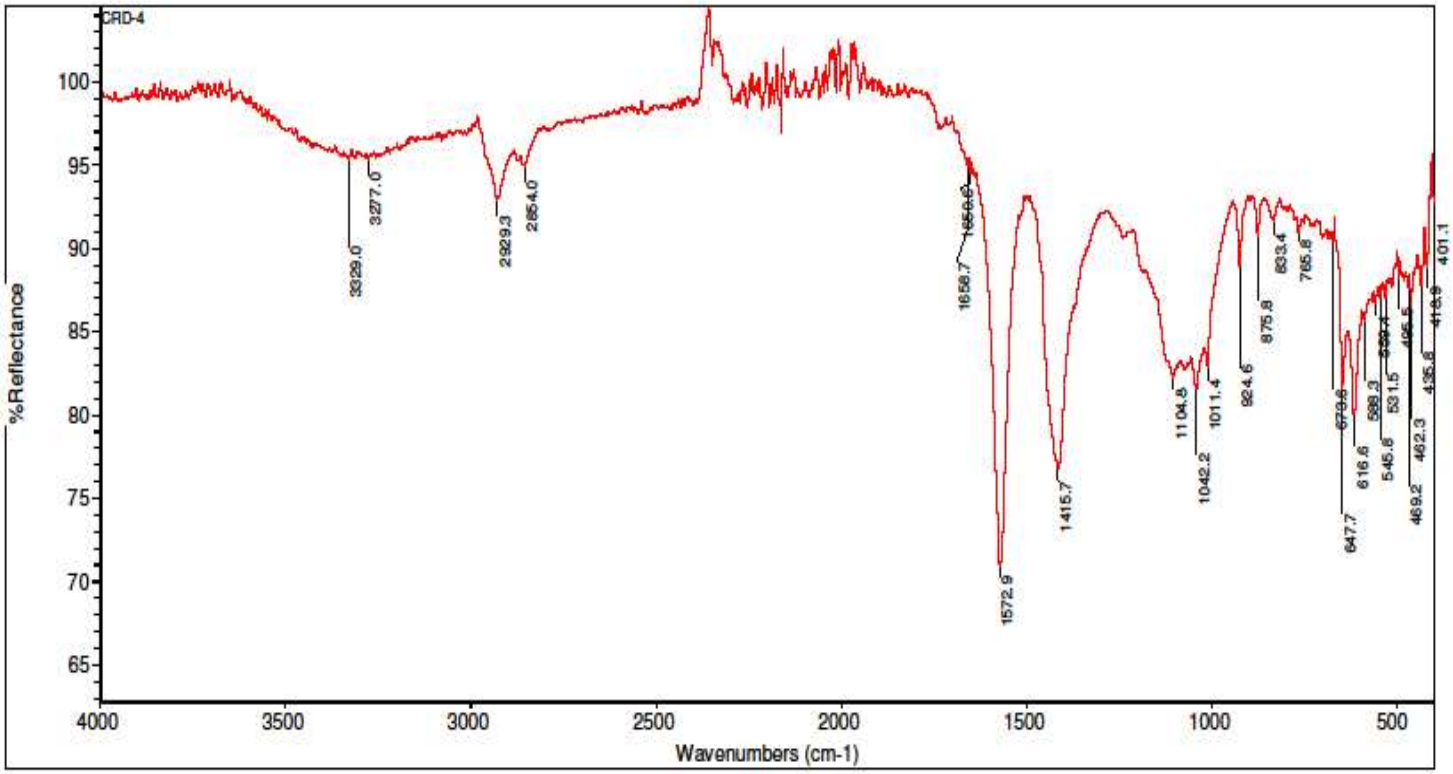
Appendix 10d– DEPT spectra of compound CR3



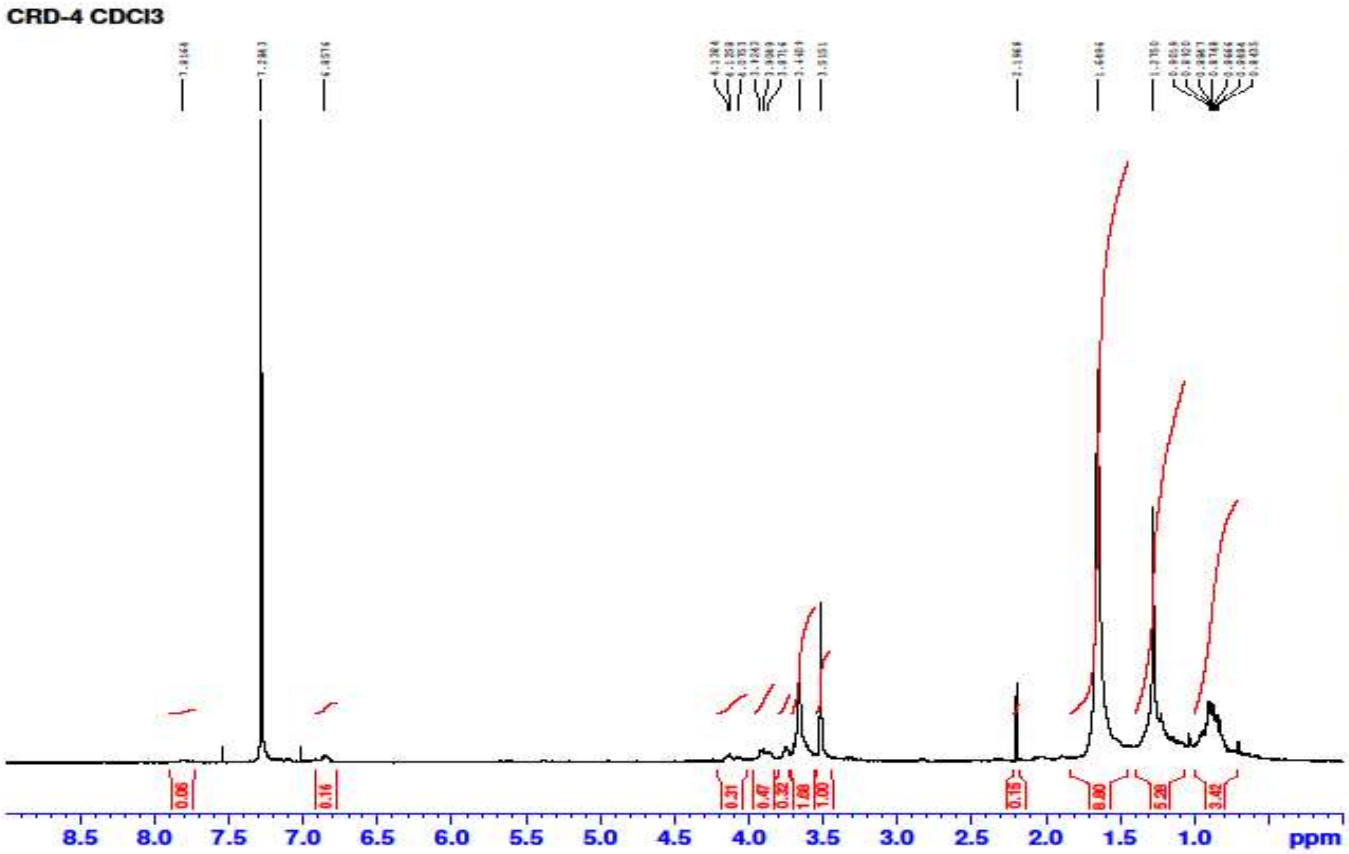
Appendix 10e– HSQC spectra of compound CR3



Appendix 11a– IR spectra of compound CR4

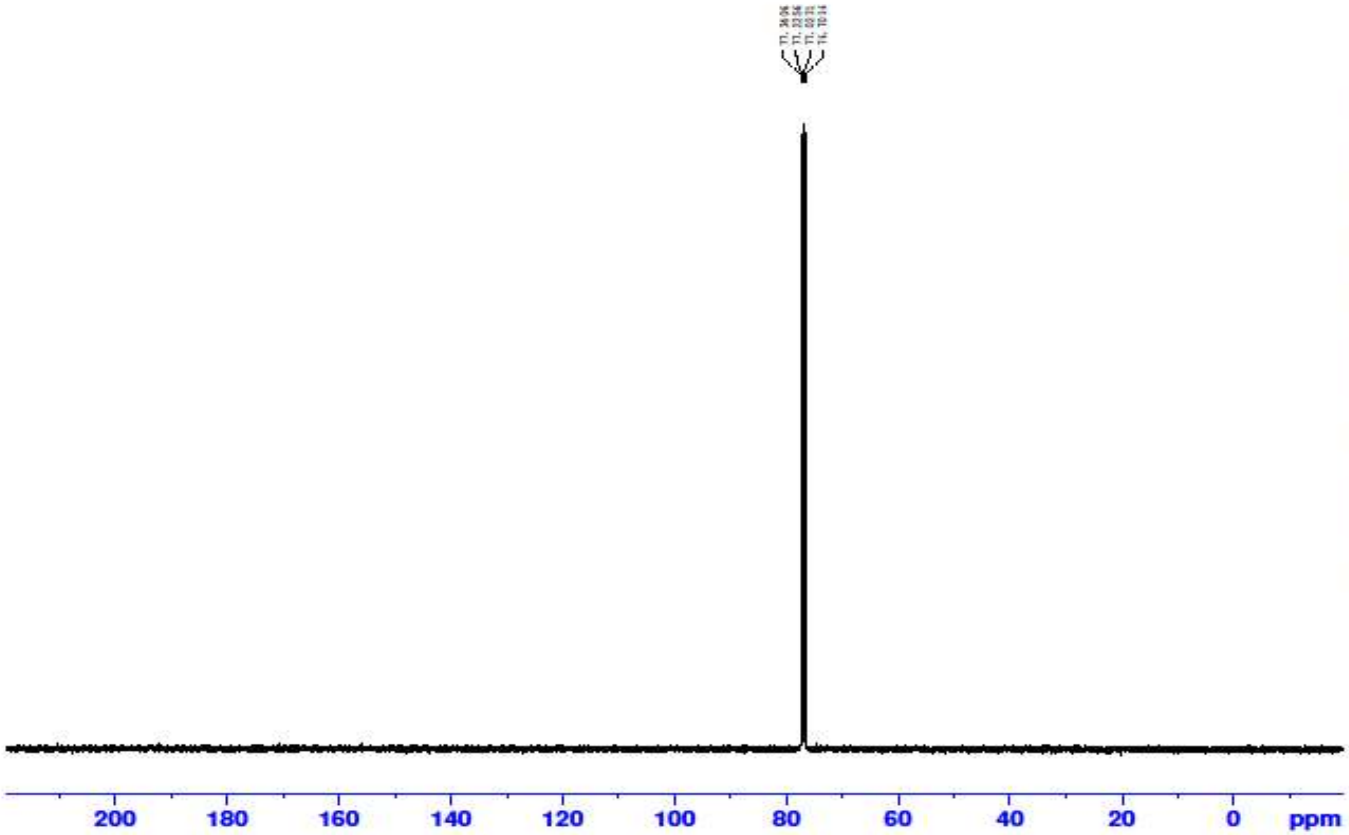


Appendix 11b– ^{1}H spectra of compound CR4

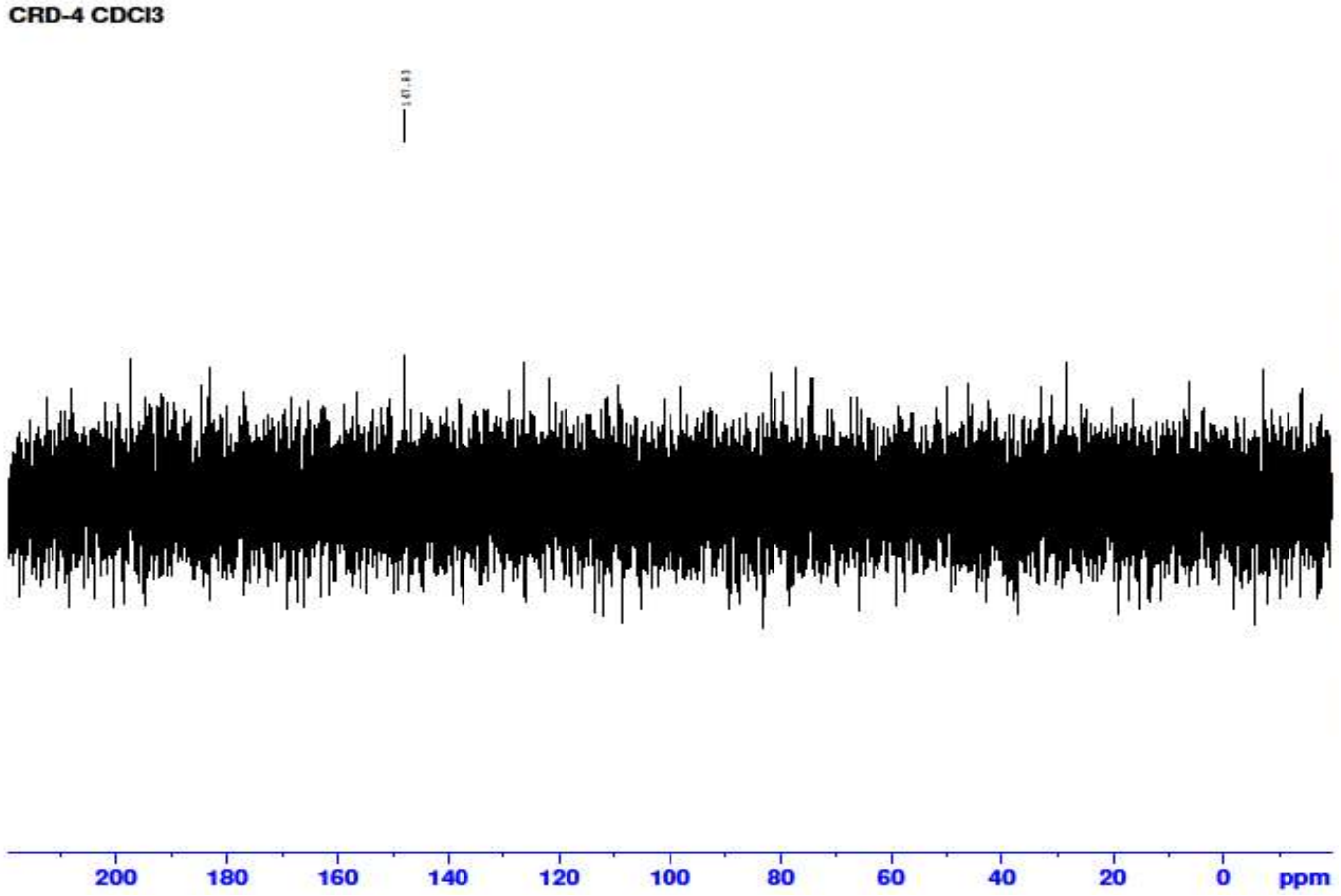


Appendix 11c– ^{13}C spectra of compound CR4

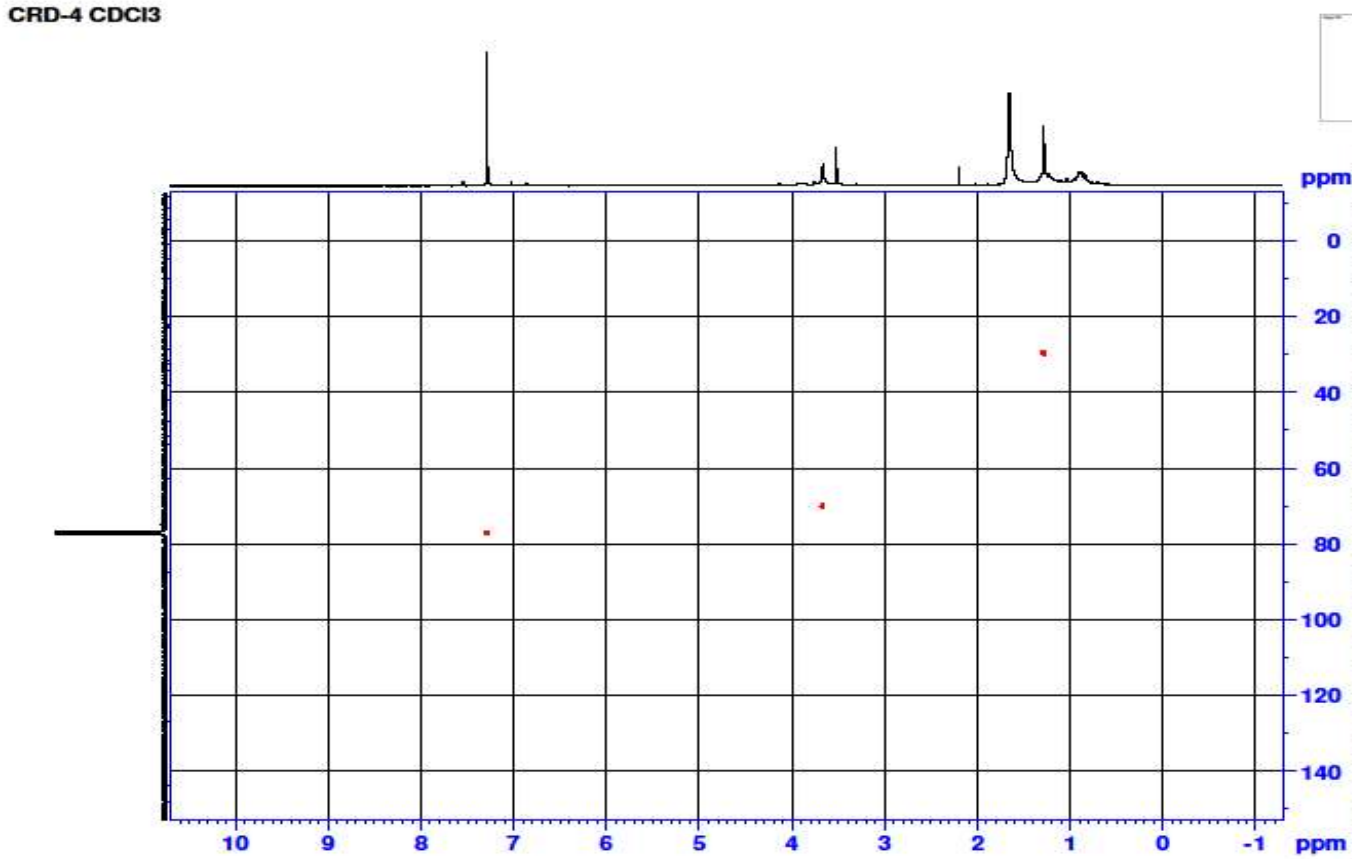
CRD-4 CDCl3



Appendix 11d– DEPT spectra of compound CR4



Appendix 11e– HSQC spectra of compound CR4



Appendix 12a – Determination of IC_{50} for *C. benthamiana* and *C. racemosum* fractions against MCF-7 carcinoma cells

Fractions	Concentration (µg/mL)	Absorbance readings and percentage viability in triplicate						IC_{50}
		Abs.	% Viab.	Abs.	% Viab.	Abs.	% Viab.	
CBN	100	1.1739	65.35817	1.3737	75.91899	1.2738	70.66537	72.75±0.18
	50	0.5673	30.59599	0.6672	35.84071	0.4674	24.68784	
CBD	100	0.2932	14.88825	0.2912	14.51101	0.2922	14.69867	10.46±0.10
	50	0.1967	9.358166	0.1987	9.263671	0.1977	9.310679	
CBA	100	0.1212	5.031519	0.1213	4.872929	0.1211	4.943269	11.28±0.08
	50	0.3415	17.65616	0.4405	22.98049	0.2425	11.86499	
CRN	100	0.3351	17.2894	0.53549	28.36907	0.435	22.84053	17.67±0.08
	50	0.3737	19.50143	0.3638	18.62945	0.3936	20.48007	
CRD	100	0.3738	19.50716	0.3938	20.33129	0.3838	19.92132	19.49±0.04
	50	0.4639	24.67049	0.464	24.31359	0.4638	24.48258	
CRA	100	0.7415	40.5788	0.9413	51.38983	0.8414	46.01175	51.35±0.08
	50	0.7309	39.97135	0.8209	44.55979	0.6409	34.58008	
Methotrexate	100	0.2113	10.19484	0.2131	10.08055	0.2122	10.13741	12.11±0.06
	50	0.3235	16.62464	0.3334	16.90492	0.3136	15.91881	
Control	0.02	1.7784	100	1.7982	100	1.7883	100	
Blank	-	0.0334	0	0.0354	0	0.0344	0	

Appendix 12b – Determination of IC_{50} for *C. benthamiana* and *C. racemosum* fractions against A549 carcinoma cells

Fractions	Concentration (µg/mL)	Absorbance readings and percentage viability in triplicate						IC_{50}
		Abs.	% Viab.	Abs.	% Viab.	Abs.	% Viab.	
CBN	100	0.666	29.67769	0.4662	22.81469	0.5661	25.86062	12.61±0.16
	50	0.1911	7.429963	0.1921	8.720691	0.1901	7.289969	
CBD	100	0.0749	1.986321	0.0747	2.684081	0.0748	1.595298	8.45±0.11

	50	0.2311	9.303851	0.2231	10.31469	0.2321	9.36435	
CBA	100	0.6204	27.54146	0.5214	25.65302	0.4224	18.76327	21.17±0.07
	50	0.4017	17.29598	0.6015	29.7717	0.5016	22.67496	
CRN	100	0.9658	43.72248	0.8659	43.36693	1.0657	50.53588	72.75±0.03
	50	1.2598	57.49555	1.0618	53.43994	1.1608	55.23287	
CRD	100	0.1609	6.015178	0.1509	6.602221	0.1709	6.34168	9.76±0.09
	50	0.3942	16.94463	0.1944	8.838955	0.2943	12.43641	
CRA	100	1.228	56.00581	1.327	67.07631	1.129	53.66227	89.10±0.08
	50	1.201	54.74094	0.921	46.20012	1.111	52.77325	
Methotrexate	100	0.1709	6.48365	0.1609	7.116413	0.1509	5.35388	9.97±0.08
	50	0.2928	12.19432	0.293	13.90889	0.2929	12.36726	
Control	0.02	2.1671	100	1.9673	100	2.0672	100	
Blank	-	0.0325	0	0.0225	0	0.0425	0	

Appendix 12c – Determination of IC_{50} for *C. benthamiana* and *C. racemosum* fractions against PC3 carcinoma cells

Fractions	**Concentration (µg/mL)**	**Absorbance readings and percentage viability in triplicate**						**IC_{50}**
		Abs.	**% Viab.**	**Abs.**	**% Viab.**	**Abs.**	**% Viab.**	
CBN	100	0.8773	35.4938	0.9763	36.85741	0.7783	29.77971	31.07±0.07
	50	0.6792	27.14009	0.879	33.08521	0.7791	29.81223	
CBD	100	0.6912	27.64612	0.7911	29.67744	0.5913	22.17932	27.90±0.04
	50	0.8116	32.72329	0.8316	31.24758	0.8216	31.53959	
CBA	100	0.2726	9.994096	0.3725	13.44886	0.3724	13.28239	16.60±0.04
	50	0.6441	25.65995	0.7341	27.46763	0.5541	20.66737	
CRN	100	0.4761	18.57553	0.5761	21.34217	0.3761	13.43278	25.15±0.03
	50	0.8775	35.50223	0.8973	33.79468	0.8874	34.21395	
CRD	100	0.5055	19.8153	0.4055	14.72823	0.6055	22.75646	30.47±0.03
	50	0.9426	38.24745	1.1424	43.29689	1.0425	40.5178	

CRA	100	0.4529	17.5972	0.5519	20.40397	0.6509	24.60169	25.49±0.02
	50	0.8441	34.09378	0.8541	32.11987	0.8341	32.04763	
METHO	100	0.2725	9.989879	0.4725	17.32573	0.3725	13.28646	12.98±0.07
	50	0.4431	17.18394	0.5432	20.06668	0.343	12.08747	
Control	0.02	2.407	100	2.605	100	2.506	100	
Blank	-	0.0356	0	0.0256	0	0.0456	0	

CBN = *C. benthamiana* hexane fraction
CBD = *C. benthamiana* dichloromethane fraction
CBA = *C. benthamiana* aqueous fraction
CRN = *C. racemosum* hexane fraction
CRD = *C. racemosum* dichloromethane fraction
CRA = *C. racemosum* aqueous fraction
METHO = Standard reference
Control = Cells + media + DMSO
Blank = Media only
Treated = Cells + media + drug

$$\% \text{ cell viability} = \left[\frac{(\text{Abs of treated cells} - \text{Abs of blank})}{(\text{Abs of control cells} - \text{Abs of blank})} \right] \times 100$$

IC_{50} = Nonlinear regression curve fit (Log of concentration versus normalised inhibitor response (graph pad prism))

Appendix 13a – Determination of IC_{50} for isolated compounds from *C. benthamiana* and *C.racemosum* against MCF-7 carcinoma cells

Compounds	**Concentration (µg/mL)**	**Absorbance readings and percentage viability in triplicate**						**IC_{50}**
		Abs.	**% Viab.**	**Abs.**	**% Viab.**	**Abs.**	**% Viab.**	

CB1	100	1.3109	66.20675	1.2119	61.68552	1.1129	56.17416	13.44±0.17
	50	0.6915	34.07853	0.7914	39.66059	0.7916	39.44065	
	25	0.437	20.87764	0.3371	15.86528	0.2373	10.57237	
	12.5	0.8101	40.2303	0.6301	31.21203	0.7201	35.71689	
CB2	100	0.8021	39.81534	0.9021	45.45883	1.0021	50.40362	26.44±0.12
	50	1.2565	63.38503	1.0567	53.55646	1.1566	58.45008	
	25	0.4411	21.09031	0.3421	16.12717	0.5401	26.34238	
	12.5	1.2611	63.62363	1.0613	53.7974	1.1612	58.68965	
CB3	100	0.5817	28.38321	0.4818	23.44437	0.6816	33.71179	21.97±0.06
	50	0.5271	25.55112	0.5469	26.85418	0.537	26.18093	
	25	1.164	58.58706	1.0641	53.94406	1.2639	64.03833	
	12.5	1.0391	52.10851	0.9401	47.44919	0.8411	42.01864	
CB4	100	0.837	41.6256	0.9369	47.28158	0.7371	36.60226	25.72±0.8
	50	0.6807	33.51834	0.4827	23.49151	0.5817	28.50893	
	25	1.263	63.72218	1.164	59.17662	1.064	53.62742	
	12.5	1.0391	52.10851	0.8411	42.26378	0.9401	47.17463	
CB5	100	0.8516	42.3829	0.7514	37.56547	0.9515	47.76835	31.37±0.70
	50	0.6753	33.23824	0.4755	23.11439	0.5754	28.18082	
	25	1.2461	62.84558	1.2462	63.48209	1.1463	57.91365	
	12.5	1.1752	59.16801	1.0762	54.57783	1.2742	64.57476	
CB6	100	1.3164	66.49204	1.1166	56.6939	1.2165	61.56971	63.87±0.17
	50	1.581	80.21682	1.361	69.49508	1.471	74.82423	
	25	1.1721	59.00721	1.0731	54.41546	1.2711	64.41331	
	12.5	0.491	23.67861	0.691	34.40184	0.591	28.99328	
CB7	100	1.4896	75.47591	1.3897	70.99832	1.5895	80.99578	7.197±0.25
	50	0.1334	5.129934	0.1343	5.243034	0.1325	5.114317	
	25	0.5483	26.65076	0.3485	16.46239	0.4484	21.56659	
	12.5	0.348	16.26122	0.339	15.9648	0.357	16.80642	
CR1	100	0.8357	41.55817	0.7367	36.79552	0.6377	31.42545	30.89±0.05

	50	0.8547	42.5437	0.7548	37.74356	0.9546	47.9298	
	25	0.9536	47.67363	0.7556	37.78546	0.8546	42.72173	
	12.5	1.462	74.0443	1.3621	69.55269	1.2622	63.94979	
CR3	100	1.7342	88.16329	1.5362	78.67169	1.6352	83.37587	198.20±0.15
	50	1.6604	84.33529	1.4606	74.71192	1.5605	79.48544	
	25	1.2828	64.74921	1.1829	60.16656	1.3827	70.22551	
	12.5	1.4718	74.55262	1.2738	64.92772	1.3728	69.70991	
CR4	100	1.0763	54.03807	0.9764	49.35051	1.1765	59.48649	87.86±0.09
	50	1.5667	79.47508	1.2669	64.56631	1.4668	74.60549	
	25	1.4331	72.54526	1.2341	62.84831	1.5321	78.00635	
	12.5	1.3141	66.37274	1.2142	61.80599	1.1143	56.24707	
METHO	100	0.4896	23.606	0.4797	23.33438	0.4995	24.2279	8.261±0.08
	50	0.5933	28.98491	0.3935	18.8194	0.4934	23.91021	
	25	0.4484	21.46896	0.3485	16.46239	0.5483	26.76944	
	12.5	0.6779	33.3731	0.4781	23.25058	0.578	28.31623	
Control	0.02	1.9624	100	1.9434	100	1.9544	100	
Blank	-	0.0345	0	0.0342	0	0.0343	0	

Appendix 13b – Determination of IC_{50} for isolated compounds from *C. benthamiana* and *C.racemosum* against A549 carcinoma cells

Compounds	**Concentration (µg/mL)**	**Absorbance readings and percentage viability in triplicate**						**IC_{50}**
		Abs.	**% Viab.**	**Abs.**	**% Viab.**	**Abs.**	**% Viab.**	
CB1	100	1.415	52.88701	1.235	49.8047	1.325	51.4044	28.97±0.09
	50	0.9718	35.88337	0.9619	38.45674	0.9817	37.70747	
	25	1.2143	45.18703	1.3142	53.09565	1.4141	54.9593	
	12.5	1.2232	45.52849	1.2412	50.06233	1.2322	47.70188	
CB2	100	0.0466	0.387493	0.0484	0.498629	0.0475	0.434887	16.16±0.10
	50	0.8047	29.47247	0.7147	28.18499	0.6247	23.46393	

	25	1.5819	59.29024	1.7419	70.86761	1.6619	64.84599	
	12.5	1.0068	37.22617	0.9068	36.16721	0.8068	30.72933	
CB3	100	0.1988	6.226741	0.1968	6.665005	0.1978	6.431535	13.46±0.07
	50	0.8627	31.69768	0.9626	38.48583	1.0625	40.93122	
	25	0.4808	17.04585	0.3908	14.72617	0.5708	21.31344	
	12.5	1.577	59.10224	1.478	59.90194	1.379	53.55889	
CB4	100	1.378	51.46749	1.178	47.43622	1.278	49.52921	19.1±0.14
	50	1.2625	47.03626	1.0627	42.64523	1.1626	44.92499	
	25	0.2808	9.372722	0.2718	9.781434	0.2898	10.10214	
	12.5	1.6464	61.76482	1.5564	63.15964	1.4664	57.04596	
CB5	100	1.3911	51.97007	1.1913	47.98886	1.2912	50.05586	22.09±0.14
	50	1.2393	46.14617	1.3392	54.13446	1.4391	55.95675	
	25	1.1339	42.10244	1.2329	49.71744	1.0349	39.83004	
	12.5	0.5889	21.19317	0.7869	31.18507	0.6879	25.98548	
CB6	100	1.1989	44.5962	1.0989	44.14942	0.9989	38.39371	16.33±0.10
	50	0.1006	2.459237	0.1004	2.659353	0.1005	2.549473	
	25	1.0575	39.1713	0.8577	34.12698	0.9576	36.74593	
	12.5	1.5611	58.49223	1.4612	59.20386	1.661	64.81009	
CB7	100	1.3501	50.39708	1.2502	50.4363	1.1503	44.43425	10.9±0.14
	50	0.5023	17.87071	0.3023	11.04878	0.4023	14.59065	
	25	1.0804	40.04988	1.1803	47.53179	0.9805	37.65959	
	12.5	0.7033	25.5822	0.5053	19.48392	0.6043	22.65002	
CR1	100	0.4989	17.74026	0.3989	15.06274	0.5989	22.43457	5.878±0.09
	50	0.6179	22.30577	0.5279	20.423	0.4379	16.01101	
	25	0.3646	12.58776	0.3844	14.46023	0.3745	13.48149	
	12.5	0.4501	15.86802	0.5501	21.34547	0.6501	24.47734	
CR3	100	1.1483	42.6549	1.3481	54.50428	1.2482	48.34025	41.78±0.10
	50	1.6468	61.78016	1.5478	62.80229	1.4488	56.34376	
	25	1.0224	37.82467	0.8225	32.66434	0.9223	35.33754	

	12.5	1.782	66.9672	1.792	72.94939	1.772	69.23875	
CR4	100	2.0651	77.82851	1.8651	75.98687	1.9651	76.94303	53.8±0.16
	50	1.4203	53.09035	1.3213	53.39068	1.2223	47.30689	
	25	1.3716	51.22195	1.3726	55.52231	1.3706	53.22375	
	12.5	1.056	39.11375	1.054	42.28372	1.055	40.63198	
METHO	100	0.9541	35.2043	0.7543	29.83047	0.8542	32.62049	9.734±0.15
	50	0.4033	14.07251	0.4013	15.16247	0.4023	14.59065	
	25	1.1804	43.88644	1.0804	43.3807	0.9804	37.6556	
	12.5	0.7042	25.61673	0.5044	19.44652	0.6043	22.65002	
Control	0.02	2.643	100	2.443	100	2.543	100	
Blank	-	0.0365	0	0.0364	0	0.0366	0	

Appendix 13c – Determination of IC_{50} for isolated compounds from *C. benthamiana* and *C.racemosum* against PC3 carcinoma cells

Compounds	**Concentration**	**Absorbance readings and percentage viability in triplicate**						**IC_{50}**
	(µg/mL)	**Abs.**	**% Viab.**	**Abs.**	**% Viab.**	**Abs.**	**% Viab.**	
CB1	100	0.3164	9.746461	0.5162	16.52681	0.4163	13.10409	8.44±0.05
	50	0.4535	14.45651	0.6515	21.14204	0.5525	17.76895	
	25	0.8278	27.31551	0.7279	23.74812	0.9277	30.61958	
	12.5	0.8464	27.95451	1.0464	34.6125	0.9464	31.26006	
CB2	100	1.433	48.10705	1.3331	44.39214	1.5329	51.34774	11.13±0.18
	50	0.6045	19.64408	0.8045	26.36103	0.7045	22.97496	
	25	1.4819	49.787	1.6619	55.60786	1.5719	52.68349	
	12.5	0.3028	9.279236	0.3038	9.281621	0.3048	9.285201	
CB3	100	0.9279	30.75443	1.1259	37.32433	1.0269	34.01719	13.23±0.10
	50	0.6498	21.20036	0.7497	24.49175	0.5499	17.6799	
	25	1.2829	42.95039	1.4829	49.50198	1.3829	46.21023	

	12.5	0.9004	29.80967	1.0003	33.03998	0.8005	26.26297	
CB4	100	1.0604	35.30644	0.9604	31.67895	0.8604	28.31455	13.13±0.09
	50	0.7039	23.05895	0.7059	22.99768	0.7049	22.98866	
	25	1.2037	40.22949	1.3036	43.38586	1.4035	46.91578	
	12.5	0.8504	28.09193	0.9504	31.33784	1.0504	34.82207	
CB5	100	1.3914	46.67789	1.1916	39.56543	1.2915	43.07977	16.71±0.13
	50	1.3744	46.09386	1.4734	49.17792	1.2754	42.52834	
	25	1.004	33.36883	1.204	39.9884	1.104	36.65788	
	12.5	0.8743	28.91301	0.8763	28.81021	0.8753	28.82488	
CB6	100	1.3805	46.30342	1.2806	42.60131	1.4804	49.54961	10.78±0.10
	50	0.6942	22.72571	0.4962	15.84459	0.5952	19.23143	
	25	1.2729	42.60684	1.3729	45.74976	1.1729	39.01771	
	12.5	1.3216	44.27992	1.2226	40.62287	1.1236	37.32918	
CB7	100	0.2622	7.88443	0.3621	11.2703	0.1623	4.404562	10.29±0.04
	50	0.2368	7.011818	0.2566	7.671579	0.2467	7.29527	
	25	1.1017	36.7253	1.0027	33.12184	0.9037	29.79758	
	12.5	1.3396	44.89831	1.5196	50.75385	1.4296	47.80971	
CR1	100	1.0879	36.2512	0.9879	32.617	1.1879	39.53146	18.59±0.09
	50	1.2006	40.12299	1.4004	46.68782	1.3005	43.38802	
	25	0.8912	29.49361	1.0912	36.14067	0.9912	32.79447	
	12.5	1.3211	44.26275	1.3112	43.64511	1.331	44.43265	
CR3	100	2.1453	72.57799	2.3451	78.91254	2.2452	75.74408	117.6±0.14
	50	2.1515	72.79099	2.2514	75.71633	2.0516	69.11327	
	25	1.8995	64.13357	1.7996	60.30495	1.6997	57.06066	
	12.5	1.6594	55.88498	1.8592	62.33797	1.7593	59.10196	
CR4	100	1.8004	60.72901	2.0006	67.16128	1.9005	63.93808	32.23.±0.13
	50	1.4719	49.44345	1.4729	49.16087	1.4709	49.22424	
	25	0.9209	30.51395	1.1207	37.14695	1.0208	33.80827	
	12.5	1.5206	51.11653	1.6106	53.85796	1.4306	47.84396	
METHO	100	0.4162	13.17507	0.616	19.9311	0.5161	16.52225	7.054±0.08

	50	0.4711	15.06115	0.4721	15.02251	0.4701	14.94674
	25	0.7804	25.6871	0.9804	32.36117	0.8804	28.99955
	12.5	0.6489	21.16944	0.6589	21.39446	0.6389	20.72816
Control	0.02	2.9435	100	2.9633	100	2.9534	100
Blank	-	0.0327	0	0.0317	0	0.0337	0

CB1 = *C. benthamiana* compound 1
CB2 = *C. benthamiana* compound 2
CB3 = *C. benthamiana* compound 3
CB4 = *C. benthamiana* compound 4
CB5 = *C. benthamiana* compound 5
CB6 = *C. benthamiana* compound 6
CB7 = *C. benthamiana* compound 7
CR1 = *C.racemosum* compound 1
CR3 = *C.racemosum* compound 3
CR4 = *C.racemosum* compound 4
METHO = Methotrexate (standard reference)
Control = Cells + media + DMSO
Blank = Media only
Treated = Cells + media + drug

$$\% \text{ cell viability} = \left[\frac{(\text{Abs of treated cells} - \text{Abs of blank})}{(\text{Abs of control cells} - \text{Abs of blank})} \right] \times 100$$

IC_{50} = Nonlinear regression curve fit (Log of concentration versus normalised inhibitor response (graph pad prism))

Appendix 14- Statistical analysis on compounds bioactivity studies

Appendix 14a- One-way analysis of variance tableon MCF-7 carcinoma cells

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	2467	8	308.4	F (2.883, 11.53) = 1.125	P=0.3774
Individual (between rows)	25128	4	6282	F (4, 32) = 22.92	P<0.0001
Residual (random)	8772	32	274.1		
Total	36367	44			

Repeated measures ANOVA summary	
F	1.125
P value	0.3774
P value summary	ns
Statistically significant (P < 0.05)?	No

Appendix 14b – Dunnett's multiple comparisons teston MCF-7 carcinoma cells

Isolated compounds	Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
CB1	METHO vs. CB1	-10.61	-43.18 to 21.96	No	ns	0.6518
CB2	METHO vs. CB2	-17.21	-49.3 to 14.87	No	ns	0.2843
CB3	METHO vs. CB3	-12.65	-42.87 to 17.57	No	ns	0.4600
CB4	METHO vs. CB4	-15.78	-44.02 to 12.47	No	ns	0.2598
CB5	METHO vs. CB5	-18.82	-51.77 to 14.14	No	ns	0.2468
CB6	METHO vs. CB6	-25.45	-70.98 to 20.09	No	ns	0.2596
CB7	METHO vs. CB7	-4.276	-58.32 to 49.76	No	ns	0.9996
CR1	METHO vs. CR1	-18.75	-47.48 to 9.984	No	ns	0.1774

Appendix 14c- One-way analysis of variance tableon A549 carcinoma cells

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	1995	8	249.4	F (3.116, 12.46) = 0.9419	P=0.4528
Individual (between rows)	32010	4	8002	F (4, 32) = 30.22	P<0.0001
Residual (random)	8474	32	264.8		

Repeated measures ANOVA summary	
F	0.9419
P value	0.4528
P value summary	ns
Statistically significant (P < 0.05)?	No

Appendix 14d – Dunnett's multiple comparisons test on A549 carcinoma cells

Isolated compounds	Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
CB1	METHO vs. CB1	-15.24	-35.4 to 4.925	No	ns	0.1186
CB2	METHO vs. CB2	-3.163	-44.55 to 38.22	No	ns	0.9996
CB3	METHO vs. CB3	-1.465	-54.2 to 51.27	No	ns	0.9999
CB4	METHO vs. CB4	-10.68	-64.56 to 43.21	No	ns	0.9189
CB5	METHO vs. CB5	-12.14	-42.58 to 18.29	No	ns	0.4971
CB6	METHO vs. CB6	-6.216	-44.17 to 31.73	No	ns	0.9632
CB7	METHO vs. CB7	-3.175	-16.91 to 10.56	No	ns	0.8608
CR1	METHO vs. CR1	7.861	-17.95 to 33.67	No	ns	0.7002

Appendix 14e- One-way analysis of variance tableon PC3 carcinoma cells

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	1303	8	162.8	F (2.186, 8.744) = 1.509	P=0.2752
Individual (between rows)	35064	4	8766	F (4, 32) = 81.24	P<0.0001
Residual (random)	3453	32	107.9		
Total	39819	44			

Repeated measures ANOVA summary	
F	1.509
P value	0.2752
P value summary	ns
Statistically significant (P < 0.05)?	No

Appendix 14f – Dunnett's multiple comparisons test on PC3 carcinoma cells

Isolated compounds	Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
CB1	METHO vs. CB1	-1.55	-11.89 to 8.791	No	ns	0.9756
CB2	METHO vs. CB2	-10.25	-44.12 to 23.62	No	ns	0.7048
CB3	METHO vs. CB3	-9.883	-24.4 to 4.634	No	ns	0.1585
CB4	METHO vs. CB4	-9.609	-21.49 to 2.277	No	ns	0.0974
CB5	METHO vs. CB5	-14.58	-40.51 to 11.35	No	ns	0.2559
CB6	METHO vs. CB6	-13.39	-36.39 to 9.613	No	ns	0.2358
CB7	METHO vs. CB7	-2.91	-30.69 to 24.87	No	ns	0.9957
CR1	METHO vs. CR1	-14.96	-38.96 to 9.048	No	ns	0.1996

Appendix 15Manuscripts from this study

Famojuro, T. I. and Taiwo O. Elufioye. 2019. Documentation of the medicinal plants used for the management of cancer by the people in three selected Local Government Areas of Ekiti State, Southwestern Nigeria

Famojuro, T. I., Elufioye, T. O., Rakesh, P. 2019. Cytotoxicityand Phytochemical evaluation of methanol extracts of nine plants used in Nigeria ethnomedicine.

Famojuro, T. I., Elufioye, T. O., Olajide, O. A., Dybek, M. and Adejare, A. 2019. Cytotoxicity of six compounds isolated from the leaves of *Caesalpinia benthamiana* (Baill.) Herend. & Zarucchi (Fabaceae) against three human adenocarcinoma cells

Tayo I. Famojuro, Taiwo O. Elufioye, Thomas. O. Idowu. 2019. Cytotoxicity of a new phenolic acid isolated from the root bark of *Combretum racemosum* P. Beauv. against three human adenocarcinoma cells.