PHYTOCHEMICAL INVESTIGATION OF THE KENYAN *Croton sylvaticus* FOR ANTI-CANCER PRINCIPLES

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DECLARATION

This thesis is my original work and has not been presented for award of a certificate in any other Institution.

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DEDICATION

I dedicate this thesis and research work to my beloved wife Julia Chelimo and my daughters, Goulder Bosibori and Winnifridah Moraa, may God bless them all**.**

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ABSTRACT

Plants offer unlimited compounds that can be developed into anticancer agents. In this study, compounds isolated from *Croton sylvaticus* Hochst were evaluated for their cytotoxicity against human drug sensitive (CCRF-CEM) and drug resistant (CEM/ADR5000) leukemia cell lines. The isolation was carried out using column chromatography (CC) and their structure elucidation was determined by use of 1D and 2D NMR spectroscopy in comparison with literature. The cytotoxicity of the compounds was evaluated using resazurin reduction bioassay. Approximately 200g crude extract from the stem bark extract (1:1 methanol in dichloromethane and 0.95:0.05 methanol to water) of *Croton sylvaticus* Hochst yielded three labdane diterpenoids namely austroinulin (**57**, 34 mg), labd-13(*E*)-ene-8*α*, 15-diol(**74,** 43 mg) and 18-*nor*-labd-13(*E*)-ene-8α,15-diol (**109,** 82 mg). The crude extract (1:1MeOH in CH_2Cl_2) was found to be active at the tested concentration of 10 μg/ml exhibiting cell inhibition of 86 %compared to the positive control, doxorubicin which showed cell inhibition of 97.36 % against the drug sensitive leukemia cells, CCRF-CEM. All the three isolated labdanediterpenoids**57**, **74** and **109** showed lower activity against the cell resistant and cell sensitive leukemia cells, with cell viabilities of44.89±2.31, 91.88±4.27 and 51.40±4.08 %, respectively against the drug sensitive CCRF-CEM cells and 53.97±0.70, 79.74±1.77 and 66.17±4.79 %, respectively against the drug resistant CEM-ADR5000 cells. While the positive control, doxorubicin had cell viability of 2.64 %and 78.97 % for CCRF-CEM and CEM-ADR500, respectively. Seemingly, the pure compounds lost their synergistic effect during isolation that is why their cytotoxicity against the cancer cells is low as compared to the extract.

TABLE OF CONTENTS

LIST OF TABLES

TABLE OF FIGURES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

CEM-ADR5000	The human drug resistant cancer cell lines
CCRF-CEM	The human drug-sensitive cancer cell lines
DCM	Dichloromethane
DEPT	Distortionless enhancement by polarization transfer
DMSO	Dimethyl sulfoxide
EtOAc	Ethyl Acetate
H,H-COSY	Homonuclear correlation spectroscopy
HMBC	Heteronuclear multiple bond connectivity
HMQC	Hetronuclear multiple quantum coherence
HSQC	Heteronuclear single quantum coherence
NCEs	New Chemical Entities
NMR	Nuclear magnetic resonance
NOESY	Nuclear overhouser exchange spectroscopy
IC_{50}	50 % Inhibition Concentration
IR	Infrared
LC_{50}	Lethal Concentration-50:Concentration that kills 50 % of test animal
MDR	Multi-drug resistant
MeOH	Methanol
TLC	Thin Layer Chromatography
UV	Ultra Violet
UV-VIS	Ultraviolet Visible
WHO	World Health Organization

CHAPTER ONE: INTRODUCTION

1.1 Background of the Study

From the field of medicinal chemistry, drug development has been described to be costly and needs patience as it is a time consuming process. The process starts from identifying new chemical entity (NCE) with therapeutic properties. The compounds with therapeutic properties are obtained majorly in two ways: they can be obtained by isolating them from nature or by carrying out structural modifications (Katiyar *et al*., 2012). Majority of the drugs used in clinical medicine these days originated from natural products as novel compounds. These novel compounds can have certain therapeutic properties with little modifications of their structures (Li-Weber, 2009; Fabricant & Farnsworth, 2001).

Up to date, development of novel compounds with therapeutic values into drugs has been influenced majorly by traditional medicine. Among these the oldest are African, Indian and Chinese traditional medicine (Gurib-Fakim, 2006). Plants have contributed greatly to drug discovery. About 25% of all the drugs in clinical use have been contributed from plants. This makes plants the key player in medicine (Gurib-Fakim, 2006; Rates, 2001). The World Health Organization has identified some of these medicinal compounds as crucial to the human health and about 11% of these medicinal compounds comes from plants (Rates, 2001). Natural products have been derived or acted as drug leads for antibiotics, anticancer anti-plasmodial and antidiabetics drugs among others (Harvey, 2008; Harvey *et al.*, 2015).

Traditionally, plant-based remedies have been prescribed in three ways: taken in as herbal tea, as crude extracts or standard enriched fractions (Rates, 2001). Pharmacologically active compounds like morphine (**1**), cocaine (**2**), pilocarpine (**3**),

codeine (**4**), quinine (**5**), reserpine (**6**) and digitoxin (**7**). These componds whose structures are shown in figure 1.1 are some of the common examples of drugs that were isolated from plants which have revolutionalized the field of medicine in one way or the other.

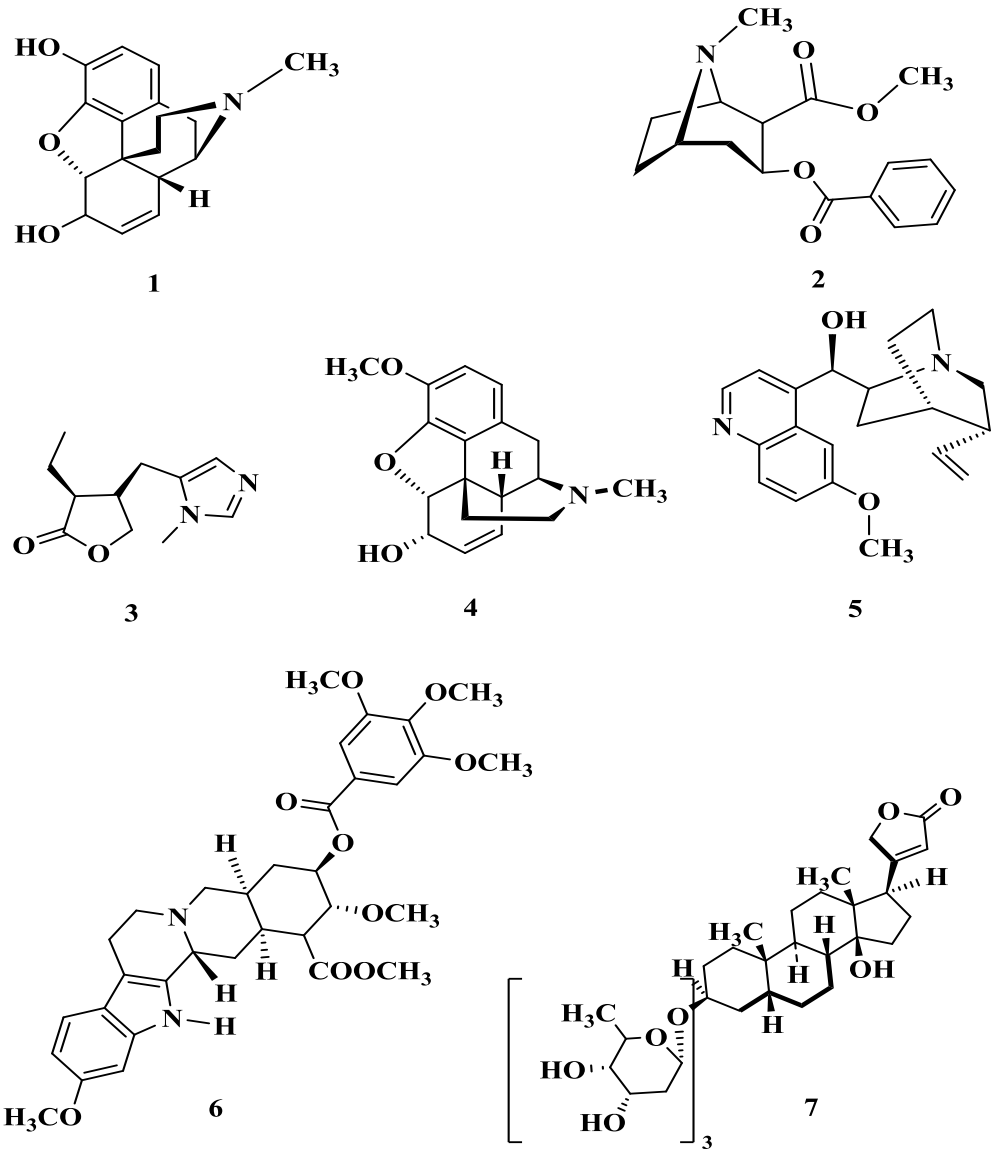


Figure 1.1: Structures of Pharmacologically active compounds isolated from plants

Majority of the people in developing countries, especially from low and middle income earners practice traditional herbal medicine to cure and treat different types of diseases. This is being influenced by reasons such as high cost of conventional medicines and low coverage of the conventional primary health care facilities. The traditional

practitioners are very flexible when it comes to payment for their services. This is also another factor that has contributed greatly towards the ethnomedicinal health care services (Liefooghe *et al.,* 2003).

However it is regrettable that the ethno-medicinal information is normally held in confidentiality by few traditional herbalists. With these herbalists have little or no scientific knowhow of the efficacy of these herbs and their application (Katiyar *et al*., 2012).

In Kenya, ethnomedicinal practices are very common in various traditional set ups (Fratkin, 1996). Majority of the ethnomedicinal practitioners have used plants in one way or the other in handling of various unhealthy conditions such as: madness, elephantiasis, malaria, common cold, microbial and cancer (Kokwaro, 2009; Maundu *et al*.,2005; Munguti, 1997).

The continual use of the traditional herbal medicine which lacks sufficient knowledge on their efficacy subjects the consumers to adverse toxic side effects. These consumers are not aware of these severe side effects. It is therefore of great importance to evaluate scientifically the pharmacological efficacy of these traditional herbal medicines using conventional methods such as biological assays. These bioassays can as well be used in evaluating the possible adverse toxic side effects. The scientific evaluation strategies involving detection, isolation, purification and characterization unveils novel bioactive molecules from extracts that traditional medicine men and women claim to be effective.

The advancement of research through Fourier transfer Infrared (FTIR), Gas Chromatography-Mass spectroscopy (GC-MS), Mass Spectroscopy (MS) and 2-dimensional NMR (2D NMR) ^{1}H and ^{13}C NMR spectroscopy. These spectroscopic techniques have greatly influenced the research on phytochemicals. Researchers have

better linkage between the biochemistry and their drug abilities. The active natural compounds now can be easily identified and isolated then can be used as drugs or drug leads. Unfortunately, only a very small portion of about up to 10% of all the plants on the earth surface have their chemical and pharmacology been studied for their medicinal value

The menace of multi-drug-resistant mechanism of different disease- causing agents has necessitated the investigation of any slightest mean available of obtaining a lead to novel compounds which have antimicrobial, antiparasitic and anticancer properties. These leads may be plants as traditional phytomedicine. Drugs derived from plants have some advantages over synthetic drugs. These advantages include less negative side-effects, they are cheaper and more accessible to the impoverished population in under-developed countries most of which are from tropical Africa like Kenya.

The connection between traditional usage of some plants in treating most illnesses most of which are infectious nature is an indication of them comprising antimicrobial, anti-malarial and anti-cancer principles (Inoussa *et al*., 2015).

Even if quite a number of traditional medicine men have formulated herbal remedies which they claim to have anticancer properties, it is only a small portion that has been substantially studied as alternative cancer drugs. The well-known anticancer drugs like paclitaxel, vinblastine and vincristine have their sources from plants, *Taxus brevifolia* (paclitaxel) and *Catharanthus roseus* (periwinkle plant). These plants have contributed richly towards current drug development (Takimoto & Awada, 2008). This shows that only limited studies have been carried out on anticancer principles of plants.

Earlier reports have indicated that plants originating from Africa, especially their extracts have exciting anticancer properties (Choumessi *et al*., 2012; Díaz-Chiguer *et*

al., 2012; Efferth *et al*., 2011; Kuete & Efferth, 2010, 2015; Kuete *et al*., 2015; Omosa *et al.,* 2015; Nyaboke *et al*., 2018). The extracts from these plants are believed to offer better anticancer properties to the resistant tumor clones than synthetic drugs. This is because the extracts can contain different compounds with different functional groups which offer a multiple of mechanisms of action on a number of targets (Efferth *et al*., 2011).

1.2 Statement of the Problem

A larger proportion of the world's population is struggling to treat communicable and non-communicable diseases such as microbial and cancer (Jemal *et al*., 2011). Systemic disease treatment has not been fruitful majorly because the cell mutation towards chemotherapy drugs leads to multidrug resistance (MDR). The multidrug resistant mechanism menace prompts for more research in order to reduce cancer incidences in the world. However, this effort that include combination chemotherapy has realized little progress in the fight against cancer (Broxterman *et al*., 2009).The mortality rate of more than 11.2 million cancer deaths per annum globally is high and unbearable and it calls for the cancer diagnosis, treatment and prevention awareness (Ferlay *et al*., 2013).

The most current chemotherapy treatment techniques for cancer which include the most current techniques such as molecular inhibitors from monoclonal antibodies which are target-specific are not readily available for the majority of the low and middle class income earners in the developing countries. The failure of the governments to put up wavering and or subsidizing policies to help them access affordable cancer treatment services has made most of them to go back and practice traditional medicine. For these reasons stakeholders have embarked on a journey to search for more affordable and easily accessed alternative drugs with equal impact as the conventional and fewer side effects from traditional medicine.

Majority of the ethnomedicinal users have limited knowledge on the efficacy and toxicity of their drugs but they believe that they have fewer side effects. Despite all the arguments on the support of herbal-based drug use, native medicine will remain unconventional because they are not scientifically evaluated on their pharmacological and toxicological profiles (efficacy, safety of therapy and raw materials and interaction with other drugs).

For the aforementioned reasons the modern scientists are researching on how to improve this kind of alternative medicine by improving on natural products especially from animals and plants which have mechanisms to manage the degenerating cancer (Kuete,2013; Cragg & Newman, 2013).

In Africa, where there isn't much information about the scientific approval of the therapeutic use of medicinal plants, majority of the population (about 80%) continues to use the plant parts as drugs to manage their ailments (Ochwang'i *et al*., 2014). It is therefore important to investigate these plants for their claimed biological values and scientifically validate them in order to institute their efficacy and see if new drugs can be isolated or their drug leads to them.

Despite varied ethno medicinal uses of *Croton* plants over the world, and considering the high diversity of the genus (over 1300 species), studies for their ethno-pharmacological relevancies are rather few. American and Asian species lead in chemistry and pharmacology reports on *Croton* genus. Some have been established to be turmeric (co- carcinogenic) and anti- HIV-1 (El-Mekkawy *et al*., 2000).

Leukemia cancer cells were used in this because of mainly two chief reasons: First, in Africa currently, with little treatment options, leukemia is among the tumor type cancer that are affecting majority of the population including infants (Stefan, 2015; WHO,

2014). Secondly compared with other solid cancer cell lines, leukemia is very sensitive to cytotoxic compounds and therefore it is very easy to observe very minimal inhibition activities even from compounds with little activity against tumor cells that can record nil inhibition effect (Zamora *et al*., 1988).

1.4 Justification of the Study

At present, interest in herbal medicines is enjoying a renaissance with a seeming emergence of a new culture of 'return to nature' among pharmaceutical companies and other stakeholders. A positive, rational and non-prejudicial approach in scientifically evaluating the potential of reputed medicinal plants as chemotherapeutics is a more realistic response to global health burden. This was a driving force behind the serendipitous, random and multidisciplinary screening approaches that were used in study.

Croton sylvaticus was the choice for this study because it has been widely linked management of a wide array of ailments such as malaria, cancer among others (Ochwang'i *et al*., 2013). Some of the compounds that have been isolated from *Croton* species, their derivatives and their models have been found to be pharmacologically useful. Some of the compounds isolated from this genus have toxic and inhibitory effects to the growth of micro- organisms. A notable example is the cytotoxicity of trachylobane diterpenes from *Croton zambesicus* (Salatino *et al*., 2007).

The high mortality rate of cancer is of great public importance and therefore should be controlled or else it will remain to be a global health burden (Kakde *et al*., 2011). Information from the World Health Organization indicates that the death rate due to cancer is about 9.6 million per annum and it is ranked second after cardiovascular diseases (Kadioglu *et al*., 2018). Therefore, to step up the fight against cancer,

alternative medicine is necessary to search for new anticancer lead structures with powerful biological mechanisms that are able to fight against the emerging multi drug resistant cancer cells. The drugs must also be target- specific in the sense that they have little or no side effects to the health cells of the body. That is it must. In this case the assured and major sources are natural products. Natural products for a long time (for more than 30 years) since 1960s have been providing drug leads for cancer chemotherapy (Newman *et al*., 2007). Most of these anticancer drugs that are used in cancer clinics were derived from natural origin from which plants have provided most of them (Newman *et al*., 2003). Traditional medicine has played an important role in the discovery of the drugs that are being used for chemotherapy in the cancer clinics today. It is from the knowledge on their uses in managing cancer related ailments that scientists pick this and advance the search for active compounds.

With continued research assurance of more drugs from natural sources that is going to be used as lead compounds and structures in future (Mann, 2002). This is the course from which currently powerful anti-leukemia drugs like vincristin, vinblastin and podophyllins were obtained.

The ethnomedicinal survey of the Kenyan plants indicates that the plant in this research, *Croton sylvaticus* is among the candidate plants used in the treatment and management of cancer and cancer-related ailments by a community that lives in the vicinity of Kakamega forest (Ochwang'i *et al.,* 2014). Further studies have proved that the extracts from the plant has good activity against various cancer cell lines. But isolation of pure compounds from the stem barks have not been evaluated for anticancer activity hence the interest to study the anticancer activity of their compounds. This study intended to evaluate the phytochemistry and anticancer activity of the Kenyan *Croton sylvaticus*

and also to support the potential formulation of new drugs that could help in management of cancer related ailments.

1.5 Objectives

1.5.1 General objective

Characterization and evaluation of cytotoxic properties of the compounds isolated from the stem bark of the Kenyan *Croton sylvaticus* against drug sensitive and drug-resistant cancer cell lines.

1.5.2 Specific objectives

The specific objectives of this study were to:

i. Isolate compounds from the stem bark of the Kenyan *Croton sylvaticus.*
ii. Characterize the structures of the isolated compounds from Kenyan *Croton sylvaticus.*
iii. Evaluate the cytotoxicity of the isolated compounds from the Kenyan *Croton sylvaticus* against drug sensitive and drug resistant leukemia cancer cells.

CHAPTER TWO: LITERATURE REVIEW

2.1 Cancer

Cancer is classified among the non-communicable disease which is described by the rapid and uncontrolled growth of cells which spread beyond their usual boundaries, invading the adjacent tissues and organs of the body (Stewart, & Wild, 2014). It is sometimes referred to as malignant; tumor or neoplasm. It is a generic term for a large group of diseases that can affect any part of the body.

The cancerous cells spread from one tissue to another either through the blood system or lymph system within the body system (Holmes *et al.*, 2007). Cancer cells can be broadly classified into: carcinomas, sarcomas, lymphomas, leukemia and adenomas. The cancerous cells that cover internal and external parts of the body such as lung, breast and colon are carcinomas while those ones that affect the supportive tissues like the bones, cartilages, fats connective tissues and muscles etc. are classified as sarcomas cancer. Lymphomas are the cancers that have their origin from the lymph nodes or immune system tissues while leukemia cancers have their origin from the bone marrow and they affect the blood system. The adenomas cancer arises from the glandular system like the thyroid, pituitary gland or adrenal glands. In mortality rating, the lung, liver, stomach, colorectal, breast and oesophageal cancers have caused the highest mortality (Stewart, & Wild, 2014).

2.1.1 Causes, diagnosis, prevention and treatment of cancer

There are three major factors that cause cancers; they include genetic predisposition, nutrition and environmental pollution. Change in human lifestyle such as high intake of sugars is also another factor that has immensely influenced the increase in cancer cases especially from the developing countries in the sub-Saharan Africa. This has caused

about 95% of cancer cases. Excessive usage of alcohol, Tobacco smoking, unhealthy food, lack of physical exercise and natural cancer-causing agents has extremely increased cancer cases in the greater part of the population in the developing nations (Ferlay et al., 2018).

There is a number of infections that have been associated with causing a number of cancers. They include such infections as Human Immunodeficiency Virus (HIV), Epstein – Barr virus (EBV), Human Papilloma virus (HPV), Hepatitis B virus (HBV), Hepatitis C virus (HCV), Human Herpes Virus 8(HHV-8) and Human T-cell Lymphotropic Virus (HTLV-1) (Parkin, 2006; zur Hausen, 2009; Adedeji, 2016), these infections are common in the sub-Saharan Africa region.

Diseases such cancer, cardiovascular and microorganism infections are among the greatest social and economic burden in the sub-Saharan Africa. Cancer is the second in leading in causing mortality in the world currently (Jemal *et al.*, 2011). The world health organization (WHO) reported that by the year 2018 the mortality rate due to cancer has increased to about 9.6 million per annum from 8.2 million per annum in 2012 (Ferlay *et al*., 2013; 2018). This mortality rate is projected to continue increasing to 11.5 million per annum by 2030 if there are no sufficient interventions made to control it (Ferlay *et al*., 2013).

Of the cancer deaths that have occurred in the world, majority (about 65%) are from the low and middle income earners of the developing countries (Ferlay *et al*., 2013). As much as cancer is one of the dreadful diseases, only a modest progress has been made on research to reduce its morbidity and mortality.

In Kenya, cancer causes 7% of the deaths that occur annually and it is the third cause of mortality after infectious diseases such as malaria and cardiovascular diseases (Langat

et al., 2017). Cancer does not spare the generation or sex it cuts across both the old, youths and children. However, the most affected are aged people who have attained 70 years and above who accounts for about 60% of those affected. Based on gender, breast cancer, oesophagal cancer and cervical cancers are the most common in women where breast cancer is the leading killer while in men the leading cancers are oesophagal and prostate cancer report from the regional Nairobi Cancer Registry (Langat *et al*., 2017).

The social economic burden which cancer is causing is not unnoticed, therefore modest effort have been put to prevent, control and treat it (Jemal *et al*., 2011). Prevention of infective cancer types involves vaccination, screening and treatment of precancerous lesions. So many campaigns have been done to teach man to be aware against poor living style especially, the dangers associated with excessive use of alcohol and tobacco smoking. This will greatly reduce the chances of getting lung, throat, mouth and liver cancer and hence will significantly control them.

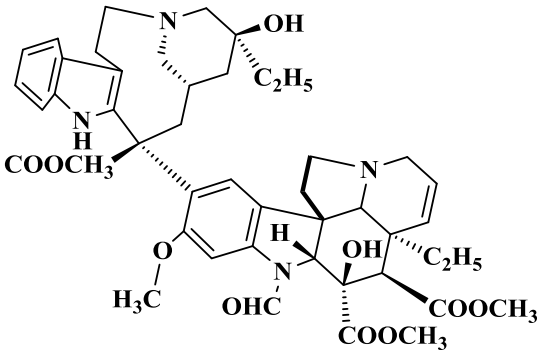
Also improvement on our diets that includes taking less fats and instead encourage taking more of fresh fruits, vegetables and whole grains that greatly improve our immunity against suffering from cancer. In addition, engaging in sufficient body physical exercises, management of blood pressure and blood sugar will also greatly reduce cancer cases (Stewart & Wild, 2014).

Another way of preventing cancer is through vaccinations. For example cervical cancer in women and liver cancer can be prevented though vaccination (Stewart & Wild, 2014).

The conventional methods for treatment and management of cancer are surgery, radiotherapy, chemotherapy, combination therapy and lastly, blood and marrow transplant. Surgery involves total removal of the cancerous cells from the affected

organs, while radiotherapy and chemotherapy involves exposing the cancerous cells to irradiations or some drugs which will normally target the cancerous cells and eventually destroy them (Stewart &Wild, 2014). In blood and marrow transplant, the donor bone marrow replaces the patient's marrow that is diseased. The objective of a chemotherapeutic agent is to reactivate the apoptotic pathway of the cancer cells. During cancer treatment the ultimate goal is either totally cure the patient or to improve and prolong the life of the patient.

Chemotherapy is the usage of drugs to treat cancer. Some of the conventional chemotherapeutic drugs that have been employed in the fight against cancer include; vincristine (**8**), Vinblastine (**9**), vindesine (**10**) vinorelbine (**11**), cyclophosphamide (**12**), doxorubicin (**13**), prednisone (**14**) and hydroxydaunorubicin (**15**) among others. Some of these drugs there structures are shown in figure 2.1.



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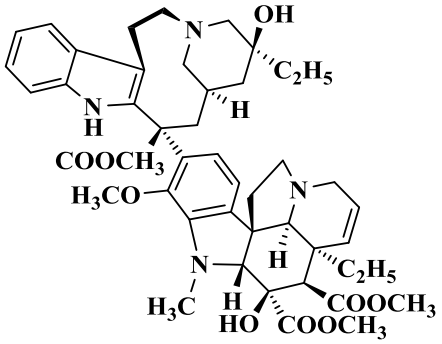

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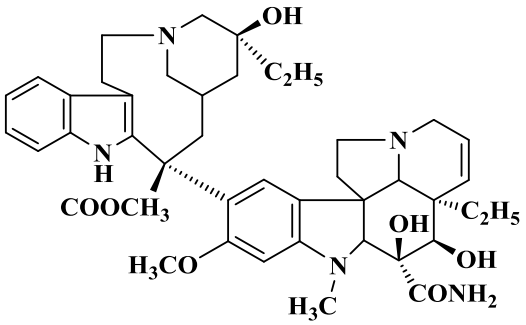

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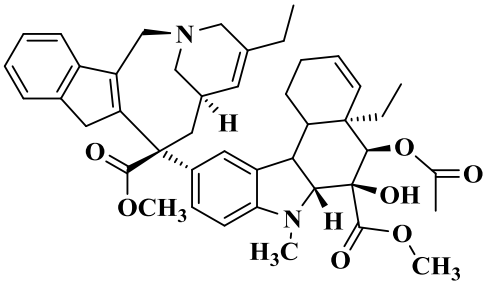

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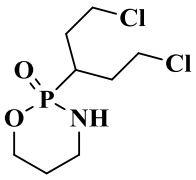

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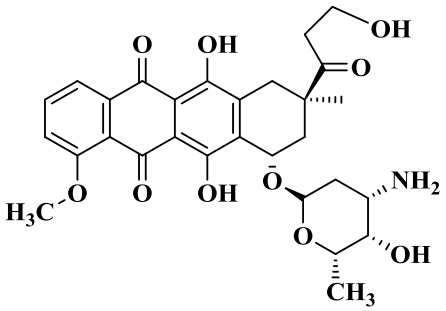

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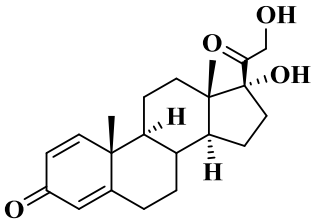

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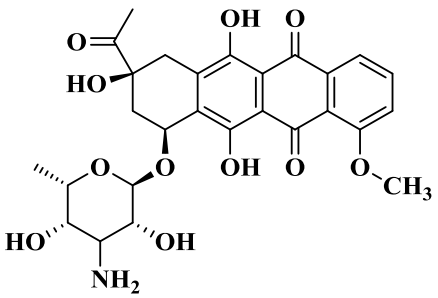


15

Figure 2.1: Structures of some anticancer Drugs in clinical use

2.1.3 Challenges of chemotherapy in the fight against cancer

The war against high cancer mortality rate has not been successful for long majorly because of the following three reasons: in countries yet to develop, chemotherapy drugs are unaffordable and unavailable to the majority of the people there especially middle and low class earners. The global health body (WHO) has indicated a small percentage (about 22%) access the drugs used in treating cancer drugs. Further, worse still, it is only 11% of the middle class and low class populace that can afford these drugs (Dzoyem *et al*., 2013).

The Second reason why chemotherapy has not been successful is because of the severe side effects of the most available and affordable anticancer drugs. Lastly the cancer cells have developed mutation and have become resistant to these chemotherapy drugs a condition known as multi- drug resistance (MDR). The resistance has led to systemic failure of cancer treatment by chemotherapeutic method (Broxterman *et al*., 2009; Efferth *et al*., 2008; Kuete & Efferth, 2010; 2015).

These encounters among others have compelled a constant quest for more effective and non-toxic chemotherapeutic drugs which can be used in the fight against cancer with fewer side effects.

The major guarantee source of anticancer drugs can be said to be nature. Medicinal plants offer alternative medicines and can be exploited for their principles which are active against these infectious diseases (Kuete, 2013).

2.1.4 Natural anticancer chemotherapy agents

Natural products have been approved to be a major source for anticancer drugs. The leading body is the USA's National Cancer Institute (NCI) that approved some anticancer drugs from natural products by 2005 (Cragg & Newman, 2013). Nature plays a very important role in providing sources for drugs for treating various ailments including cancers. Majority of the drugs, about 60%, currently used as anticancer agents are obtained as a result from natural sources(Butler, 2004; Cragg &Newman, 2013). Some of these natural sources include animals, plants, and marine micro- organisms.

2.1.5 Plant Based Anticancer Agents

These drugs have been classified into the following groups:

(i). *Vinca alkaloids*: These compounds were first isolated from *Catharanthus roseus.* They include; vincristine (**8**) and vinblastine (**9**), it is almost 60 years since their discovery in 1960s and are being used in the fight against leukemia and the Hodgkin's disease in cancer clinic (Devita *et al*., 1970).

(ii). *Taxanes*: These drugs are effective against ovarian, breast and lung cancers. They include; taxol (first isolated from *Taxus brevifolia*) commonly known as paclitaxel (**16**) and its analogue docetaxel (**17**) (Wani *et al*., 1971).

(iii). *Epipodophyllotoxins*: this class consists of podophyllotoxin (**18**) and its analogues, etoposide (**19**) and teniposide (**20**). Podophyllotoxin (**18**) which was first isolated from *podophyllum petatum* was discouraged from being used due to its toxic effects. However, its analogues, etoposide (**19**) and teniposide (**20**) are well known for its efficacy against leukemia, lung, testis, ovarian, and brain cancer (Bhanot, *et al*., 2011).

(iv). *Camptothecin (***21***)*:isolated from *Camptotheca accuminata* was modified to more active derivatives: topothecan **(22)** and irinothecan **(23).**They are well known for their fight against colon, pancreas, liver, breast and prostate cancers (Creemers *et al*., 1996).

Other anticancer drugs are anthracyclines, doxorubicin (**13**), vindesine (**10**), vinorelbine (**11**), daunorubicin (**24**), epirubicin (**25**) and idarubicin (**26**). These plant based anticancer agents, their sources and uses are summarised in table 2.1 and their structures shown in figure 2.2.

Table 2.1: List of Plant based anticancer agents

Plant sources	**Compound**	**Treatment uses**	**References**
Catharanthus roseus	Vincristine (**8**) and	Leukemia, lymphoma, breast, lung, pediatric solid cancers	Devita *et al*., 1970
	Vinblastine (**9**)	Breast, lymphoma, germ-cell and renal cancer	Devita *et al*., 1970
Taxus brevifolia	Paclitaxel (**16**) and Docetaxel (**17**)	Ovary, breast, lung, bladder head and neck cancer Breast and lung cancer	Wani *et al.*, 1971
Podophyllum petatum	Podophyllotoxin (**18**), Etoposide (**19**) and Teniposide (**20**)	Leukemia, lung, testis, ovarian, and brain cancer	Bhanot *et al.*, 2011
Camptotheca acuminata	Camptothecin (**21**)	Ovarian, lung and pediatric cancer	Creemers *et al*., 1996
	Topotecan (**22**) and Irinotecan (**23**)	Colorectal and lung cancer	Creemers *et al*., 1996

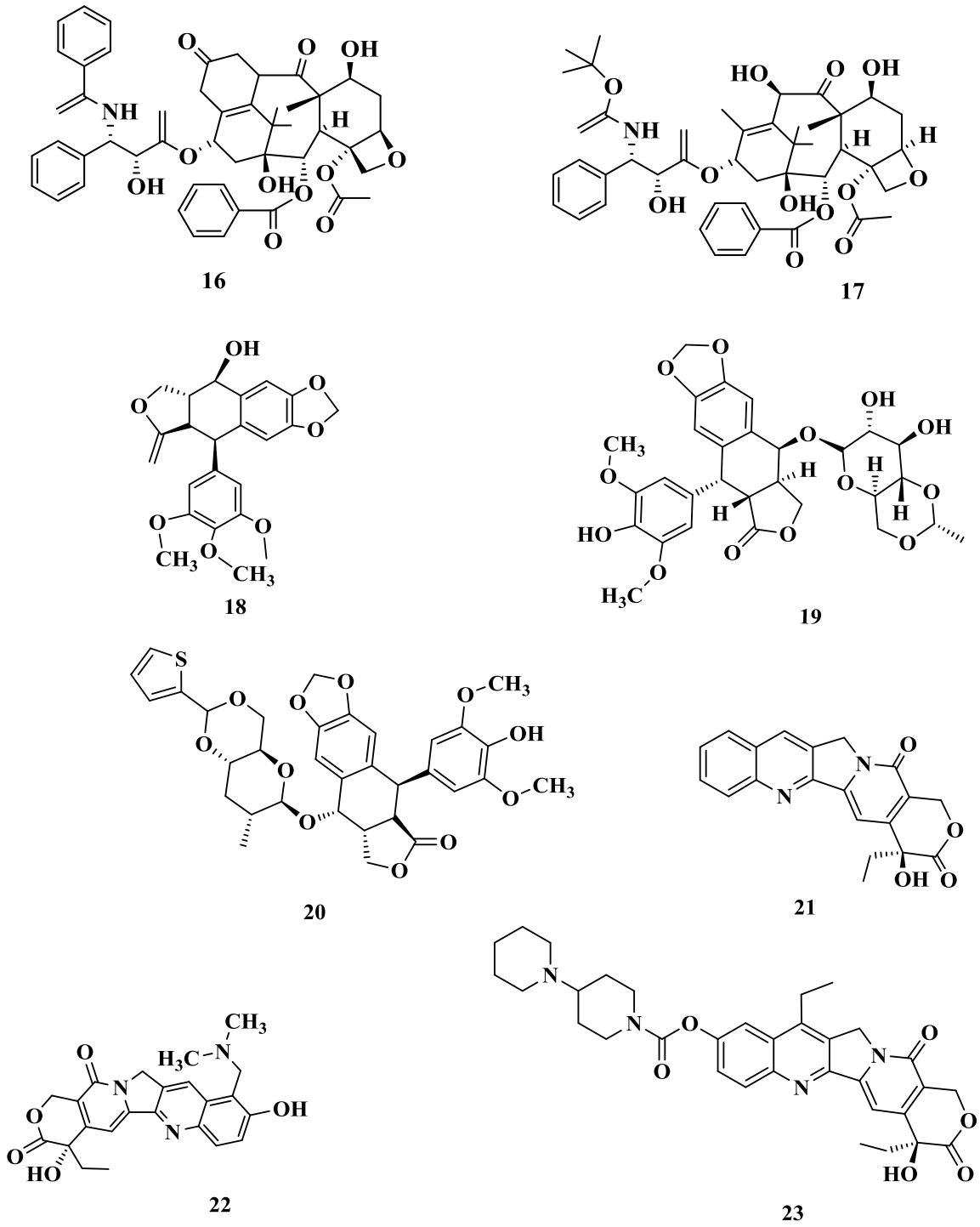
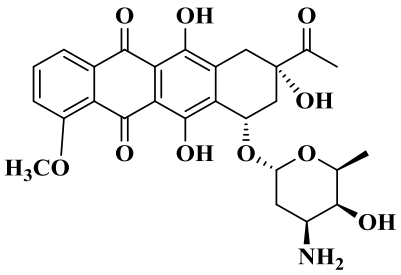


Figure 2.2: plant derived anticancer drugs



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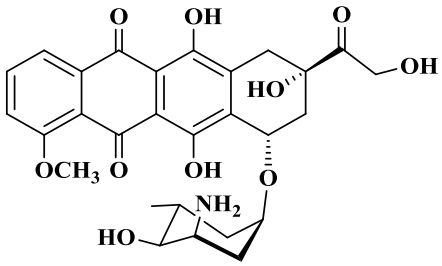

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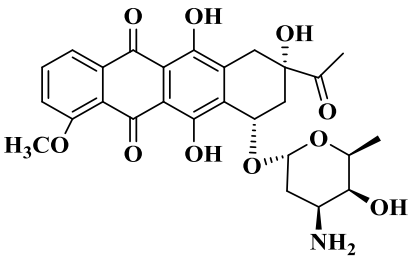

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Figure 2.2 continue**:** Plant- Derived anticancer drugs continue

2.2 Genus *Croton*

2.2.1 Botanical information

Plants in the genus C*roton* belongs to the family *Euphorbiaceae* and sub family *Crotonoideae.* The genus consists of trees, shrubs and herbs which can make up to 1,300 species(Berry *et al.*, 2005; Webster, 1967). Plants in this genus are distributed in both the tropical and subtropical regions around the world. These plants thrives well mainly in the warm tropical regions than the temperate regions (Salatino *et al*., 2007).

In Kenya a total of fifteen species of the genus *Croton* have been identified among them are; *C. dichogamus* pax. *C. bonplandianus* Baill, *C. alienus* Pax., *C. megalocarpus* Hutch., *C. menyhartii, C. macrostachyus, C. polytrichus, C. pseudopulchellus, C. sylvaticus,* and *C. zambesicus*. Several of these *croton* species contains volatile oil constituents which are indicated by their aromatic smell (Ciiaveiho *et al*., 1981; Magalhães *et al*., 1998). As expected, from the members of the *Euphorbiaceae* family.

Some of the species from the genus *Croton* are not excluded from containing secondary metabolites associated with medicinal characteristics commonly known as latex.

2.2.2 Ethno-medicinal uses of the genus *Croton*

Several kinds of the genus *Croton* have been utilized as traditional medicine globally. It has been used among traditional medicinal plants in Africa, Asia and South America. The *Croton* species have been involved in curing a great number of diseases such as leukemia, balsamic, narcotic, rheumatism, stomach ache and tonic, bronchitis, diarrhea, leprosy, psoriasis, urticarial, malaria and ulcers(Gupta *et al*., 1993). The various parts of the plants that have been used are the fruits, leaves and the barks of roots and stem (Salatino *et al*., 2007). African *Croton* species and their ethnomedical uses are summarized in table 2.2.

Table 2.2: Some ethnomedical uses of different *Croton* species in Africa

S/No	*Croton* Species	Region	Part used	Ethnomecinal Uses	References
1	*C. gratissmus* Burch	Western and Southern Regions of Africa	Leaves and stem bark	Rheumatism, perfume, dropsy, lever, bleeding gum, carthatic, eruptive irritant, respiratory condition, intercostals neuralgia, indigestion, pleurisy, uterus disorder and fish poison	Watt & Breyer-Brandwijk, 1962; Wickens & Oliver-Bever, 2007
2	*C. macrostachyus* Hochst. ex Delile	Madagascar, Somalia, Sudan, Eritrea, East Africa, Angola Guinea, Liberia, Malawi, Zambia and Zimbabwe	Entire plant and Seeds	Malaria, dysentry, rheumatism, venereal diseases, infertility, skin rashes, constipation, anthelmintic, vermifuge, stomach pains, diabetics chest pains, bloat, wound healing, and mumphs	Mazzanti *et al*., 1987; Klauss & Adala, 1994; Schmelzer & Fakim, 2008; Chan *et al*., 2012
3	*C. alienus* Pax	Kenya	Whole plant	Body weakness	Kamau *et al*., 2016
4	*C. dichogamus* Pax	Kenya, Uganda, Tanzania, Rwanda and	Leaves, roots and	Decoction used to treat fever, chest ailments,	Jeruto *et al*., 2008;

		Ethiopia	whole plant	stomach diseases, tuberculosis, impotence and malaria	Kokwaro, 2009
5	*C. jatrophoides* Pax	Tanzania	Roots	Colds, intestinal worms and stomachache	Schmelzer & Gurib-Fakim, 2008; Kokwaro, 2009
6	*C. lobatus* Vell.	Ivory Coast, Togo, Benin, Senegal, Eritrea and Ethiopia	Leaves and roots	Pregnancy disorders, aching, whooping cough, infections involving eyes and mouth, female infertility, malaria, dysentery, laxative antispasmodic in case of threatening miscarriage and hiccups	Attioua *et al*., 2007; Neuwinger, 2000; Schmelzer & Fakim, 2008
7	*C. longiracemosus* Hutch	Gabon	Roots	Anthelimintic and anti-inflammatory	Akendengue & Louis, 1994
8	*C. mayumbensis*	Gabon, Cameroon and	Stem bark	Microbial infections, and	Yamale *et*

	J. Léonard	Central African Republic	and leaves	human parasitic diseases such as amoebiasis	*al*., 2009
9	*C. megalobotrys* Müll. Arg.	Zimbabwe	Stem bark, roots, seeds	Purgative, used to treat malaria, abortion and tapeworms	Nyazema, 1984
10	*C. megalocarpus* Hutch	Kenya, DRC, Mozambique Malawi and Zimbabwe	Entire plant, stem bark root and leaf sap	Gall bladder, chest pains, internal swellings, malaria, anthelmintic, whooping cough, pneumonia and bleeding wounds	Beech *et al.*, 2017; Chan *et al*., 2012; Johns *et al*., 1994; Kokwaro, 2009; Wickens & Oliver-Bever, 2007
11	*C. macrostachyus* Hochst. ex Delile	Kenya	Entire tree	Psychotherapeutic	Kokwaro, 2009
12	*C. sylvaticus* Hochst	Ethiopia, South Africa, Gabon and Angola	Stem bark, roots, and	Decoctions and infusion used to treat abdominal	Kokwaro, 2009; Mcgaw

			leaves,	disorders, tuberculosis, rheumatism, fish poison, gall sickness in cattle, kwashiorkor and malaria	*et al*., 2000; Neuwinger, 2000; Watt & Breyer-Brandwijk, 1962
13	*C. steenkampianus* Gerstner	Tanzania, Mozambique and Southern Africa	Fresh leaves	Vapor inhalation relieves body pains	Adelekan *et al*., 2008; Schmelzer & Fakim, 2008
14	*C. enduliflorus* Hutch	Sierra Leone, Eastwards to Nigeria, Central African Republic, Gabon, Ghana, Nigeria and Cote d'Ivoire	Roots, seeds, and Stem bark	Stomach-aches, labor pains, headaches, menstrual disorders, fever, uterine, tumors, and Stomach complaints	Adesogan, 1981; Anika & Shetty, 1983; Schmelzer &Gurib-Fakim, 2008
15	*C. polytrichus* Pax	Kenya	Roots	Reliefs labor pains and headache	Kokwaro, 2009
16	*C. pseudopulchellu*	Mali, Tanzania, Nigeria, Somalia,	Leaves, roots and	Anthrax, insecticide, Syphilitic ulcers, chest	Langat *et al*., 2012; 2008

	s Pax	Kenya, Ethiopia, Angola, Zimbabwe, Mozambique and South Africa	stem barks	infections, tuberculosis, asthma, colds, viral and tissue infections and condiment, is used to flavor fresh milk	
17	*C. scheffleri* Pax	Tanzania	Roots	Insanity, Remedy for miscarriage	Mathias, 1982; Watt & Breyer-Brandwijk, 1962
18	*C. zambesicus* Müll. Arg.	Guinea, Congo and Tropical Africa	Roots and leaves	Menstrual pains, aperients, anti-malarial, anti-diabetic, fevers, dysentery, convulsions, hypertension, urinary infections and antimicrobial	El-Hamidi, 1970; Mohamed*et al*., 2009; Ngadjui *etal*., 1999; Watt & Breyer-Brandwijk, 1962

2.2.3 Phytochemistry of the genus *Croton*

Different classes of secondary metabolites have been isolated and characterized from the genus *Croton*. They include; alkaloids, terpenoids and flavonoids (Ndunda, 2014; Ndunda *et al.*, 2015). Among these classes of the secondary metabolite isolated and characterized from this genus, the diterpenoids are the most abundant (Block *et al*., 2004). The diterpenoids that have been isolated from the genus *Croton* includes kauranes, clerodanes, neoclerodanes, labdanes, phorbol esters and trachylobane.

2.2.3.1. Terpenoids

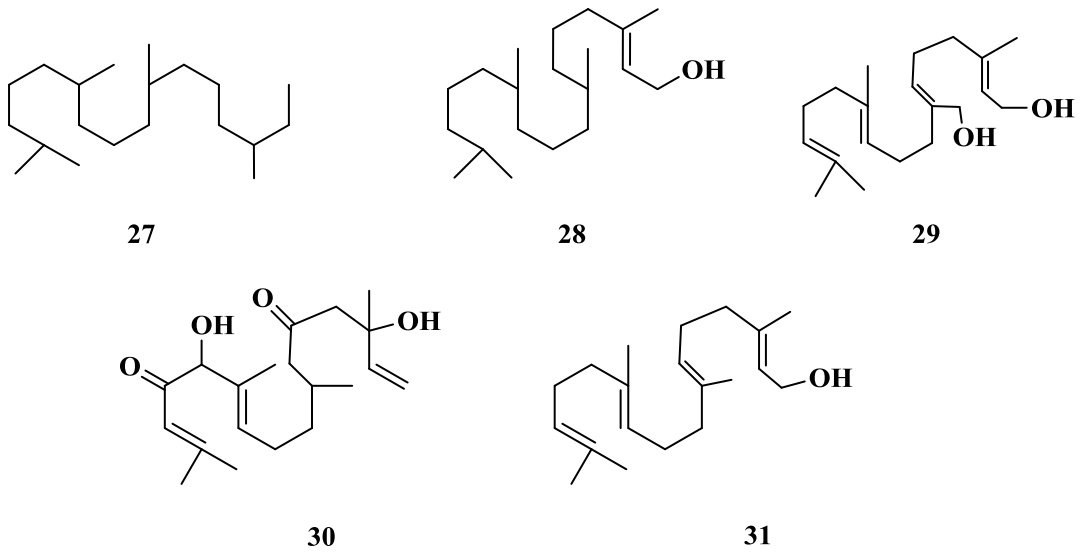
Terpenoids are the secondary metabolites type of hydrocarbons produced as resins in form of turpentine. They are derived from head-to-tail coupling of the C_5- isoprene units. These coupling results to the formation of five carbon (C_5) compounds called hemiterpenoids, ten carbon (C_{10}) compounds- monoterpenoids, 15- carbon (C_{15}) compounds- sesquiterpenoids, 20- carbon (C_{20}) compounds diterpenoids, 30- carbon (C_{30}) compounds triterpenoids and others.

a) **Diterpenoids from the genus *Croton***

These classes of compounds have either cyclic or acyclic hydrocarbon skeleton structures. Diterpenoids isolated from the genus *Croton* have been reported to be bioactive against trypanocidal parasite, HIV and cancer tumors (Díaz-Chiguer *et al*., 2012; El-Mekkawy *et al*., 2000; Grynberg *et al*., 1999).

i. Acyclic diterpenoids reported from the genus *Croton*

Acyclic diterpenoids are linear and the simplest types of skeleton which may have cyclic or lactone groups included in their structures. They include phytane (**27**), phytol (**28**)and its isomers, derivative, plaunotol (**29**) (Wungsintaweekul & De-Eknamkul, 2005), 3, 12- dihydroxy-1, 10, 14- phytatrien-5, 13-dione (**30**) (Tansakul & De-Eknamkul, 1998) and geranylgeraniol (**31**) (Attioua *et al*., 2007; Chabert *et al*., 2006). Some of the structures for acyclic diterpenoids are shown in figure 2.3.



27 **28** **29**

30 **31**

Figure 2.3: Acyclic diterpenoids isolated from the genus *Croton*

ii. Cyclic diterpenoids

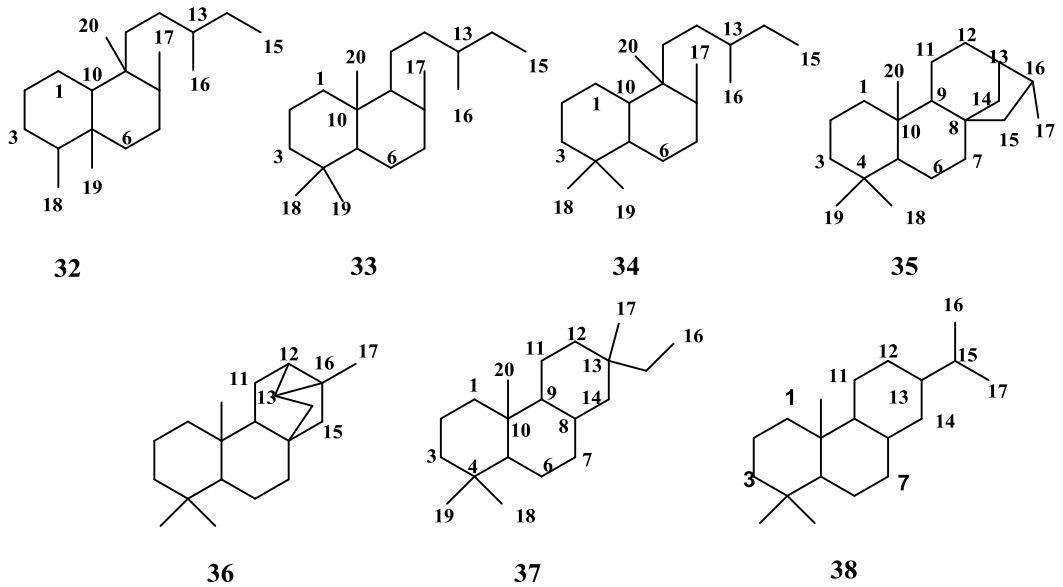
They contain a number of rings which are fused together to form *di-*, *tri-*, *tetra-* and *penta-* hydrocarbon skeleton. The most commonly types of cyclic diterpenoids that have been isolated from the genus *croton* belong to clerodane (**32**), labdane (**33**), halimane (**34**) kaurane (**35**), trachylobane (**36**), pimaranes (**37**) and abiatane (**38**) depending on the shifting of the methyl groups and the number of fused cyclic skeletons(De Heluani *et*

al., 2000; Kapingu *et al*., 2000), amongst others (figure 2.4). Of these, the clerodanes and labdanes are major types of compounds that has been reported from the genus *Croton*.

The configuration of cyclic diterpenoids at C-5, C-9 and C-10 is used to classify the compounds further into two distinctive enantiomeric groups that are referred to as '*normal*' and '*ent'* series as they are summarized in table 2.3.

Table 2.3: Classification of diterpenes skeletons on the basis of configuration

Class of diterpenes	Series	Specific rotation	Selected NOESY resonance correlation
Labdane (**33**)	*Normal*	+	H-5α and H-9α; H-5α
	Ent-	-	H-5β and H-9β; H-5β
Pimaranes (**37**)	*Normal*	+	H-5α and H-9α; H-5α
	Ent-	-	H-5α and H-9α; H-5α
Abiatane (**38**)	*Normal*	+	H-5α and H-9α; H-5α
	Ent-	-	H-5β and H-9β; H-5β



32 **33** **34** **35**

36 **37** **38**

Figure 2.4: Skeletal structures of diterpenoids common from the genus *Croton*

1. Clerodane diterpenoids from the genus *Croton*

They are the most common types of diterpenoids that have been isolated from the genus *Croton.* Some of them include; *trans*- dehydrocrotonin (**39**), *cis*-dehydrocrotonin (**40**), crotocorylifuran (**41**) isoteucvin (**42**), jatropholdin (**43**), teucvin (**44**), chiromodine (**45**), epoxychiromodine (**46**) and crotepoxide (**47**). These compounds and their sources have been summarized in table 2.3. Their structures are also shown in figure 2.5**.**

Table 2.3: **C**lerodane compounds isolated from the genus *Croton*

Compound	**Sources from *Croton* species**	**References**
trans- Dehydrocrotonin (**39**) and *cis*-Dehydrocrotonin (**40**)	*C. cajucara* and *C. schieddeanus*	Maciel *et al*., 2000; Babili *et al*., 1998; Grynberg *et al*.,1999; Rodríguez *et al.*,2004
Crotocorylifuran (**41**)	*C. zambesicus* and *C. haumanianus*	Tchissambou *et al.*, 1990; Ngadjui *et al*., 1999
Isoteucvin l (**42**), Jatropholdin (**43**), and Teucvin **(44**)	*C. jatrophoides*	Mbwambo *et al*., 2009
Chiromodine (**45**) and Epoxychiromodine (**46**)	*C. megalocarpus*	Addae-Mensah *et al*., 1989
Crotepoxide (**47**)	*C. macrostachyus*	Addae-Mensah *et al*., 1989; Kapingu *et al*., 2000

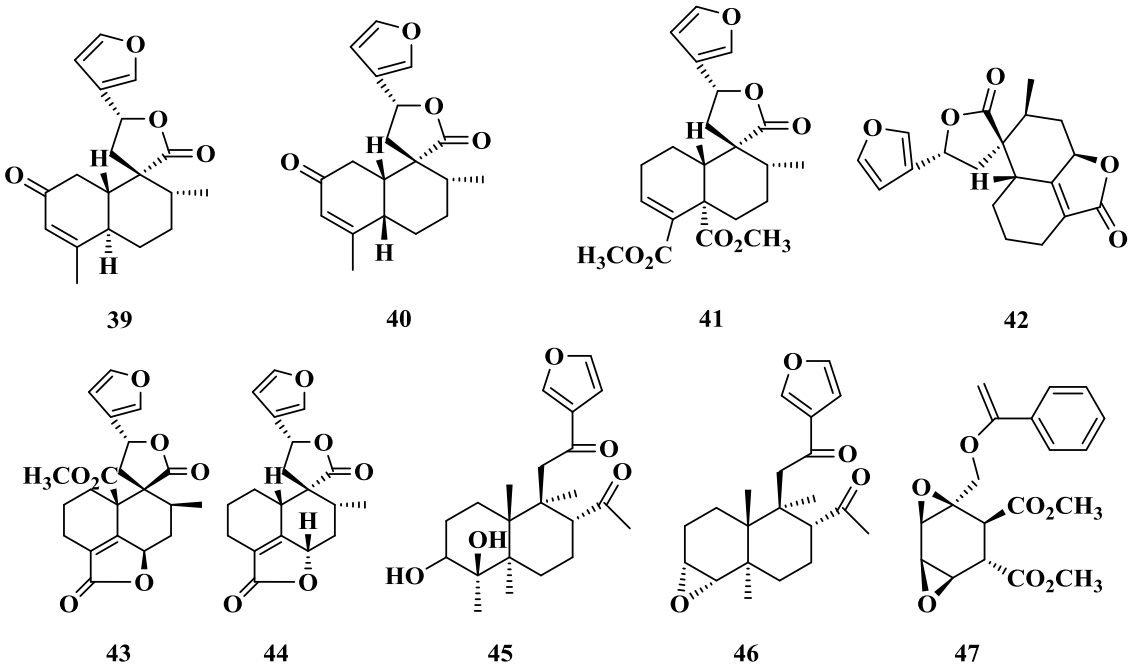


Figure 2.5: Structures of some Clerodane diterpenoids from the genus *Croton*

2. Halimane and Indane derivatives

Biosynthetically, diterpenoids possessing the structure of halimane (**34**) that have been isolated and reported from the genus *croton* include:- centrafine (**48**), penduliflaworosin (**49**), 3α,4β- dihydroxy- 15,16-epoxy- 19-nor- 12-oxo-cleroda- 5(10), 13(16), 14-triene (**50**) and neoclerodane-5, 10-en-19,6β,20,12-diolide (**51**) from *C. membranaceous, C. sjatrophoides, C. penduliflorus* Hutch, *C. hovorum, C. sylvaticus* and *C. megalocarpus* (Adesogan, 1981; Addae-Mensah *et al*., 1989; Schneider *et al*., 1995; Krebs &Ramiarantsoa, 1996, 1997; Peres *et al*., 1998; Mbwambo *et al*., 2009; Kuete *et al*., 2015).

2,6- dimethyl-1-oxo-4-indanecarboxylic acid (**52**) is an indane derivative that was isolated from *C. steenkampianus* (Adelekan *et al*., 2008). The structures of halimane and indane derivatives have been shown in figure 2.6.

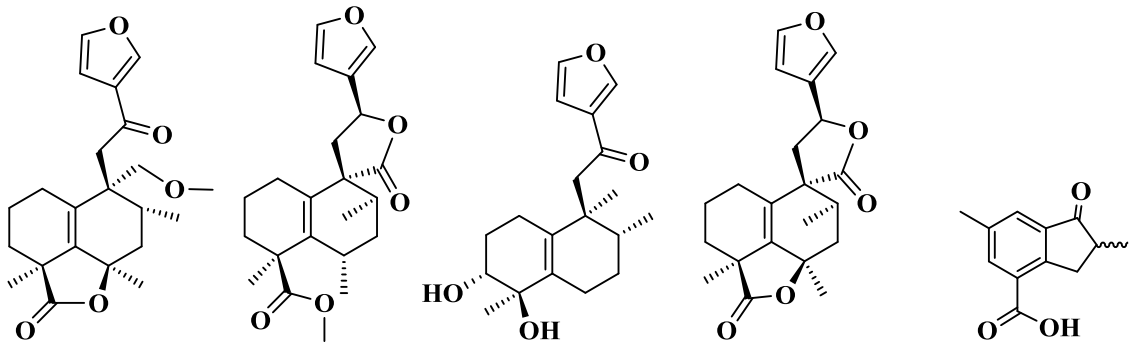


Figure 2.6: Structures of Halimanes and Indane from the genus *Croton*

3. Labdane diterpenoids

Some of the reported labdane diterpenoids that have been isolated and reported from the genus *Croton* have been summarized in the table 2.4 together with their source. Figure 2.7, shows the structures of these labdane diterpenoids

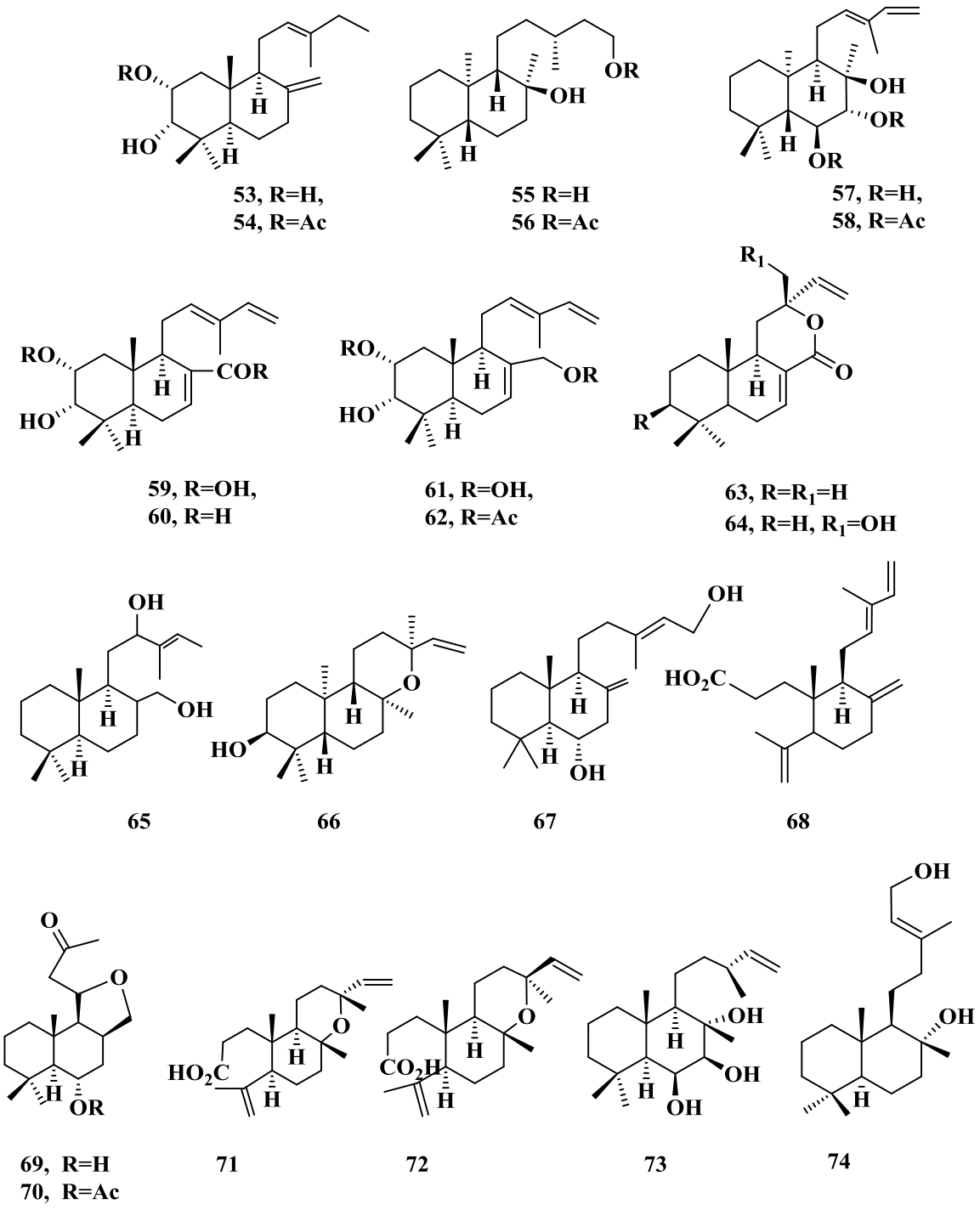


Figure 2.7: Structures of Labdane diterpenoids isolated from the genus *Croton*

Table 2.4: Labdane diterpenoids reported from genus *Croton*

Labdane diterpenoid	**Croton *species***	**Reference**
2α,3α- Dihydroxylabd- 8,12,14-triene (**53**) 2α-Acetoxy-3α-dihydroxylabd-8,12,14-triene (**54)**	*Croton ciliatoglanduliferus*	Nabeta *et al*., 1995
Labdane-8α, 15-diol (**55**) 15-Acetoxylabdan-8α-ol (**56**)	*C. eluteria*	Vigor *et al*., 2001
Austroinulin (**57**) 6-*O*-Acetylaustroinulin (**58**)	*C. glabellus*	Morales-Flores *et al*., 2007
Labda-7,12(*E*),14-trien-17-oic acid (**59**) Labda-7,12(*E*),14-trien-17-al (**60**) 17-Hydroxylabda-7,12,14-triene (**61**) 17-Acetoxylabda-7,12,14-triene (**62**) Labda-7,13-dien-12,17-olide (**63**) 15-Hydroxylabda-7,13-diene-12,17-olide (**64**) 12,17-Dihydroxylabda-7,13-diene (**65**) *ent*-3α-Hydroxymanoyl oxide (**66**)	*C. oblongifolius*	Sommit *et al*., 2003
Crotonadiol (**67**)	*C. zambescus*	Ngadjui *et al*., 1999
Maruvic acid (**68**)	*C. motourensis*	Chaichantipyuth *et al*., 2005
Crotonlaevin A (**69**), and Crotonlaevin B (**70**)	*C. laevigatus*	Huang *et al*., 2014
Geayinine (*ent*-8,13-epoxylabd-14-ene) (**71**) Isogeayinine (**72)**	*C. geayi*	Radulović, *et al* 2006
Crotomachlin (**73)**	*C. macrostachyus*	Addae-Mensah *et al*., 1989
Labd-13(*E*)-ene-8*α*, 15-diol (**74**)	*C. sylvaticus*	Ndunda, 2014

(b) Triterpenoids from *Croton*

Triterpenoids with either pentacyclic or steroid structures. Also some of these compounds have been reported from the genus *Croton.* Some of these triterpenoids includes: acetylealeuritolic acid (**75**) and lupeol (**76**) from *C. zambesicus, C. megalocarpus, C. gratissimus* and *C. haumanianus* (Addae-Mensah *et al*., 1989;

Tchissambou *et al*., 1990; Ngadjui *et al*., 1999, 2002; Mulholland *et al*., 2010), 3β-*O*-acetoacetyllupeol (**77**), Betulin (**78**), from *C. megalocarpus* (Addae-Mensah *et al*., 1989), Friedelin (**79**), lβ-amyrin (**80**) from *C. hovarum* (Krebs & Ramiarantsoa, 1996, 1997), α-Amyrin (**81**) from *C. hieronymi*, α-Amyrin acetate (**82**) from *C. hieronymi*, *C. tonkinensis* (Addae-Mensah *et al*., 1989; Block *et al*., 2004) and 3-*oxo*-20β-hydroxytarastane (**83**) from *C. betulaster* (Salatino *et al*., 2007). These structures are shown in figure 2.8.

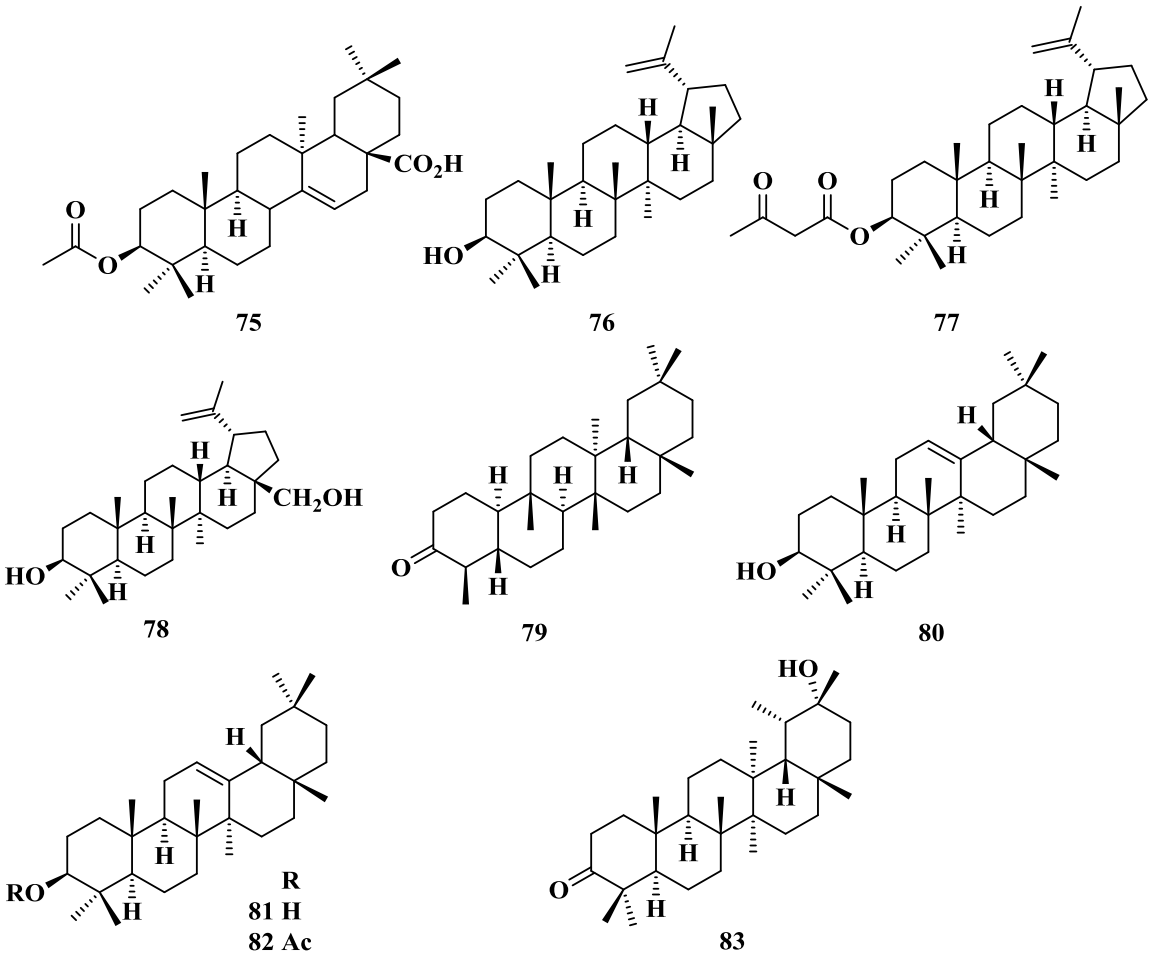


Figure 2.8: Triterpenoids reported from genus *Croton*

2.1.3.2 Alkaloids:

Though alkaloids are not common in the genus *Croton*, some species of the genus have produced this type of phytochemicals. The species which have produced alkaloids include; *C. draco*, *C. flavens*, *C. lechleri*, *C. muscicapa* Müll. Arg. and *C. palanostigma*. The most frequently encountered class of alkaloids isolated from the genus *Croton* are related with a skeleton of benzylisoquinoline (**84**), mostly those of the morphinane (**85**) and protoberberine (**86**) (Salatino *et al*., 2007).

Other classes of alkaloids that have been reported from the genus *Croton* includes aporphine (**87**), proaporphine (**88**), glutarimide (**89**) (Aboagye*et al.*, 2000; Suárez*et al.*, 2004) and guaiane (**90**) hydrocarbon skeletons (Bhakuni *et al.*, 1970; De Araújo-Júnior *et al*., 2005). Examples of glutarimide alkaloids includes julocrotine (**91**) isolated from *C. sylivaticus* and *C. membranaceus* (Aboagye *et al*., 2000; Kapingu, 2005; Bayor *et al*., 2009), crotonimide A (**92**), crotonimide B (**93**), julocrotone (**94**), isojulocrotol (**95**) and julocrotol (**96**) isolated from *C. pullei* and *C. cuneatus* (Suárez *et al*., 2004; Blenkiron *et al*., 2007). Most of these alkaloids whose structures are shown in figure 2.9 have cytotoxic properties (Montopoli *et al*., 2012).

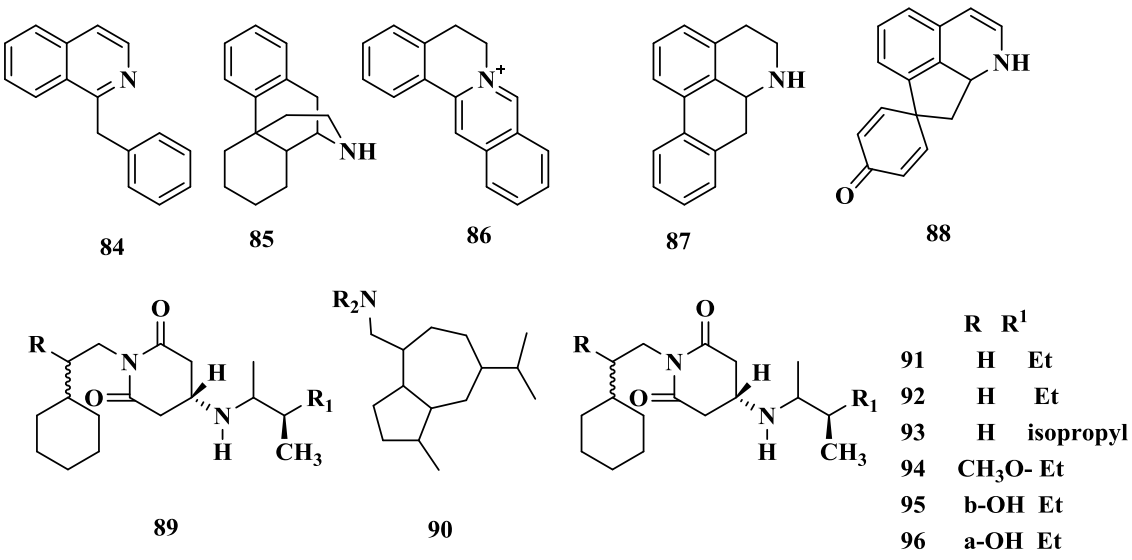


Figure 2.9: Alkaloids reported from genus *Croton*

2.1.3.3 Flavonoids from the genus Croton

Flavonoids are water- soluble glycoside natural compounds which are derived from phenols. Some species the genus *Croton* like *Croton steenkampianus, C. schiedeanus, C. cajucara* and *C. menthodorus* have produced this class of secondary metabolites. Common flavonoids that have been reported from the genus *Croton* include: quercetin (**97**), taxmarixetin (**98**), eriodictyol (**99**) (Adelekan *et al*., 2008; Schmelzer &Gurib-Fakim, 2008), Quercetin-3,7-dimethyl ether (**100**) (De Garcia *et al*., 1986), Kaempferol-3-O-rutinoside (**101**) (Nascimento *et al*., 2017; Zou *et al*., 2010) and Kaempferol-3,4′,7-trimethylether (**102**) (Capasso *et al.*,1998). The structures of flavonoid reported from *Croton* are shown in figure 2.10.

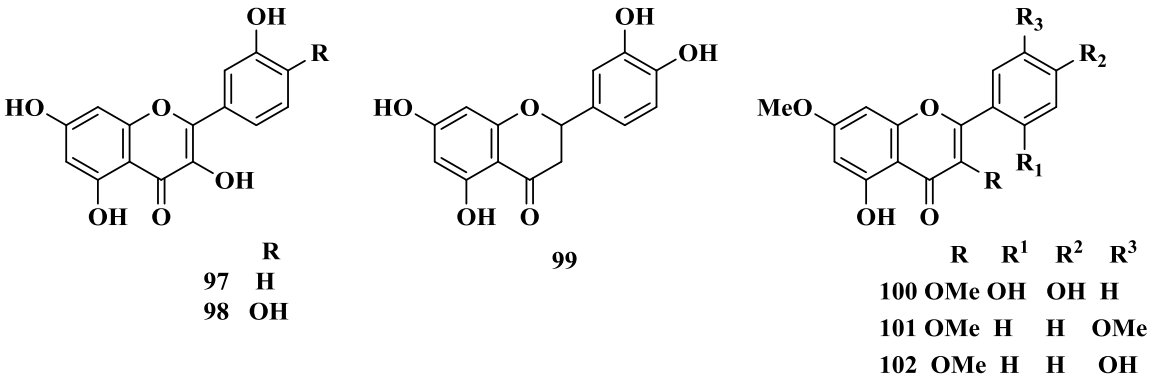


Figure 2.10: Flavonoids from the genus *Croton*

2.2. Croton sylvaticus

2.2.1 The historical and Botanical information

The word *''Sylvaticus*" means sylvan, because this species is associated with dense woodland or sometimes a founding species in woodland forests. Ancient names which were used to describe *C. sylvaticus* are: *Croton sylvaticus* Pax, *Croton bukobensis* Pax, *Croton elskensi* De Wild., *Croton oxypetalus* Möll. Arg., *Croton sphaerocarpum* Kuntze, *Croton stuhlmannii* Pax and *Croton verdickii* De Wild. This plant is commonly known as woodland *Croton* or forest fever berryin English, msindusi in Swahili and in South Africa Afrikaanas calls it Boskoorsbessie; Xhosa call it Umfeze or umagwaqane while the Zulu call it umzilanyoni, umhloshazane, ugebeleweni or indulambahlozi.

Croton sylvaticus (figure 2.11) i*s* a semi-deciduous plant which normally grows in the woodland savannah and coastal forests. It normally doesn't grow as an isolated plant but likes where there are other kinds of plants along the rivers rocky slopes.it is an outcrop at an altitude of up to 1700m above sea level. *Croton sylvaticus* grows up to 30 m tall, with a dense spreading crown. Its stem can grow a girth of up to one metre in diameter and is straight. Its young branches have smooth, grey bark but the stems are

grey and roughly fissured. *C. sylvaticus* is normally strongly aromatic characteristic for the members of the Euphorbiaceae family.

Figure 2.11: photo of Croton sylvaticus

C. sylvaticus is rare in West Africa but it is commonly distributed along the tropical regions of Africa (Schmelzer &Gurib- Fakim, 2008). It is one of the native plants to Ethiopia, Kenya, Lesotho, South Africa and Uganda (Orwa , 2009).

2.2.2 Ethno medicinal uses of *C. sylvaticus* Hutch

Croton sylvaticus is an important herbal medicine. It has demonstrated to have considerable pharmacological properties in different parts of Africa along tropical region in countries like Kenya, Tanzania, Democratic Republic of Congo (DRC), South Africa and Gabon. The various parts of *Croton sylvaticus* that have shown pharmacological activities in various traditional set ups include: leaves stem bark and root bark.

Informal markets in South Africa sell the bark of *Croton sylvaticus* as herbal medicines together with the barks of *Acacia sieberiana* DC., *Acacia xanthophloea* Benth and *Albizia adianthifolia* Schum (Grace *et al*., 2003).

In East African countries like Uganda, Kenya and Tanzania, the leaf, stem bark and root decoction is used to treat swollen body caused by kwashiorkor or tuberculosis (oedema). These decoctions can also treat and control hypermenorrhea (excessive menstrual bleeding) malaria and inflammations (Beentje, 2016; Kokwaro, 2009; Kamatenesi-Mugisha & Olwa-Odyek, 2007). The seeds of *Croton sylvaticus*, in Kenya, are used as an ethno veterinary medicine; it is used to repel ticks hence controlling tick infestation in cattle (Wanzala *et al.*, 2012).

The most common mode of application is taking these decoctions orally. Other modes of application includes as purgatives, poultices and infusions of the various parts. In countries like Gabon, Tanzania and Kenya seeds, oil seed, leaf and root infusion are taken as a laxatives (Beentje, 2016; Kokwaro, 1976; Burkill, 1985). In Tanzania, a leaf and its sap extract plays a very important role treating ear infections,cancer and malaria (Minja, 1994; Kapingu *et al.*, 2005; 2012).

The roots can be used as poultices for boils (Lovett *et al.*, 1994). In the Democratic Republic of Congo (DRC) the wood shavings treats elephantiasis (Burkill, 1985) and the seeds together with its oil are used as purgatives (Burkill, 1985). When the leaves of *Croton sylvaticus* are mixed with those of other *Croton* species it can be used for the treatment of insomnia and abdominal disorders (Mcgaw *et al*., 2000; Stafford *et al.*, 2005).

In South Africa, the stem bark, leaf or root decoctions of *Croton sylvaticus* have a wide range of uses as herbal medicine ranging from treatment for abdominal disorders, dropsical swellings (Veale *et al.*, 1992), febrile(Gerstner, 1938), fever, indigestion, internal inflammation, intestinal disorders(Schmidt *et al.*,2002), pleurisy (Gerstner,

1941), rheumatism (Palmer & Pitman, 1972; Schmidt *et al.,* 2002) and uterine disorders(Veale *et al*., 1992). The stem bark and roots are used in treating the following conditions: abdominal and internal inflammations, uterine disorders, as a purgative, for pleurisy, indigestion, tuberculosis and rheumatism (Stafford *et al*., 2005; Verschaeve & Van Staden, 2008). In Swaziland a powdered stem bark is used to treat gall sickness in cattle (Watt & Breyer-Brandwijk, 1962), while in Mozambique it is used as a fish poison (Palmer & Pitman, 1972). These ethnomedicinal uses of *C. sylvaticus* in Africa are summarized in table 2.5.

Table 2.5: Some reported ethnomedicinal uses of *Croton sylvaticus*

Country practiced	**Plant part in use**	**Use and practice**	**References**
Kenya	Leaf and root	Decoction treats oedema:- Body swelling due to kwashiorkor and tuberculosis	Kokwaro, 1976; Beentje, 2016
	Leaf, root, seeds and seed oil	Infusion used as purgative	Kokwaro, 1976; Burkil, 1985; Beentje, 2016
	Seeds	Tick prevention and control	Wanzala *et al.*, 2012
	Bark, leaf or root decoction	malaria	Kokwaro, 1976; Beentje, 2016
	Decoction from roots and stem bark when taken orally	Tuberculosis	
	Leaf or root decoction taken orally	inflammation	
Tanzania	Leaf, roots or stem bark decoction taken orally	Tuberculosis	Kokwaro, 1976; Beentje, 2016; Lovett *et al*.,2006; Ngobolua *et al*.,2014
	Fermented leaf, root , seeds or seed oil	Purgative	Kokwaro, 1976; Burkill, 1985; Bentje, 2016
	Roots	used as poultices to treat boils	Lovett *et al.*, 2006
	Leaf	Decoction is taken orally for cancer treatment	Kapingu *et al.*, 2006 & 2012
	Bark, leaf and root	Decoction is taken orally to treat malaria	Kokwaro, 1976; Beentje, 2016; Lovett *et al.*, 2006
	Leaf	Leaf sap is used to treat ear infections	Minja, 1994.
	Leaf and root	Washing the decoction treats oedema	Kokwaro, 1976; Beentje, 2016; Lovett *et al.*, 2006

Uganda	Leaf decoction	Hypermenorrhea	Kamatenesi-Mugisha *et al*., 2007
Democratic	Leaf, root and stem bark decoction	Tuberculosis	Ngobolua *et al.*, 2014
Republic of Congo (DRC)	Wood shavings	Elephantiasis	Burkill, 1985
South	Bark decoction	Abdominal disorders	Bryant, 1966
Africa	Root decoction	Indigestion	Gerstner. 1938.
	Leaves, roots made into poultices	Pleurisy	Gerstner. 1938; Schmidt and Mc Cleland, 2002; Richardson & King, 2010;
	Bark decoction	Uterine disorders	Bryant, 1966
	Bark decoction	Rheumatism	Schmidt & McCleland, 2002; Richardson & King, 2010
	Leaf fumes	When inhaled treats insomnia	Stafford *et al.,* 2005
	Bark decoction	Intestinal disorder	Schmidt *et al*., 2002
	Bark decoction taken orally	Febrile	Gerstner, 1938
	Root decoction	When taken orally controls body fever	Gerstner. 1941
	Bark	Decoction taken orally treats bleeding gums, chest complaints and dropsical swellings	Watt & Breyer, 1962; Bryant, 1966; Pujol, 1990
	Leaf and root	Decoction taken orally treats inflammation	Bryant, 1966; Kokwaro, 1976; Beentje, 2016
Swaziland	Powdered bark	Gall sickness in cattle	Watt & Breyer, 1962
Gabon	Leaf, root, seeds and seed oil	Infusions are taken as Purgative	Kokwaro, 1976; Burkil, 1985; Beentje, 2016

2.2.3 Biological activities of the plant extracts from *C. sylvaticus*

A number of biological studies have been carried out on different extracts from *C. sylvaticus.* These extracts have shown activities antifungal, antibacterial, anticancer, antimalaria and mutagenic properties.

2.2.3.1 Antibacterial Activities

Escherichia coli, Enterococcus faecalis, Staphylococcus aureus, Bacillus subtillis and *Pseudomonas aeruginosa* are among the bacteria species which have been studied against the various crude extracts obtained from *Croton sylvaticus* and found active at different concentrations. Leaf extracts made from *n*-hexane, dichloromethane, ethyl acetate, acetone and methanol of *C. sylvaticus* inhibits weakly against *Enterococcus faecalis, Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus* at minimum inhibitory concentration of 1.25mg/ml (Selowa *et al.*, 2010). *Bacillus subtillis,* a bacteria strain is sensitive towards a methanol extract of the stem bark (Ndunda, 2014).

These findings somehow confirm that this plant species' ethnomedicinal uses as antibacterial agent. Therefore its use to cure of boils and tuberculosis in Tanzania, Kenya and DRC is justified (Kokwaro, 1976; Lovett *et al.,* 1994; Ngbolua *et al.*, 2014; Beentje, 2016;)

2.2.3.2 Antifungal activities

Various extracts from *C. sylvaticus* are active against fungi infections. For instance leaf and root extracts have shown average activity (minimum inhibitory concentration of 0.07 and 0.36 for 24 hours and 48 hours) towards *Cryptococcus neoformans*, (Mokoka

et al., 2010). There is higher activity from root and stem bark extracts against *Candida albicans* (Ndunda, 2014).

2.2.3.3 Anti-inflammatory

Traditional uses of *C. sylvaticus* for the treatment and management of inflammatory conditions such as skin infections and oxidative stress related diseases such as insomnia, abdominal disorders and internal inflammations. The extract of ethanol of the bark of *C. sylvaticus* has shown some inhibition against cyclooxygenase. Aqueous and methanol extracts from roots displayed 5-lipoxygenase inhibitory activity. (Jager *et al*, 1996; Frum & Viljoen 2006)

2.2.3.4 Antioxidant

Different parts of *C. sylvaticus* have been tested for antioxidant and found to have varied activity depending on the part of the plant and the type of the solvent used in extraction and isolation. Aqueous and methanol root extracts of *C. sylvaticus* are active as evaluated by (Frum, 2006). However, Ndunda (2014) evaluated the antioxidant activities of methanol stem bark extract and the extract of the stem bark of *C. sylvaticus* were found to have low antioxidant potential. The documented antioxidant activities of root as confirmed by Frum & Viljoen (2006) and stem bark (Ndunda, 2014) extracts are probably due to the presence of flavonoids and phenolics (Ndhlala *et al.*,2006).

2.2.3.5 Effects on the central nervous system (CNS)

Stem bark extracts and compounds isolated from *C. sylvaticus* have effective activities against neurodegenerative disorders (Aderogba*et al.*, 2013). These neurodegenerative disorders includes Alzheimer's' disease, myasthenia gravis, Parkinsonism and senile

dementia (Natarajan *et al.*, 2009). These findings of Aderogba *et al* (2013) have corroborated the traditional use of *C. sylvaticus* as herbal medicine for insomnia.

2.2.3.6 anti-larvicidal Activity

Extracts of chloroform, methanol and petroleum ether from root and stem bark of *C. sylvaticus* are active against the malaria vector, *Anopheles gambiae* and could be useful leads in the search for new and biodegradable plant derived anti- larvicidal products (Kihampa *et al*., 2009).

2.2.3.7 Toxicity and mutagenic activity

The toxicity and mutagenicity of various crude extracts of *Croton sylvaticus* have also been studied. Extracts from the bark of roots and stems, leaf and twig of *C. sylvaticus* have potential genotoxicity that is they DNA (Elgorashi *et al*., 2003; Taylor *et al*., 2003; Langat *et al*., 2012). However, some of the compounds that have been isolated from *C. sylvaticus* have mutagenic activity (Aderogba *et al*., 2013). In the most recent study the methanol: dichloromethane extract from the stem bark has anti-proliferative activity against the drug-resistant leukemia cells (Omosa *et al.*, 2015).

A further research has shown that aqueous extract of the stem bark of *C. sylvaticus* prolonged ether anaesthesia, reduced exploratory activity, exhibited muscle relaxant activity and analgesic activity on mice. (Mwangi *et al.*, 1998). Aqueous ethanol from the whole stem extract of *C. sylvaticus* is toxic against brine shrimp (Moshi *et al*., 2004).

Pure compounds isolated from *C. sylvaticus* have too been tested for their cytotoxicity properties. For example, 2-[*N*-(2-methylbutanoyl)]-*N*-phenyl-ethylglutarimide isolated from the leaves showed high *in vitro* cytotoxic activity against brine shrimp (*Artemia salina*) larvae (Kapingu *et al*., 2006; 2012). The biological importance or activities of *Croton sylvaticus* have been summarized in table 2.6.

Table 2.6: Parts and biological activities of *Croton sylvaticus*

Plant part	**Extract**	**Biological activity**	**References**
Leaves	Acetone	Antifungal aginst *Cryptococcus neoformans*	Mokoka, 2010
	Dichloromethane,	5-lipoxygenase inhibitory and antioxidant	Frum & Viljoen, 2006; Lans, 2007
	Methanol and water	Antiviral, antitussive treat asthma and syphilitic sores	Langat, 2013
	Ethanol	Toxicity against *Artemia salina.* potentially useful for anticancer	Kapingu *et al*., 2012
Root and stem bark	Dichloromethane	Antibiotic	Ndunda *et al*., 2014

2.2.4 Phytochemistry of *Croton sylvaticus*

Phytochemical studies of *C. sylvaticus* has been done on various parts of the plant that includes leaves (Mwangi *et al*., 1998; Kapingu *et al*., 2006; 2012; Langat *et al*., 2008; Langat, 2009), roots (Ndunda*et al.*, 2014) and stem bark (Mwangi *et al*., 1998; Langat, 2009). From this as expected the classes of secondary metabolites from terpenoids, alkaloids and flavonoids have been reported (Mwangi *et al*., 1998; Kapingu *et al*., 2006; 2012; Langat *et al*., 2008; Aderogba *et al*., 2013; Ndunda *et al*., 2014).

According to literature, the amount and type of the class of compounds isolated from *C. sylvaticus* depends upon the part of the plant extract is and the solvent used for extraction. For instance, sterols and terpenoids extracts from stem bark and the roots especially extracts obtained from more polar solvent like methanol and dichloromethane. On the other hand the other types of phytochemicals are inn small amounts (Ndunda, 2014).

So far the types of compounds isolated from *C. sylvaticus* includes an alkaloid julocrotine (**91**) (Kapingu*et al*., 2012), two halimane diterpenoids; penduliflaworonsin (**103**), and hardwickiic acid (**104**), a clerodane diterpenoid (Kapingu*et al*., 2005) and β-caryophyllene oxide (**105**) α-humulen-1,2-epoxide (**106**). Some of the triterpenoids includes; lupeol (**76**) β-sitosterol (**107**) (Mwangi *et al*., 1998) and 2′-(3″,4″-dihydroxyphenyl)-ethyl-4-hydroxybenzoate (**108**) (Aderogba *et al*., 2013; Mutalib, 2013). These compounds have been summarized in table 2.7 and their structures shown in figure 2.12.

Table 2.7: Some compounds reported from *Croton sylvaticus*

Compound	Source	Reference
Lupeol (**76**)	Roots	Kapingu *et al*.,
Jurocrotine **(91)**		2012
Penduliflaworosin (**103**)		
(-)-Hardwickic acid (**104**)	Leaves	Mwangi *et al*.,
β-caryophyllene oxide (**105**)		1998
3 α -Humulen-1,2-epoxide (**106**)		
β-Sitosterol (**107**)		
2′-(3″,4″-Dihydroxyphenyl)-ethyl-4-hydroxybenzoate (**108**)		Mutalib, 2013

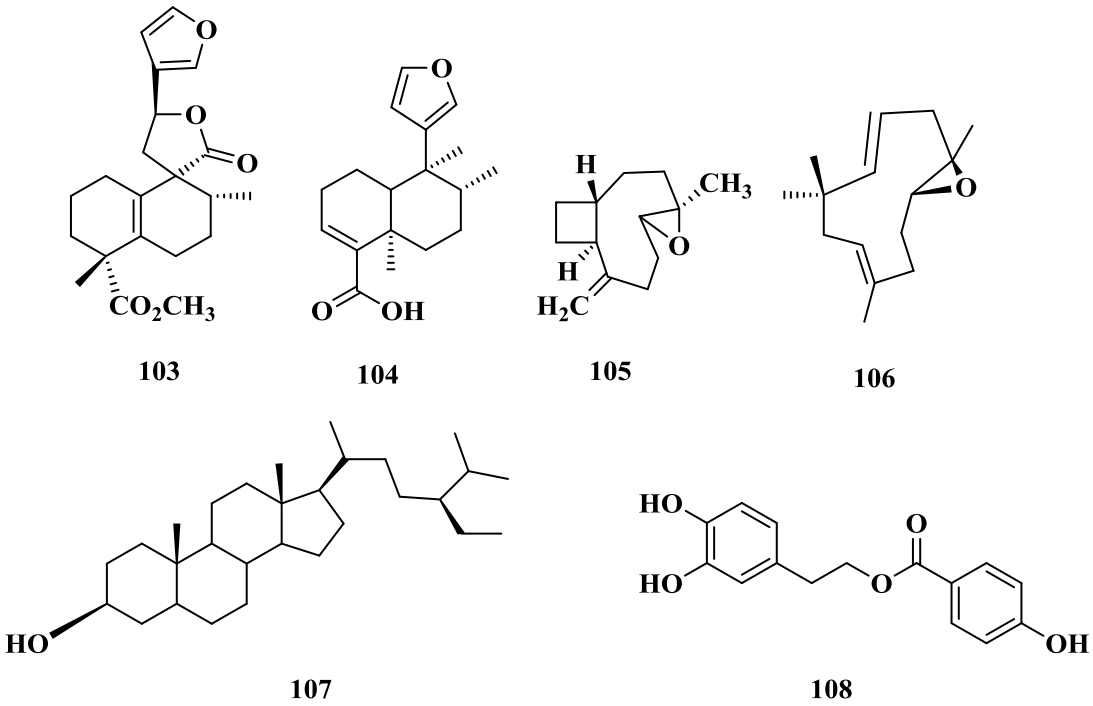


Figure 2.12: Structures of some compounds isolated from *C. sylvaticus*

CHAPTER THREE: MATERIALS AND METHODS

3.1 General Experimental Procedures

The improvement of the spectrometric metthods of structural determination of compounds has made it easier for the elucidation of structures of ner compounds isolated. These spectroscopic methods include fourier transfer infrared (FTIR), mass spectrometry (MS), gas chromatography-mass spectrometry (GC–MS) and 3D nuclear magnetic resonance (1D, 2D, ^{1}H and ^{13}C NMR). Lately the isolation, purification, characterization and identification of novel compounds from natural products has been made easier.

In this research, extraction and Isolation of pure compounds from the crude extracts was carried out in the college of Biological and Physical Sciences, University of Nairobi, Chiromo campus. Isolation was carried out by subjecting the crude extracts to chromatographic separations using mainly column chromatography (CC) packed with silica gel using Merck silica gel 60 (0.063-0.200mm) as a stationary phase and gravity gradient elution by ethyl acetate in *n*-hexane as the mobile phase. The purity of solvents used in extraction and isolation was ascertained by first distilling the commercially provided solvents in the laboratory before using them. The preparative Thin Layer Chromatography (Prep-TLC) was also used in isolation. Details of chromatographic separations were monitored on analytical thin layer chromatography (TLC). Analytical TLC was carried out using factory pre- prepared aluminium plates (0.25mm) coated with high purity silica gel grade Merck grade 9385, pore size230-400 mesh. Observation was done on UV lamp 254 nm and 366 nm for UV inactive compounds like phenolic for detection. The TLCs were exposed in ammonia solution and iodine crystals.

Structure elucidation of the isolated compounds were determined by the organic spectroscopic technique, Nuclear Magnetic Resonance (NMR), 2-Dimensional NMR techniques were used to completely assign the protons and carbons as well as stereochemistry of the compounds obtained.

For 1D NMR, the ^{1}H-NMR spectra were perfomed on a 600 MHz FT NMR bruker Spectrometer on (AM-300,AM-400 and AM-500 FT), the internal standard used was tetramethylsilane (TMS). The ^{13}C-NMR spectra were recorded at 75MHz on the same instrument. The 2-Dimensional NMR spectroscopy like COSY, NOESY, HMQC and HMBC spectra were also recorded on Bruker spectrometers operating at 300, 400 and 500 MHz. The chemical shift values were given in parts per million (ppm) (δ). Relative to the internal standard Tetramethylsilane (TMS) and coupling constants were measured in Hertz (Hz).

3.2 Material Collection

Croton sylvaticus was identified and the stem bark was collected from Kakamega forest in Kakamega County, Kenya(GPS coordinates: 0.259434, 34.915133) (map: see appendix 4) with the help of a taxonomist in the School of Biological Sciences, University of Nairobi (UoN).The voucher specimen of this plant, KO-Sept-2016, was deposited at the University of Nairobi (UoN) herbarium for future reference.

3.3 Extraction

The barks which were sampled from the stem were sliced into small portions and air dried under shade for two weeks. A weight of 2.4 kg was produced. The dried stem bark

was then ground into a fine powder using an electric mill and then left to air dry further before the extraction process. It resulted to a total weight of approximately 2005g.

About 2000g of this was then sequentially soaked for 24 hours in three liters of (1:1 v/v) methanol (CH_3OH) in dichloromethane (CH_2Cl_2) solvent and then filtered to obtain the filtrate. To ensure exhaustive extraction the material was soaked and filtered for two more times each after 24hours. After exhaustively extracting using the MeOH: CH_2Cl_2 solvent the same material was soaked in a more polar solvent composed of 0.05:0.95 v/v water in methanol and the same process of soaking for 24 hours at a temperature of about 25^0C and then was filtered. The procedure of soaking and filtering was repeated for thrice.

The resultant filtrates were concentrated *in vacuo* using a rotary evaporator to yield two brown extracts of 150g and 90g for 1:1 methanol (MeOH) : dichloromethane (CH_2Cl_2) and 0.05: 0.95 water (H_2O) : (MeOH), respectively.

3.4 Isolation of Compounds from the extract

The two extracts obtained in section 3.3 above were analyzed using thin layer chromatography (TLC). Their profiles were compared and were found to be similar and for the purpose of isolation of compounds from them, the two extracts were combined together to form a total 240g of extract.

Column chromatography (CC) was run to isolate the compounds from the extract. An 80mm diameter column was packed with 2kg of silica gel using *n*-hexane and the packed column was left standing overnight. Then 200g of the extract was adsorbed onto a similar amount of silica gel and loaded onto the packed column.

Elution was done using ethyl acetate-hexane solvent system as a mobile phase. Initially elution started with neat *n*-hexane then subsequently its polarity was increased by gradually increasing the amount of ethyl acetate (EtOAc) and reducing the amount of *n*-hexane as follows: 0.02:0.98, 0.04:0.096, 0.06:0.94, 0.10:0.90, 0.12:0.88, 0.15:0.85, 0.20:0.80, 0.25:0.75, 0.30:0.70, 0.35:0.65, 0.40:0.60, 0.5:0.5, 0.6:0.4, 0.7:0.3, 0.8:0.2, 0.9:0.1 ethyl acetate: hexane solvent system and finally 100 % ethyl acetate. The fractions were collected at a volume of approximately 500ml. These fractions were concentrated using a rotary evaporator *in vacuo*.

The initial fractions from the main column that were eluted between 2 % to 10 % ethyl acetate in hexane yielded only fatty substances. The fractions of the major column eluted at 0.20: 0.80v/v EtOAc: *n*-hexane yielded white amorphous solid of labd-13(*E*)-en-8α,15-diol (**74**, 43 mg) while from the same column at 0.25:0.75 EtOAc, 18-*nor*-labd-13(*E*)-en-8α,15-diol (**109**, 50 mg) crystallized in the flask, these crystals were purified by filtering them out through the whatman filter paper and washing them with distilled *n*-hexane.

The rest of the fractions were combined based on the similarity of their TLC profiles into the following fractions; KNO-1A (18.2g), KNO-2A (20.4g), KNO-3A (15.5g) and KNO-4A (25.6g. Fraction 2A with an interesting TLC profile of only two spots were subjected to further purification using column chromatography and silica gel as the stationary phase eluting with EtOAc/*n*-hexane solvent system. The earlier fractions (1-20) of the minor column crystallized and afforded a white crystal. This was filtered and purified by washing with *n*-hexane and afforded austroinulin (**57**, 34 mg).

3.5 Bioassay Evaluation

3.5.1 General overview of the evaluation

In this section the cytotoxic potential of the isolated compounds from the Kenyan *Croton sylvaticus* was evaluated by screening the compounds against drug -sensitive (CCRF-CEM) and drug resistant (CEM/ADR5000) human leukemia cancer cell lines. This evaluation was carried out to ascertain the possibility of getting a leukemia chemotherapy source from this plant and also ascertain the claimed ethnomedicinal anticancer properties of *Croton sylvaticus* (Ochwangi *et al*., 2014).

3.5.2 Culturing of the experimental cells

The human drug-resistant (CCEM/ADR5000) and drug-sensitive (CCRF-CEM) leukemia cell lines were cultured in RPMI 1640 with a favourable environment for the growth of the cells at a humidified 5% CO_2 atmosphere and at a body temperature of 37 °C. All tests were done with cells in the logarithmic growth period.

3.5.3 *In vitro* anticancer evaluation

The anti- cancer activity of the crude extract and the isolated compounds was carried out using the resazurin assay as described by O'Brien *et al* (2000). This technique assessed the cytotoxicity of the samples towards the two human leukemia cancer cell lines. Resazurin bioassay is a technique of determining the percentage composition of live cells in a sample by fluorescence. The dark blue resazurin dye indicator with low natural fluorescence is reduced to a highly fluorescence pink colored resorufin by live cells during metabolism (O'Brien *et al*., 2000). The percentage fluorescence is directly proportional to the number of live cells in the sample.

First, the samples of the extract and the pure isolated compounds were prepared by dissolving 0.0047g of the samples in 235µl of Dimethyl Sulfoxide (DMSO) and then they were diluted to obtain a concentration of 20µg/ml by adding the RPMI medium.

A volume of 100ml of the cultured drug-resistant and drug-sensitive viable leukemia cells equivalent to 10000 cells were seeded into a 96-well plate. The adherent cells were allowed to attach to the wells overnight before 100 ml of the early prepared 20 µg/ml samples were added into the wells containing the viable cells. This reduced the concentration of the samples to 10µg/ml finally in each well. A recognized anticancer drug, doxorubicin was also included in the bioassay evaluation served as a positive control and DMSO as a negative control. This was followed by incubating the plates in an environment of 5 % carbon dioxide, with a relative humidity of 95 % and a temperature of 37°C for further 72 hours. Then to each treated well containing the viable cells and the samples was added 20 µl of resazurin (Sigma-Aldrich, Schnelldorf, Germany) 0.01 % w/v in double-distilled water (ddH_2O) and they were incubated for four more hours in the same conditions to allow for the reduction of the resazurin dye indicator to highly fluorescent resorufin. The fluorescence was recorded on a plate reader (Tecan, Crailsheim, Germany) using an excited wavelength of 544nm and emission wavelength of 590nm. The assay was repeated twice for each sample with six replicates for each. The viability of the cells in the assay was evaluated by comparing the treated and the untreated cells. IC_{50} values of the samples were calculated using the calibration curve by linear regression using the Microsoft excel software whatever results was obtained was interpreted as results of growth aftermath of cell proliferation and cell death.

CHAPTER FOUR: RESULTS AND DISCUSSION

4.1 Description of the Plant and its Ecology

Croton sylvaticus trees were found near a permanent stream in Kakamega forest of Kakamega county, Western Kenya. This was the same locality that was described earlier by the traditional practitioners (Ochwang'i *et al*., 2013).

4.2 Yields of the extracts and the compounds

The crude extract of the stem bark from *Croton sylvaticus*, that was prepared and obtained as described in Section 3.3, yielded about 150g (6.25 %) of 1:1 methanol (MeOH): dichloromethane (CH_2Cl_2) v/v and 90g (3.75 %) of 0.05: 0.95 water (H_2O): methanol (MeOH) v/v crude extract of the 2.4kg of dry material which is about 10 %yield with respect to the dry material used.

This extract yielded three compounds which were characterized to be having labdane diterpenoid skeletons, they included: austroinulin (**57**, 34 mg), labd-13*E*-ene-8*a*, 15-diol (**74**, 43 mg), and 18-*nor*-labd-13(*E*)-ene-8*α*, 15-diol (**109**, 50 mg). The detailed spectroscopic characterizations of these compounds are discussed in Section 4.3.

4.3 Structural Elucidations

4.3.1 Structure of austroinulin (57)

Compound (**57**) was identified as austroinulin, its isolation was as a product of white crystals from the column chromatography. Its molecular formula as proposed from ^{1}H and ^{13}C NMR data spectra is $C_{20}H_{34}O_3$.

^{1}HNMR spectrum (appendix 1a) shows four olefinic proton with chemical shifts at δ_H 6.34 (*dd*, *J*=12, 6.0Hz, 1H), 5.58 (*t*, *J*=6.0Hz, 1H) and a pair at 5.03 (*d*, 1H); 4.85 (*d*,

1H) for H-14, H-12 and H-15 respectively. Also there are two protons attached to oxygenated carbon that have their signals at δ_H 4.32(*dd*, 1H), and another a triplet at 3.33 (*d*, 1H) for H-6 and H-7 respectively. Four methylene protons whose chemical shifts appeared at δ_H 2.47 (2H), 1.52 (2H); 0.92 (2H), 1.73; 1.43 and 1.35; 1.20 for H-11, H-3, H-2 and H-1 respectively. Two methine protons appear at δ_H 1.30 (1H) and 0.99 (*d*, *J*=6Hz, 1H) for H-10 and H-5 respectively. Lastly there are five methyl protons signals corresponding for methyl groups at δ_H 1.77 (3H); 1.30 (3H); 1.25 (3H); 1.23 (3H) and 1.00 (3H) for H-16, H-17, H-20, H-19 and H-18 respectively.

The ^{13}C NMR spectrum (appendix 1b) indicates that this compound has 20 carbon resonances in which there are four sp^{2}^{13}C signals at δ_C 143.4, 138.6, 132.8 and 110.0 for C-14, C-12, C-13 and C-15, respectively. There are three oxygenated carbons with signals at δ_C 82.0, 77.9 and 72.4 for C-7, C-8 and C-6 respectively, two quartenary carbons at δ_C 62.3 and 57.2 for C-9 and C-5 respectively. Four methylene carbons appear at δ_C 45.1, 43.8, 24.5 and 19.8 for C-1, C-3, C-11 and C-2, respectively.

The suggested relative configuration for compound **57** was supported by the correlations observed in the NOESY (appendix 1g) spectrum between H-6 with H-18, H-7 with H-5, H-6 and H-9 and H-12 with H-15. And also H-18 and H-20.The 2D NMR spectral data correlation experiment observation of HSQC (appendix 1c), HH COSY (Appendix 1d), HMBC (appendix 1e), DEPT(appendix 1f), and NOESY (appendix 1g) spectrum in comparison with literature (Morales-Flores *et al*., 2007) showed that the compound had a labdan-8-hydroxylabdan-13-ene skeleton as shown in Figure 4.1.

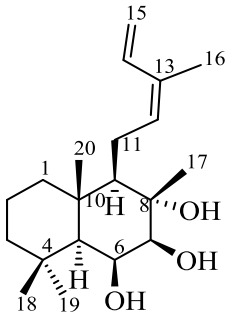


Figure 4.1: Structure for Austroinulin (**57**)

Table 4.1: NMR Correlation Data of Austroinulin (**57**)

POS	$^{13}C(\delta)$,	HSQC(δ)	HMBC	H-H COSY(δ)
1	45.1	1.19(*J*=4.2); 1.35,	10; 5; 9; 11; 2	1.73
2	19.8	1.43(*t, J*=4.2); 1.73 (*t, J*=4.14)	1; 3; 10; 4	1.41; 1.52
3	43.8	1.53(*d, J*=14.58); 0.92	1; 10; 20	1.73
4	35.2			
5	57.2	0.99 (*d, J*=2.28)	6; l9; 1; 10; 5	4.32
6	72.4	4.32 (*dd*)	7; 8; 5; 10; 4	3.33; 1.02
7	82.0	3.33 (*d*)	8; 17	4.32
8	77.9			
9	62.3	1.30 (*d, J*=2.28)	10; 1;	2.29; 2.47
10	40.7			
11	24.5	2.47 (*d, J*=19.56); 2.29 (*m*)	9; 10; 12; 13	1.77; 5.58
12	138.6	5.58 (*t,* J=6.0)	16; 15	2.47; 2.29
13	132.8			
14	143.4	5.0 (*dd*); 4.87 (*dd*)	16; 13; 12	4.87; 5.0
15	110.0	6.35 (*d*)	15; 13	6.35
16	12.1	1.77 (*s,*3H)	15; 12; 13	
17	19.0	1.30 (*s,* 3H)	8; 7; 10	
18	33.9	1.00 (*s,* 3H)	19 ; 10	
19	24.4	1.23 (*s,* 3H)	18; 4; 5	
20	17.4	1.25 (*s,* 3H)	2; 4; 9	

4.3.2 Structural Elucidation of Labd-13(*E*)-ene-8*α*,15-diol (74)

Labd-13(*E*)-ene-8*α*,15-diol (**74**) was determined to have a molecular formula of $C_{20}H_{36}O_2$ as proposed from the NMR data.

The analysis of the ^{13}C NMR spectrum (appendix 2a) revealed 20 carbon signals, based on Distortionless Enhancement by Polarization Transfer (DEPT-90) spectrum (appendix 2b) these carbon signals were assigned to: five methyl carbons that appear at δ_C 15.6, 16.6, 21.7, 24.1 and 33.6, for C-20, C-16, C-19, C-17 and C-18, respectively. Seven methylene carbons appearing at δ_C 18.7, 20.8, 23.7, 39.9, 42.2, 43.1 and 44.7 for C-6, C-2, C-11, C-1, C-3, C-12 and C-7, respectively, two sp^3-hybridised methine carbons that appeared at δ_C 56.3 for (C-5) and 61.3(C-9), two quaternary carbons observed at δ_C 33.4 (C-4) and 39.4 (C-10). Further, analysis of the carbon NMR indicated the presence of two oxygenated carbons one, a primary alcohol, at δ_C59.4 (C-15), and a quaternary hydroxyl group, observed at δ_C 74.3 (C-8). Finally, two olefinic carbons revealed at δ_C 123.4 (C-14) and δ_C 141.0 (C-13).

The ^1H, ^1H COSY spectrum (appendix 2c)showed that the olefinic protonatδ_H5.40 (H-14)was coupled with the stereotopic protons observed at δ_H 4.12 and 4.09 (H-15). The other HH COSY connections were witnessed among the protons at 1.38 (H-6) and 1.83 (H-7), 1.02 (H-9) with the proton signal at 1.33 (H-11).

The proton (^1H) spectrum (appendix 2c) confirmed that there was an olefinic proton at δ_H 5.40 (1H, *t*, *J*=6Hz H-14), two protons at δ_H 4.12 and 4.09 (H-15) attached to a hydroxyl-bearing carbon. The proton facing towards the sp^2 is more shielded due to anisotropic effect hence appears more downfield at δ_H 4.12, seven methylene protons which appeared at δ_H 2.06 (H-12, 2H, *t*, *J*=6Hz), 1.83 (H-7, 2H, *dt*, *J*=6.0, 12.0Hz), 1.63 and 1.60 (H-1, *dt*, *J*=12.0, 6.0Hz, 2H), 1.33 (H-11) and 1.23 (H-2, 2H, *tt*, *J*=6.0Hz), two sp^3 methine protons at δ_H 1.02 (H-9, 1H, *m*) and0.90 (H-5, *dd*, 1H). Finally, five singlet

methyl protons at δ_H 1.66 (H-16, 3H), 1.09 (H-17) 0.85 (H-18), 0.76 (H-20), and 0.75 (H-20).

The assignment of the protons to their carbons was completed by HMQC spectrum data (appendix 2e).The HMBC spectrum (appendix 2f) showed that there was a correlation between the methyl protons with the carbons as follows: the proton at δ_H 1.09 (H-17) correlates with δ_C 74.3, 61.3 and 44.7 for C-8 C-9 and C-7 respectively. While the proton at chemical shifts of δ_H 0.75 (H-20) correlated with the carbons at δ_C 21.7, 33.4, 33.5 and 39.4 for C-21, C-11, C-19, and C-10 respectively. There was also correlation between the olefinic proton at δ_H 5.4 and the carbons observed at δc 16.6, 43. 1 and 59.4; the oxymethine protons at δ_H 4.12 and 4.09 have their cross peaks with the carbons at δc 123.4 and δc 141.0.

The correlations observed from carbon and proton NMR data and literature in comparison identified this structure as a labdane diterpenoid carbon skeleton (Ngadjui *et al*., 1999), and therefore was identified to be labd-13(E)-ene-8*α*,15-diol (**74**). First was reported previously from *Cistus creticus* (Koukoulitsa *et al*., 2008). And it has also been reported from the root bark of *Croton sylvaticus* (Ndunda, 2014).Table 4.2shows the summary of the 1D and 2D NMR spectra for compound **74**

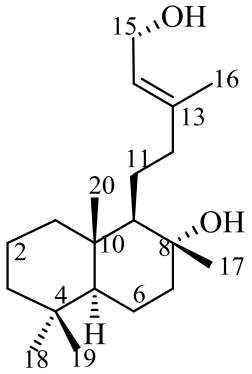


Figure 4.2: Structure for Labd-13(*E*)-ene-8*α*,15-diol (**74**)

Table 4.2: NMR spectra data correlation of Labd-13(*E*)-ene-8*α*,15-diol (**74**)

POS	^{13}C Literature from Koukoulitsa *et al*., 2008	Experimental ^{13}C(δ)	HMQC(δ)	HMBC	H-HCOSY(δ)
1	40.0	39.9	1.63 (*t*); 1.60	3; 5; 10;	1.23 (*J*=6.0)
2	18.7	20.8	1.23 (*tt*)	10;	1.63 (*J*=6.0)
3	42.2	42.2	1.35(*t*)	5	
4	33.5	33.5			
5	56.3	56.3	0.90 (*t*)	20; l9; l1; l3	
6	20.8	18.6	1.38 (*dt*)	5; 10; 7; 19	
7	44.7	44.7	1.83(*t*); 1.81(*t*)	16	1.34; 1.23; 1.66; 1.60;
8	74.3	74.3			
9	61.5	61.3	1.02 (*m*, 1H, *J*=12.0, l6.0Hz)	5; 8; 11	1.35
10	39.5	39.4			
11	23.8	23.7	1.35 (*dt*)	13; 8; 9; 5	2.08; 1.01
12	43.1	43.1	2.08 (*t*)	11; 16; 9; 13; 14	1.33
13	141.2	141.0			
14	123.4	123.4	5.40 (*m*, 1H)	16; 12;15	4.10; 1.66
15	59.5	59.4	4.12 (*m*, 1H); 4.08(*m*, 1H)	14; 13	5.4 (*J*=6.0)
16	16.7	16.6	1.66 (*s*)	13;14; 12	
17	24.2	24.1	1.09 (*s*)	8;9; 7	
18	36.6	33.6	0.83 (*s*)	1; 5	
19	21.2	21.7	0.75 (*s*)	2;	
20		15.7	0.76 (*s*)		

4.3.3 Structural elucidation of 18-n*or*- Labd-13(*E*)-ene-8*α*, 15-diol (109)

The compound was isolated as white crystals with a molecular formula of $C_{19}H_{34}O_2$ as determined using NMR spectral data compared with literature.

The ^{1}H NMR spectrum (appendix 3a) exhibited an olefinic proton that was observed at δ_H 5.45 (H-14, 1H, *ddt, J*=8.16, 5.65, 1.23Hz). This proton was coupled with the proton that was attached to an oxygenated carbon observed at δ_H 4.14(*t, J*=6.87, 2H, H-15); the methylene protons had their resonances appearing at δ_H 2.12(*t, J*=8.21Hz), 1.87 (*dt, J*=12.06, 3.13Hz), 1.72 (*m*),1.72 (*m*), 1.65(*m*), 1.55 (*m*) and 1.41 (*m*) for H-12, H-7, H-1, H-6, H-1, H-2 and H-11 respectively. The methylene proton at δ_H 2.12 (*t, J*=8.12Hz, 2H) is downfield shifted due to the anisotropic effect of the adjacent sp^2 carbon. The proton signal at δ_H 2.12 was coupled to two stereotopic protons with chemical shifts at δ_H 1.55 and 1.41 as shown by HSQC spectrum (appendix 3c).

The other methylene proton had chemical shift δ_H 1.87(*dt,* 2H). There were three methine protons observed at δ_H 1.08 (*t*), 1.00 (*m*) and 0.91 (*m*), for H-9, H-5 and H-4 respectively. While the four methyl protons from which three of them are from quaternary carbons were observed at δ_H 1.72, 1.15, 0.91 and 0.84 for H-16, H-17, H-20 and H-19, respectively.

^{13}C NMR spectrum (appendix 3d) reveals nineteen resonances including; two olefinic carbons at δ_C 140.5 and δ_C 123.4 assigned to C-13 and C-14, respectively, two oxygenated sp^3 hybridized carbons with signals appearing at δ_C 73.8 and 59.1 for C-8 and C-15, respectively, three methine carbons with chemical shifts at δ_C 61.3, 56.1 and 33.1 for C-9, C-5, and C-4, respectively. A quaternary carbon was appearing at δ_C 39.2 for C-10.

Seven methylene carbons were appearing at δ_C 44.6, 43.0, 42.0, 39.8, 23.7, 20.5, and 18.5assigned to C-7, C-12, C-3, C-1, C-11, C-6, and C-2, respectively. There were also four methyl carbons with their chemical shifts at δ_C 23.7, 21.2, 16.1 and 15.3 for C-17, C-19, C-16 and C-20, respectively.

The 2D NMR data which include ^{1}H ^{1}H COSY (appendix 3b), HSQC (appendix 3c) and HMBC (appendix 3e) spectra were correlated and suggested *nor*18-labdane diterpenoid skeleton.

The HSQC (appendix 3c) correlated carbons to their attached protons as follows: olefinic proton, at δ_H 5.45 to a carbon at 123.4, the proton observed at δ_H 4.14 correlated with a carbon at δ_C59.1, proton at δ_H 2.12 correlated with a carbon at δ_C43.0 The two stereotopic protons observed at δ_H 1.87 (*t*) and δ_H1.41 (*m*) correlated with the carbon at δ_C44.6, the proton at δ_H 1.55 had its cross peak with the carbon atδ_C23.7. The doublet for two methyl groups at δ_H 0.84 correlated with the two carbons at δ_C 15.28 and 21. 24. The singlet at δ_H 0.91 was correlated with the methylene carbon at δ_C 33.1. The methylene carbon observed at δ_C 18.46 is correlated with two protons at chemical shifts at δ_H 1.65 and 1.44.

From the ^{1}H, ^{1}H COSY(appendix 3b) spectral data correlation, the doublet of doublet of a triplet proton at δ_H5.45 showed a cross peak with the protons at δ_H4.14, 2.12 and 1.55 while the doublet of a triplets proton at δ_H 1.87 correlated with the proton at δ_H 1.32. The multiplet at δ_H 1.00 also had its cross peak with the proton observed at δ_H 1.32. The proton at δ_H 1.55 showed a cross peak with the protons at δ_H 2.12 and 1.08 while the proton at δ_H 1.65 correlated with the proton at δ_H 1.21. The triplet at δ_H2.12 (J=8.21Hz) was correlated with the protons at δ_H 1.55.

The HMBC spectrum (appendix 3e) showed that the proton at δ_H 5.45 (H-14) had cross peaks with carbons resonating at δ_C 59 .1 (C-15), 43.0 (C-12) and 16.1(C-16). The proton at δ_H 4.14 has its cross peaks with δ_C at 140.5 (C-13) and 123.4 (C-14). The triplet at δ_H 2.1 showed its cross peaks with δ_C 140.5 (C-13), 123.4 (C-14), 61.3 (C-9), 23.7 (C-11) and 16.1 (C-16).The proton at δ_H 1.08 (H-9) had cross peaks observed with

the carbons at δ_H 73.8 (C-8), 56.1 (C-5), 43.0 (C-11) and 39.1 (C-10).The multiplet proton at δ_H 1.00 (H-5) showed cross peaks with carbons at δ_C 61.3 (C-9), 44.6 (C-7), 39.2 (C-10), 33.1 (C-4),21.2(C-19), and 15.3 (C-20). The doublet proton at δ_H 0.84 (H-19) had cross peaks with the carbons whose chemical shifts were observed at δ_C56.1 (C-5), 42.0 (C-3) and 33.1 (C-4), the HMBC is summarized in figures 4.3

The complete 2D NMR correlations are summarized in table 4.3 and as shown by the structure in figure 4.4 which is similar to that of 18-*nor*-labdane diterpenoid skeleton.

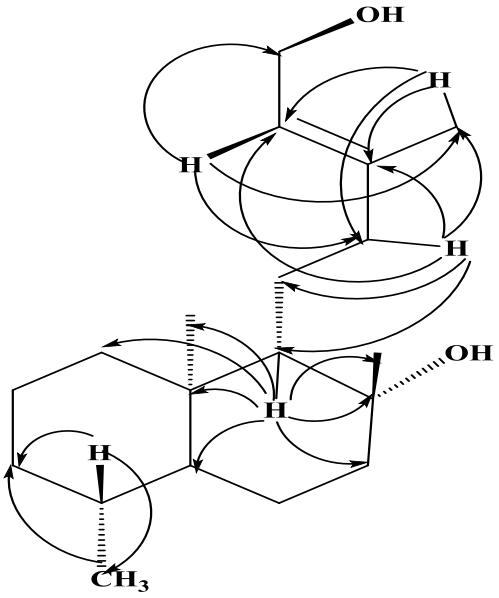


Figure 4.3: Important HMBC correlations of the **109**

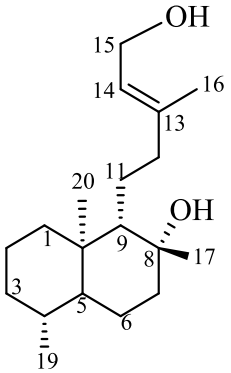


Figure 4.4: **S**tructure of 18-*Nor*- Labd-13(*E*)-ene-8*α*, 15-diol (**109**)

Table 4.3: NMR spectra data for 18-*Nor*- Labd-13(*E*)-ene-8*α*, 15-diol (**109**)

POS	^{13}C (δ),	HSQC (δ),	HMBC	H-H COSY (δ),
1	39.8	1.72; 1.00 (*m*)	10; 3; 20	1.32; 1.87
2	18.5	1.44(*m*); 1.65(*m*)	4; 18;3;	1.21
3	42.0	1.41(*m*); 1.21(*m*)	18; 1; 5; 19	1.15; 1.08; 1.55
4	33.1	0.91(*s*)		
5	56.1	1.00(*m*)	7; 3; 18; 19	1.32; 1.72
6	20.5	1.32(*m*)	5; 8; 7; 4	0.96
7	44.6	1.87(*dt*); 1.41	7; 9; 5; 4	1.41; 1.32
8	73.8			
9	61.3	1.08(*t*)	8; 5; 7; 12	1.44; 1.32
10	39.2			
11	23.7	1.55(*m*); 1.41		2.12; 1.08
12	43.0	2.12(*t*)	13; 14; 9; 16	1.55; 1.41; 5.45; 4.14
13	140.5			
14	123.4	5.45(*ddt*)	15; 12; 16	4.14; 2.12; 1.55
15	59.1	4.14(*t*)	13; 14	5.45; 2.12; 1.55
16	16.1	1.72(*m*)	12; 13; 14	
17	23.7	1.15(*s*)	8; 9; 7;	2.12; 1.08
18	-	-	-	-
19	21.2	0.84 (*d*)		
20	15.3	0.84(*s*)		

4.4 Bioassay Results

The bioassay results for the compounds are tabulated in table **4.4**

Table 4.4: Cell viability against CCRF-CEM and CEM/ADR5000

Samples	Cell Viability (%)	
	CCRF-CEM	CEM/ADR5000
Crude extract	13.04±4.86	22.43±2.84
57	44.89±2.31	53.97±0.70
74	91.88±4.27	79.74±1.77
109	51.40±4.08	66.17±4.79
Doxorubicin	2.64±0.86	78.97±2.89

Extract-1:1: MeOH: DCM, **57**-Austroinulin, **74**-labd-13(*E*)-en-18α,15-diol, **109**-18-*nor*-labd-13(*E*)-en-18α,15-diol and positive control -doxorubicin

The crude extract of the stem bark of *C. sylvaticus* (50 % MeOH in CH_2Cl_2) and isolated compounds were evaluated for their potential to inhibit drug-sensitive (CCRF-CEM) and drug-resistant (CEM-ADR5000) human leukemia cancer cell lines. Doxorubicin, a commonly used anticancer drug was used as the reference drug. The

results are shown in table 4.4 above. Compounds are considered active if they cause cell inhibition of more than 70 % at 10µg/ml leaving less than 30 % of the cell viability (Kuete & Efferth, 2015).

Using the above criteria of evaluation of cytotoxicity potential (Kuete & Efferth, 2015), the 50% MeOH in CH_2Cl_2 extract of the bark of the stem of *C. sylvaticus* was more active against the drug sensitive leukemia cells, CCRF-CEM at 10µg/ml exhibiting cell inhibition of 86.96% compared to the positive control, doxorubicin which showed cell inhibition of 97.36% against the drug sensitive leukemia cells, CCRF-CEM.

However, all the three labdane diterpenes isolated from the stem bark of C*roton sylvaticus* namely austroinul in (**57**), labd-13(*E*)-ene-8*α*, 15-diol(**74**) and 18-*nor*-labd-13(*E*)-ene-8α,15-diol (**109**) exhibited cell viability of more than 30 % and therefore were considered inactive against the evaluated leukemia cells. Compounds **57**, **74** and **109** exhibited cell viabilities of 44.89±2.31, 91.88±4.27 and 51.40±4.08 %, respectively against the drug sensitive CCRF-CEM cells and 53.97±0.70, 79.74±1.77 and 66.17±4.79 %, respectively against the drug resistant CEM-ADR5000 cells. While the positive control, doxorubicin had cell viability of 2.64 % and 78.97 % for CCRF-CEM and CEM-ADR500, respectively. Seemingly, the pure compounds lost their synergistic effect during isolation that is why their cytotoxicity against the cancer cells is low as compared to the extract.

CHAPTER FIVE: CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

The three compounds which were isolated from the combination of approximately140 g of the 1:1 MeOH in CH_2Cl_2 and 90g 0.95:0.05 MeOH in H_2O *Croton sylvaticus* stem bark extracts isolated were labdane diterpenoids that included: austroinulin (**57**), labd-13(*E*)-en-18α,15-diol (**74**), and 18-*nor*-labd-13(*E*)-en-18α,15-diol (**109**). These compounds were elucidated using 1D, 2D NMR spectroscopy and compared with data in literature. The stem bark (50 % MeOH in CH_2Cl_2) was active against the two human leukemia cell lines tested at an inhibition of 86.96. However, the isolated compound including **57**, **74** and **109** were inactive against the drug sensitive (CCRF-CEM) at inhibition of 55.11%, 8.12% and 48.60% respectively. Inhibition for drug resistant (CEM-ADR5000) leukemia cell lines at 10µg/ml was 46.03%, 20.26% and 33.83% for **57, 74** and **109** respectively.

This is the first time austroinulin (**57**) and 18-*nor*-labd-13(E)-en-18α,15-diol (**109**) have been reported from the *Croton sylvaticus,* labd-13(E)-en-18α,15-diol (**74**) was reported by Ndunda (2014) with its activity in malaria.

5.2 Recommendations

1. The isolated labdane diterpenoids should be tested for other biological activity like antiplasmodial, antimicrobial and antidiabetes
2. The compounds isolated in this study that have low activity against leukemia cell lines should be isolated and tested against other cancer cell lines to evaluate their activity.
3. In order to enhance anticancer activity of these compounds isolated from the stem bark of this plant, structure modification of these compounds isolated is recommended.

4. The doings of gynergism and antagonism interaction of crude plant extract was not taken into consideration during this study. Future studies should observe adherence to bioassay guided fractionation approach.

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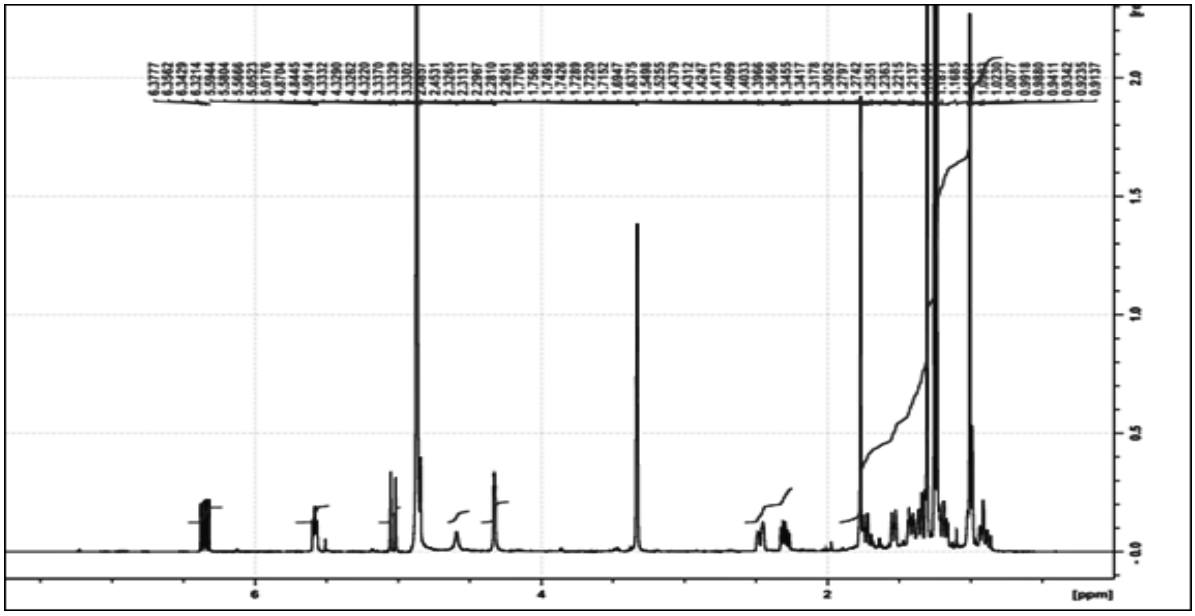
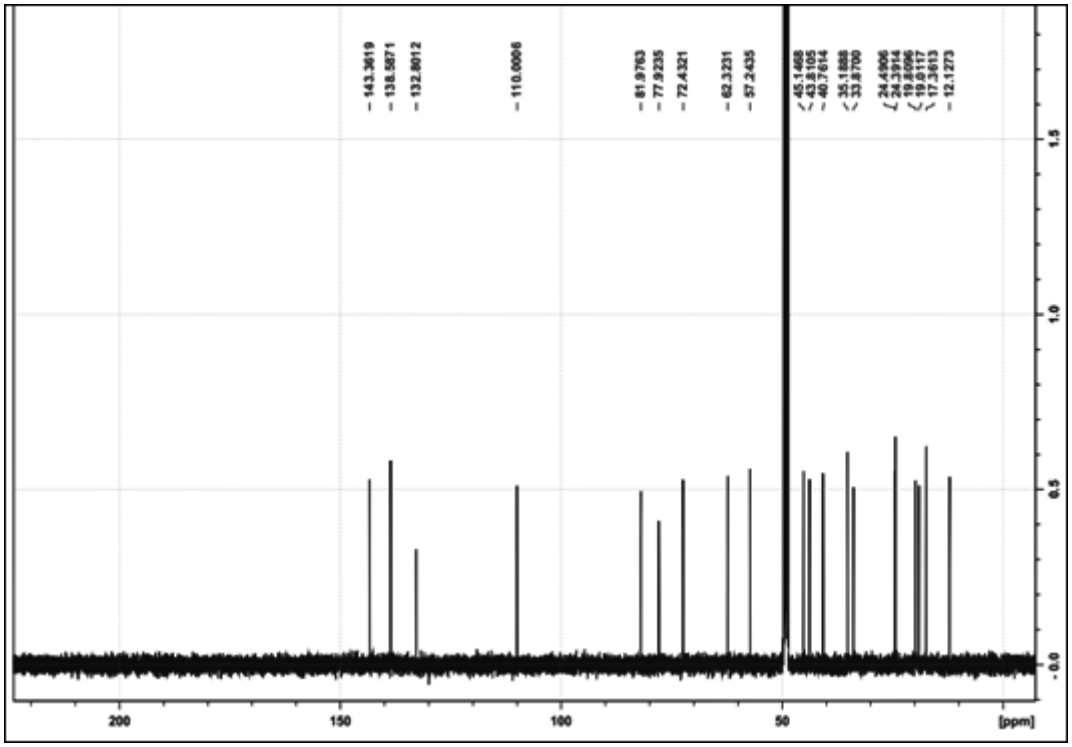
APPENDICES

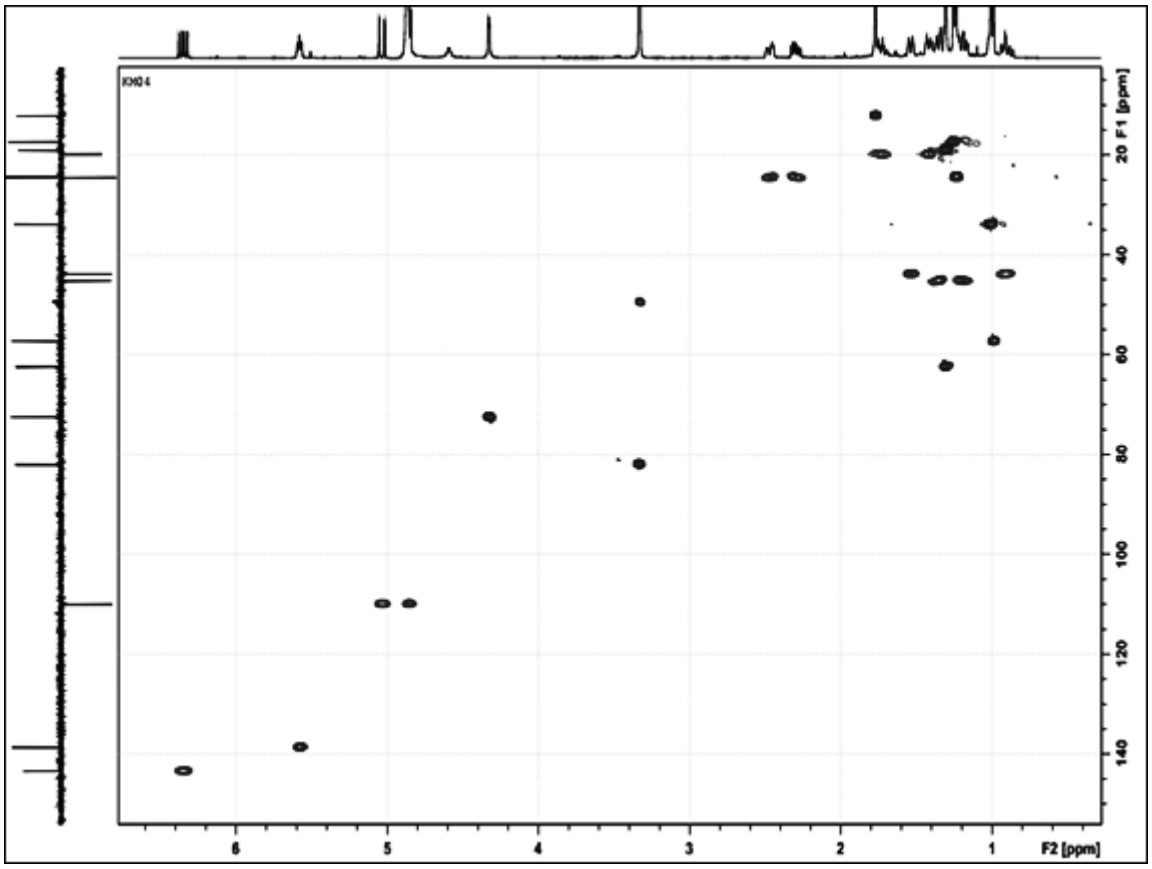
Appendix 1a: ^{1}HNMR spectrum for compound (**57**)



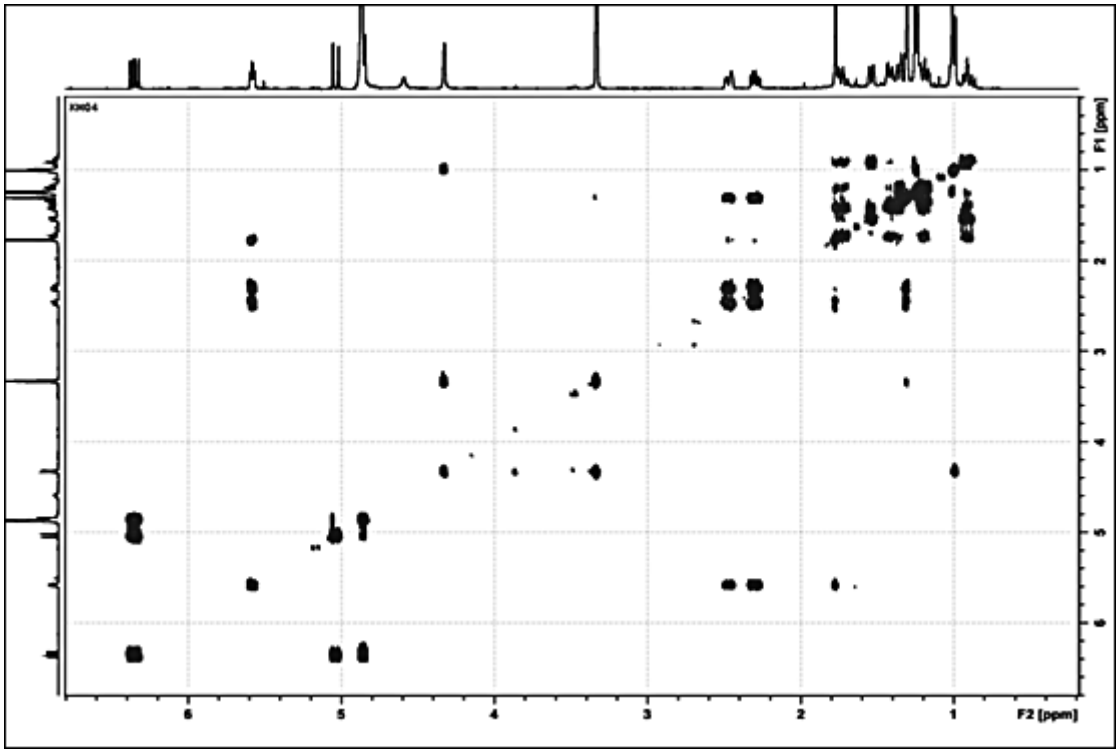
Appendix 1b: ^{13}C Spectrum for Austroinulin (**57**)



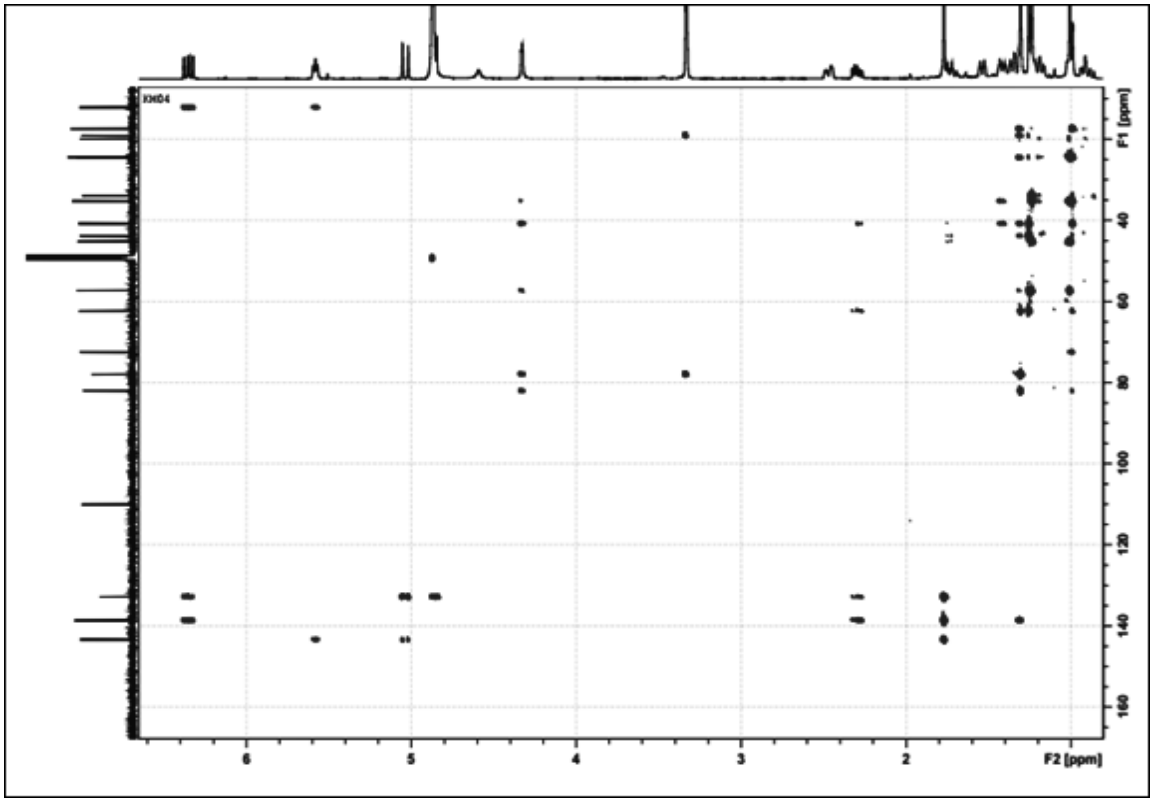
Appendix 1c: HSQC NMR spectrum for austroinulin (**57**)



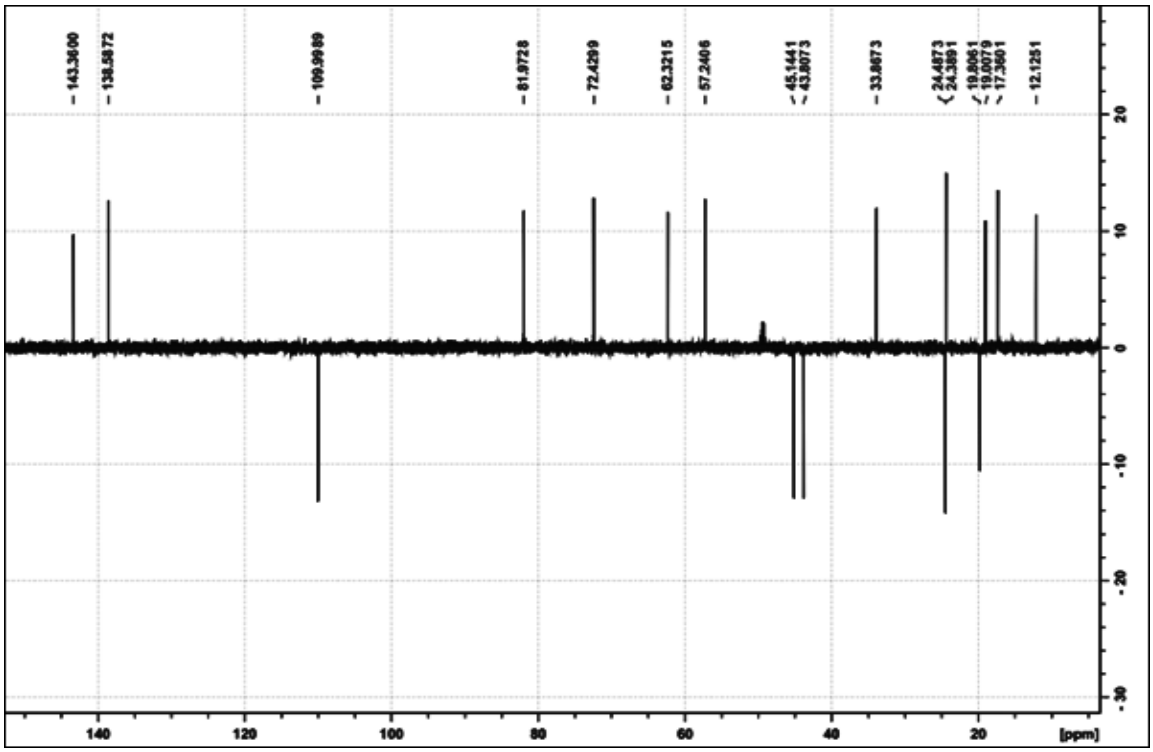
Appendix 1d: HH COSY Spectrum for austroinulin (**57**)



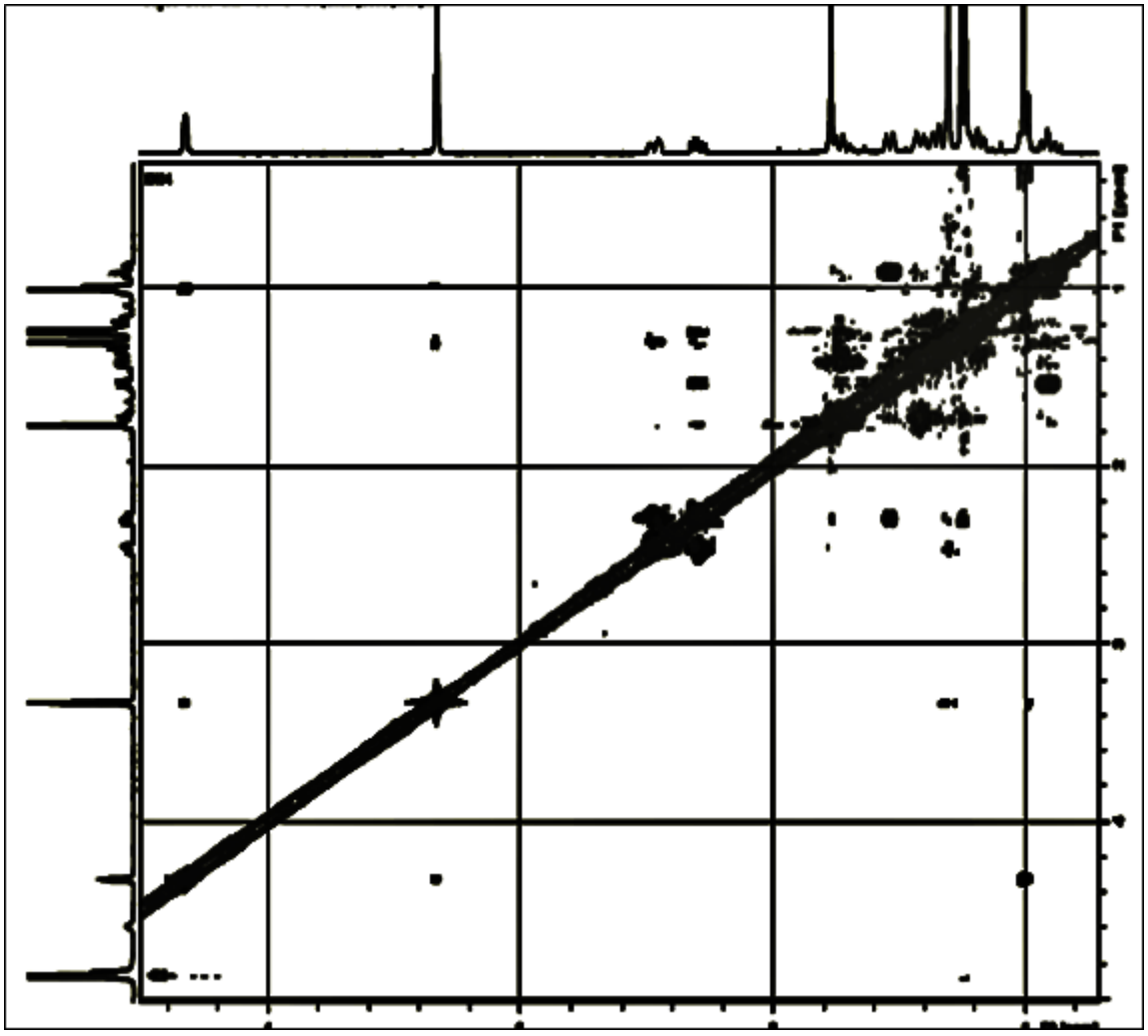
Appendix 1e: HMBC Spectrum for austroinulin (**57**)



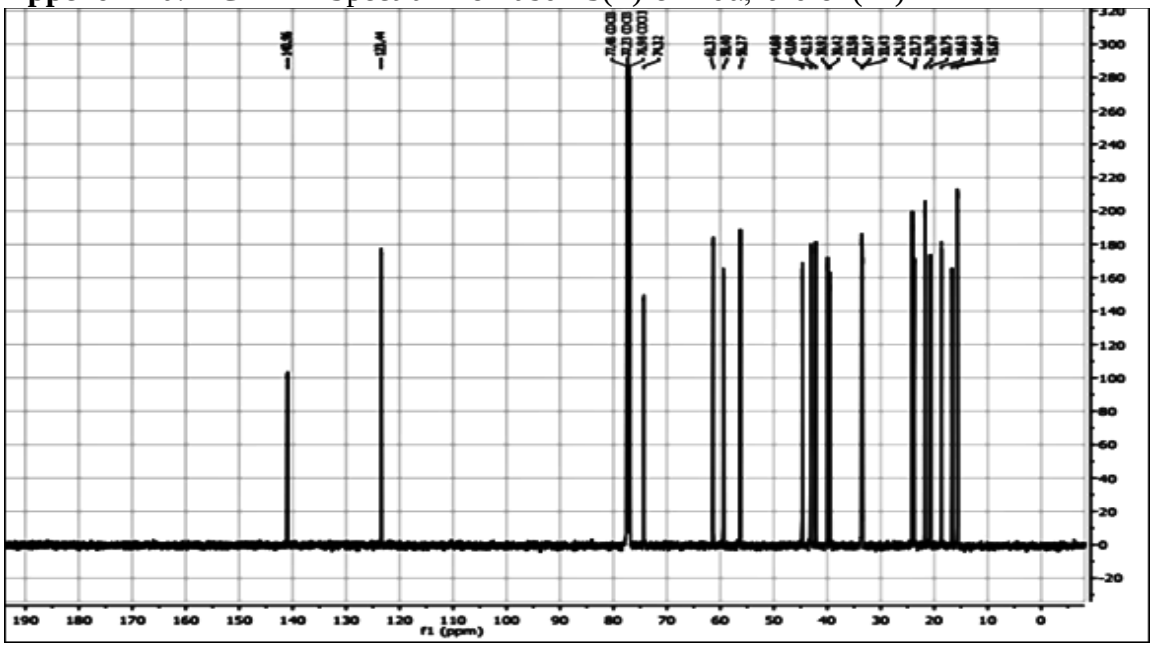
Appendix 1f: DEPT spectrum for Austroinulin (**57**)



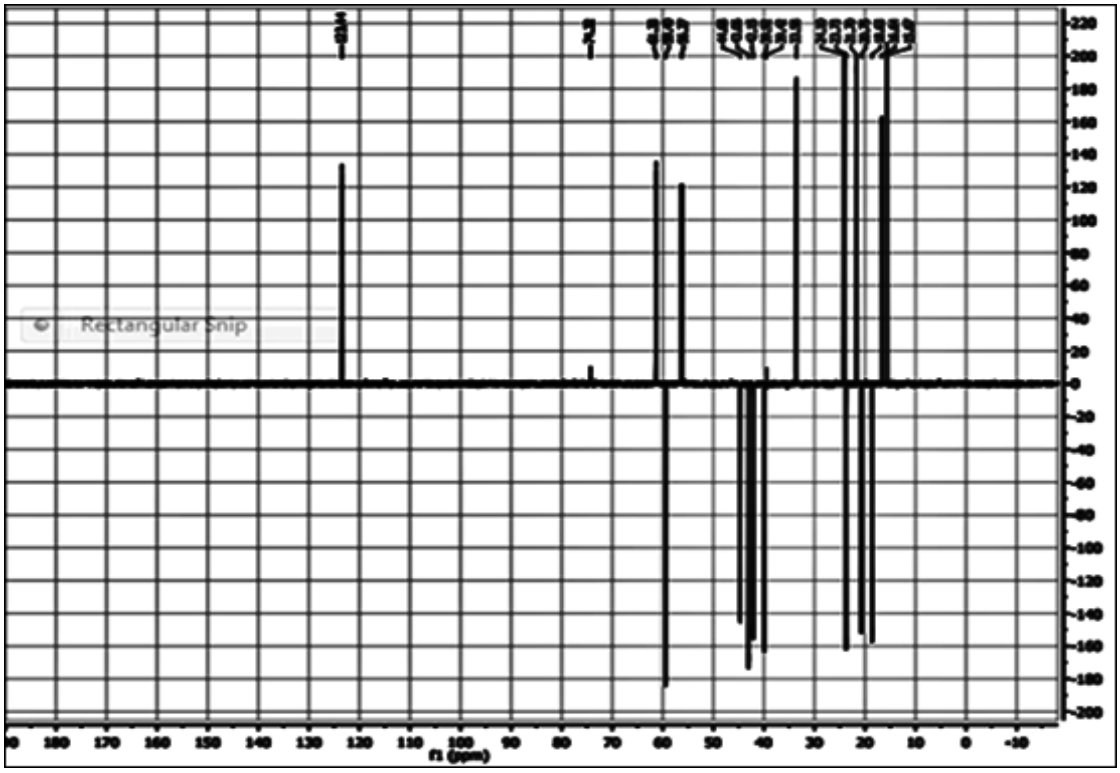
Appendix 1g: NOESY Spectrum foraustoinulin (**57**)



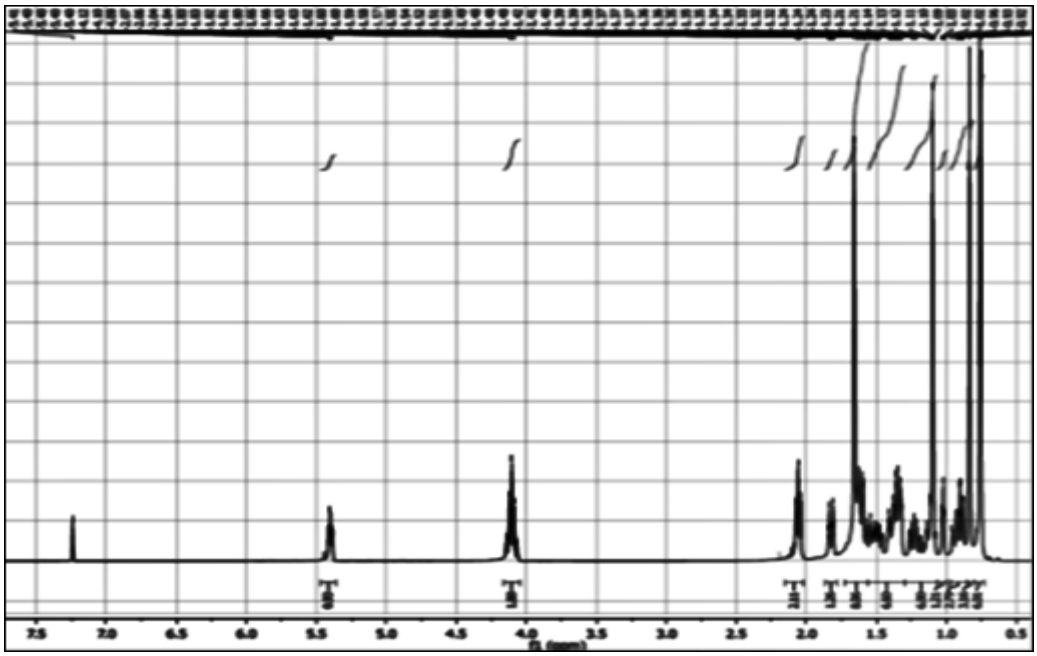
Appendix 2a: ^{13}C NMR Spectrum for labd-13(E)-en-18α,15-diol (74)



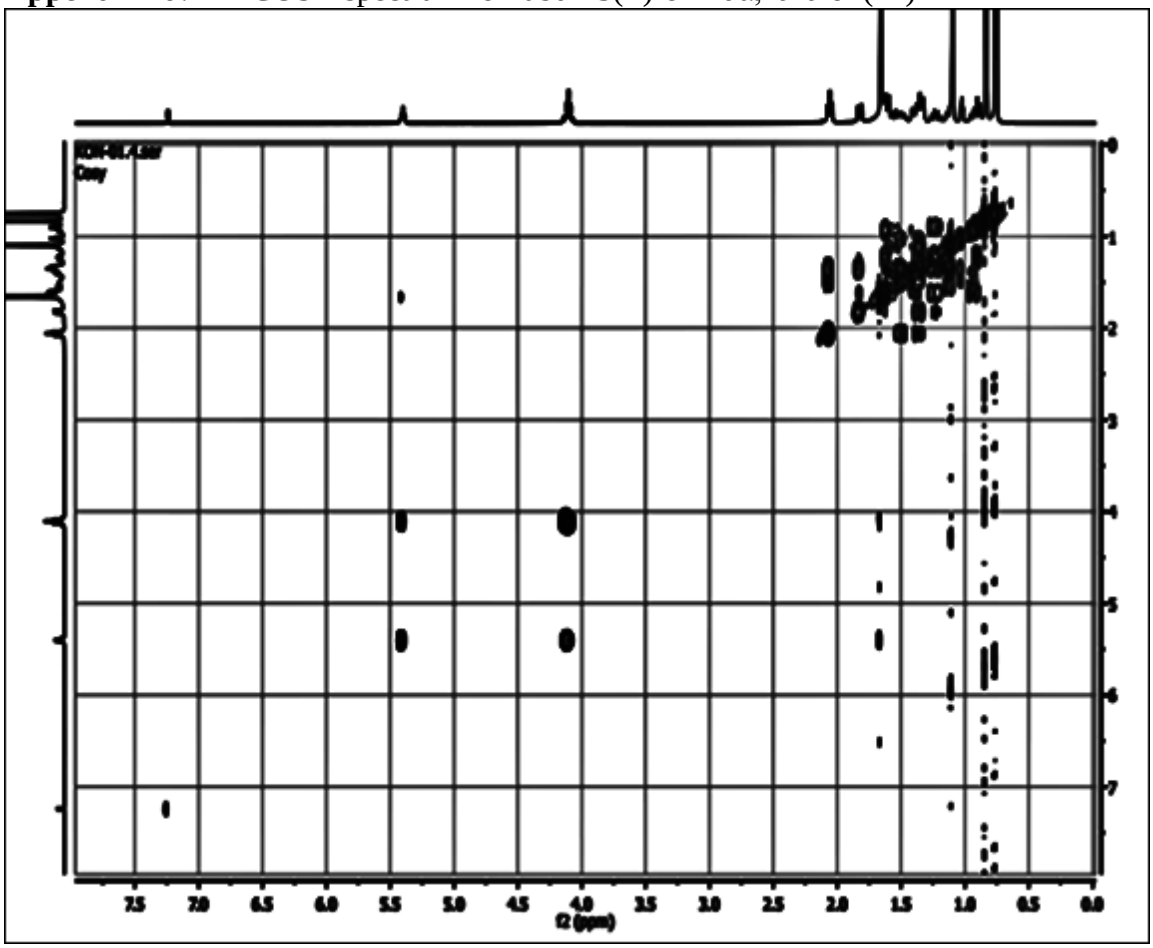
Appendix 2b: DEPT 90 spectrum for labd-13(E)-en-18α,15-diol (**74**)



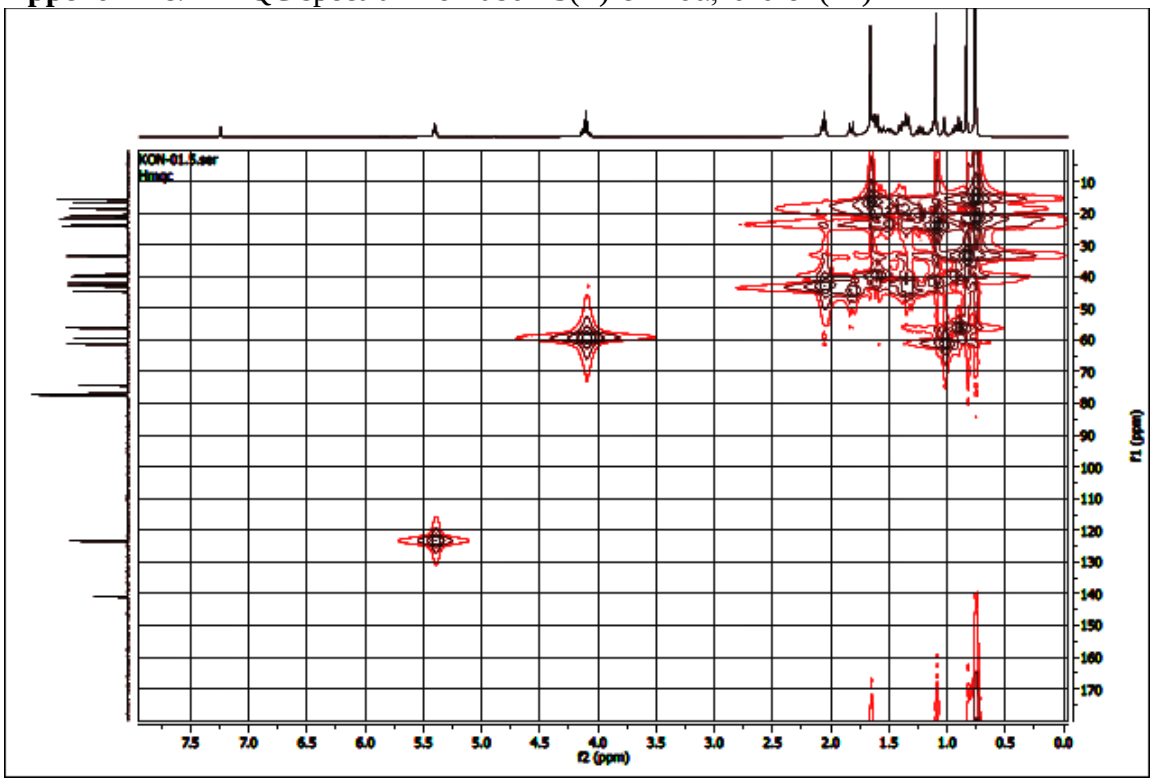
Appendix 2c: ^{1}H NMR spectrum for labd-13(E)-en-18α,15-diol (**74**)



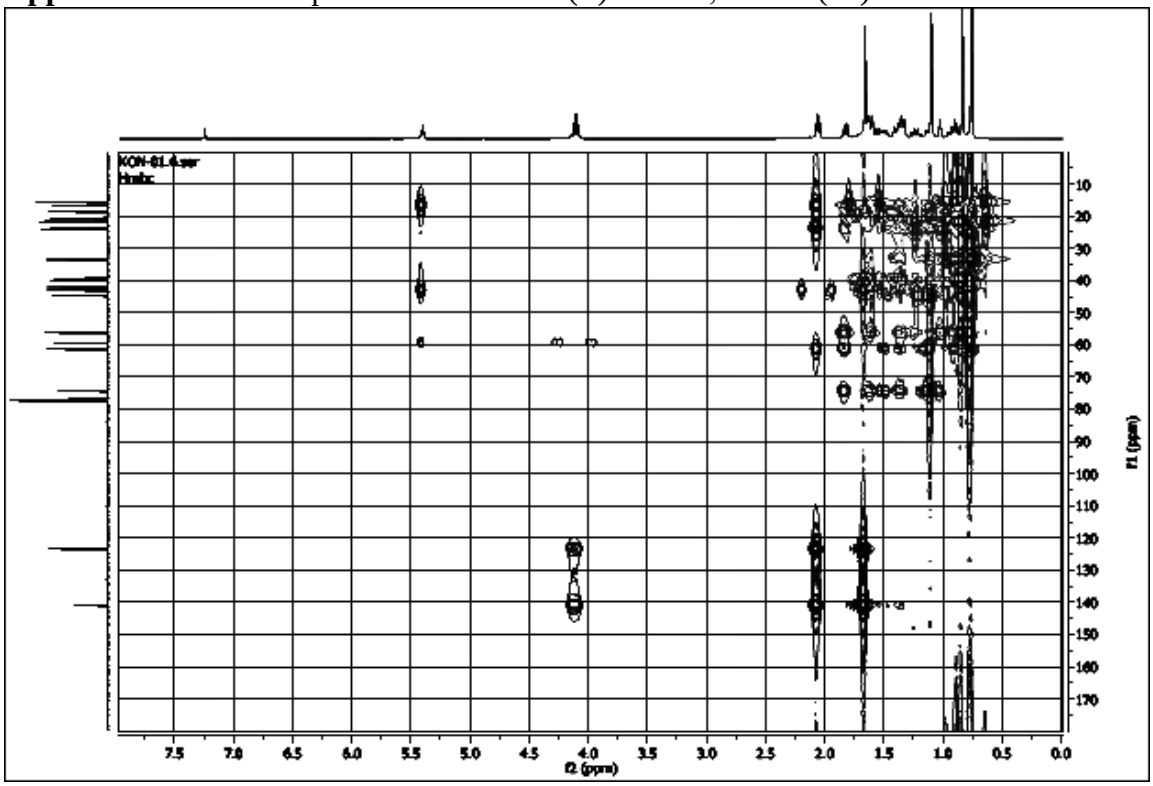
Appendix 2d: HH COSY spectrum for labd-13(E)-en-18α,15-diol (**74**)



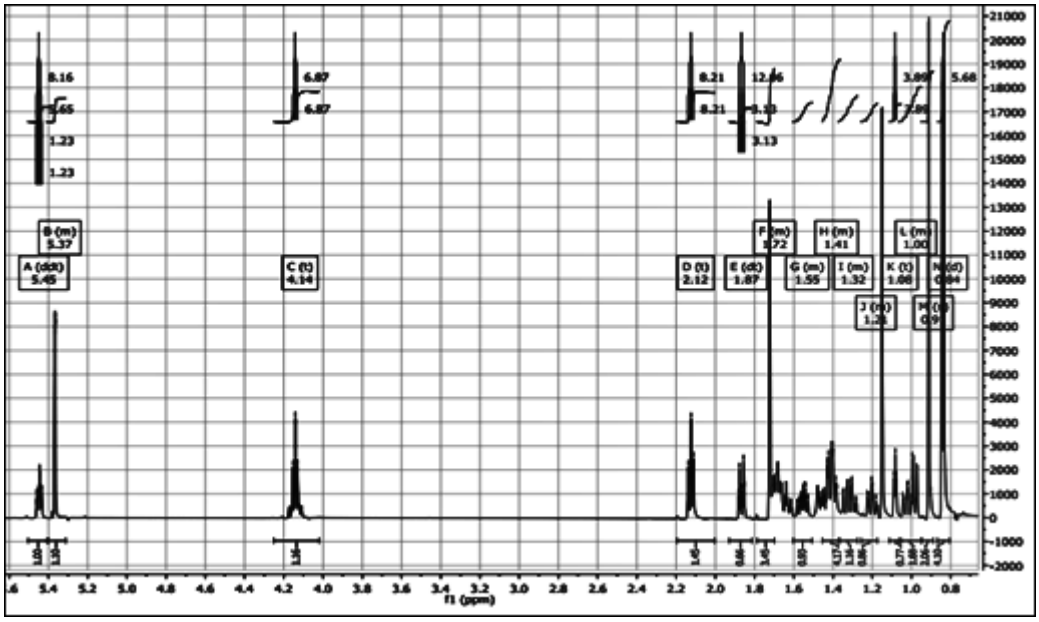
Appendix 2e: HMQC spectrum for labd-13(E)-en-18α,15-diol (**74**)



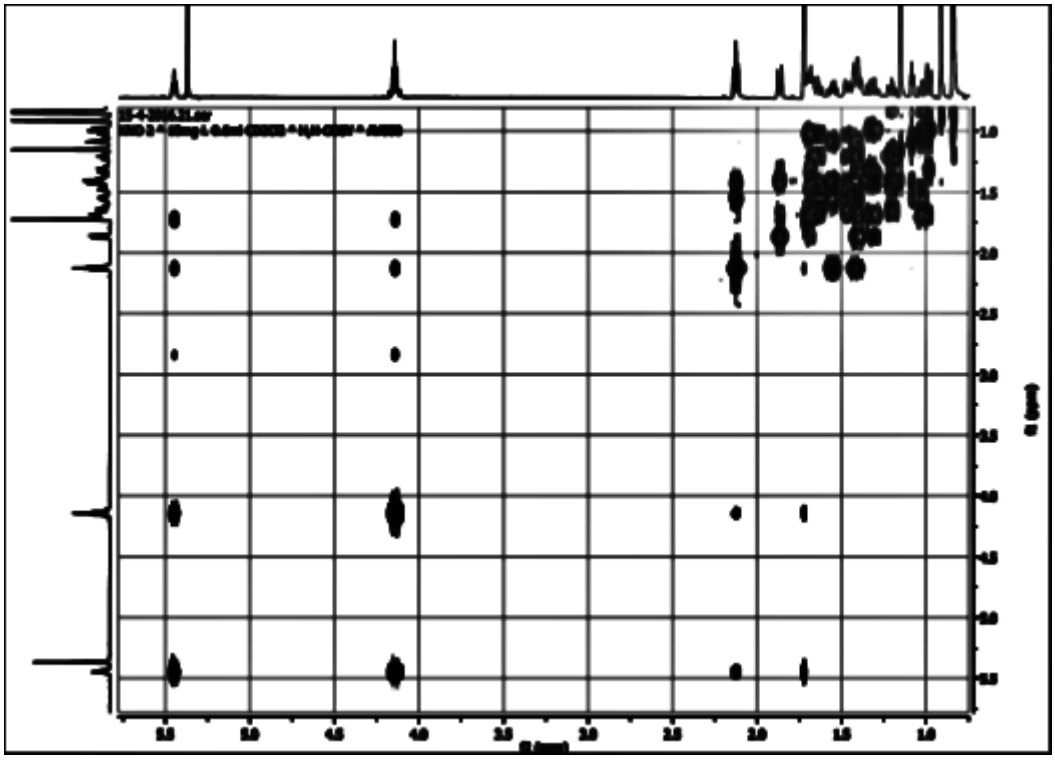
Appendix 2f: HMBC spectrum for labd-13(E)-en-18α,15-diol (**74**)



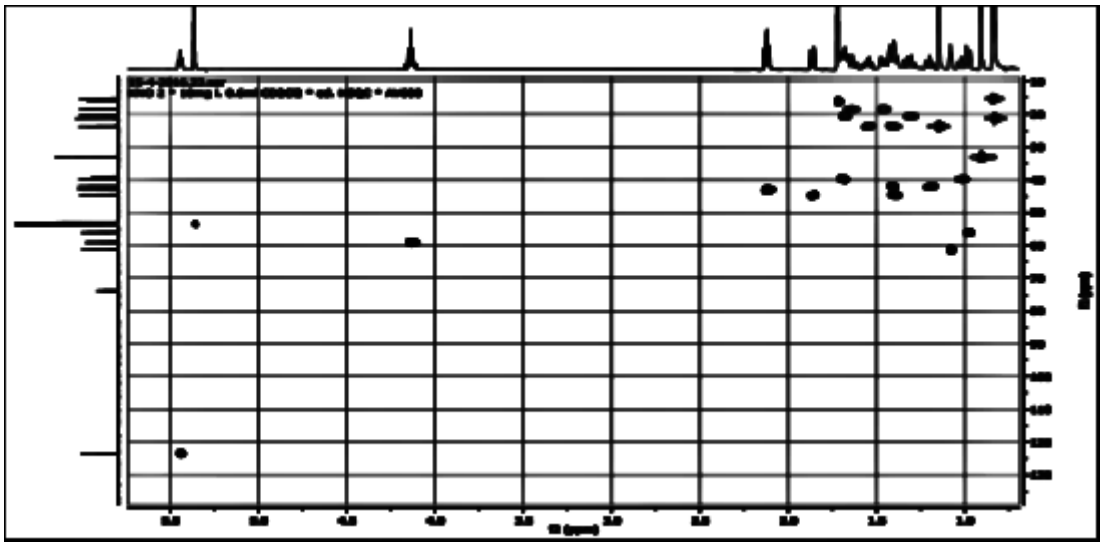
Appendix 3a: ^{1}H NMR spectrum for 18-*Nor*- Labd-13(E)-ene-8*α*, 15-diol (**109**)



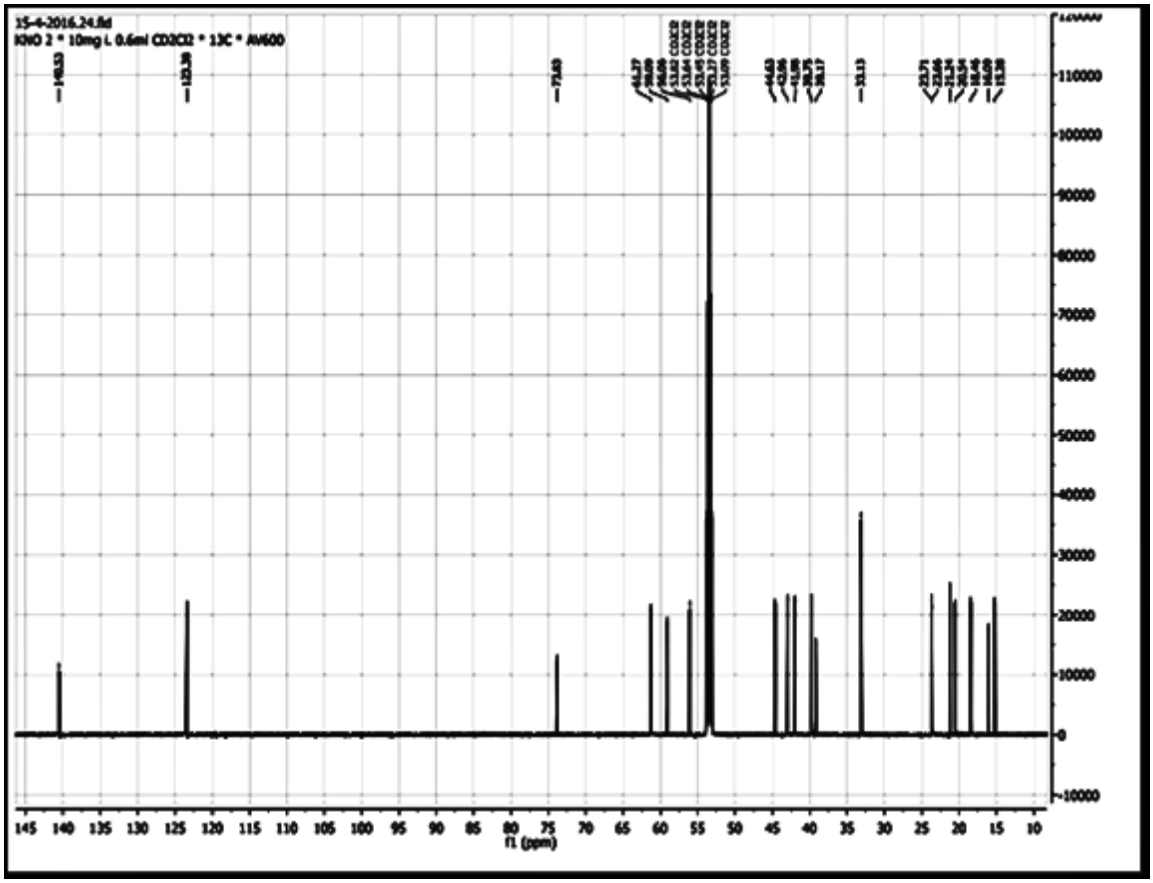
Appendix 3b: HH COSY spectrum for 18-*Nor*- Labd-13(E)-ene-8*α*, 15-diol (**109**)



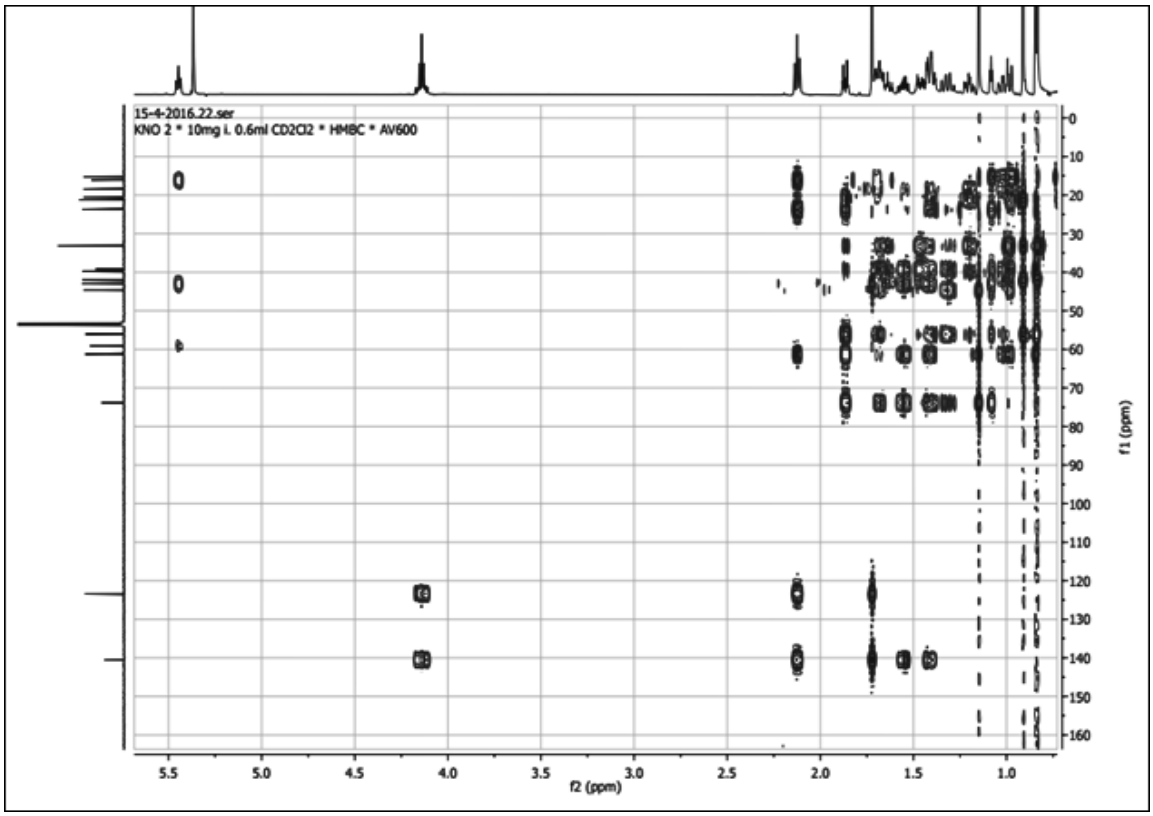
Appendix 3c: HSQC spectrum for 18-*Nor*- Labd-13(E)-ene-8*α*, 15-diol (**109**)



Appendix 3d: ^{13}C NMR spectrum for 18-*Nor*- Labd-13(E)-ene-8*α*, 15-diol (**109**)

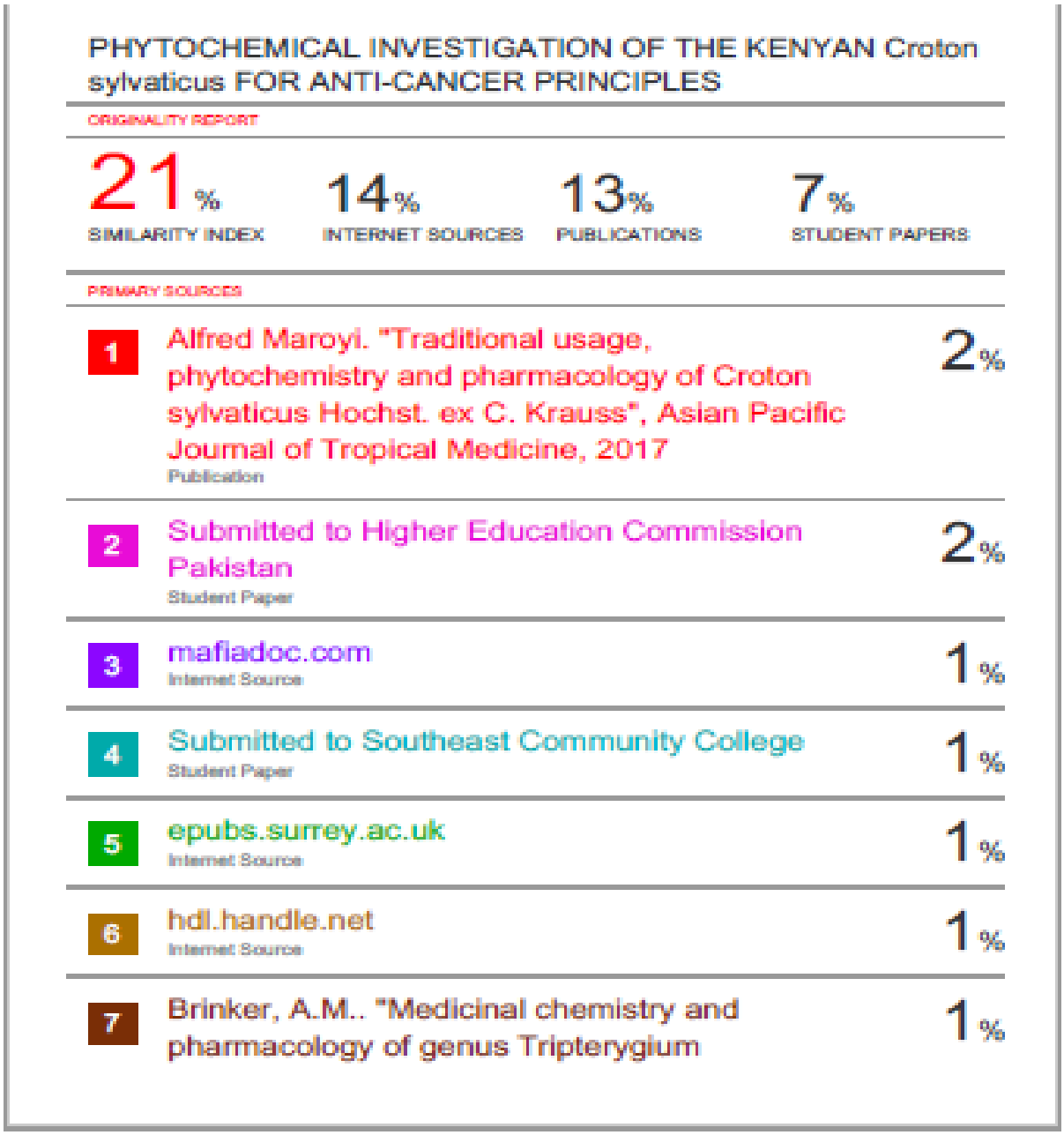


Appendix 3e: HMBC Spectrum for 18-*Nor*- Labd-13(E)-ene-8*α*, 15-diol (**109**)



Appendix 4: Map of Kakamega forest

Appendix 5: Report on plagiarism



PHYTOCHEMICAL INVESTIGATION OF THE KENYAN Croton sylvaticus FOR ANTI-CANCER PRINCIPLES

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