SCREENING OF SOME SELECTED ETHIOPIAN MEDICINAL PLANTS FOR CYTOTOXIC AND ANTIVIRAL ACTIVITIES

By: Akalu Terfa Sukesa

A Thesis Submitted to Department of Applied Chemistry

School of Applied Natural Sciences

In Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Medicinal Chemistry

Office of Graduate Studies

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APPROVAL SHEET

This is to certify that the thesis prepared by Akalu Terfa Sukesa, entitled: S**creening of Some Selected Ethiopian Medicinal Plants for Cytotoxic and Antiviral Activities**, submitted in partial fulfillment of the requirement for the degree of Doctor of Philosophy in **Medicinal Chemistry** complies with the regulation of the University and meets the accepted standards with respect to originality and quality.

Approved by the examining committee:

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_____ Post graduate Dean	_____	_____

DECLARATION

I declare that the work presented in this dissertation, entitled "S**creening of Some Selected Ethiopian Medicinal Plants for Cytotoxic and Antiviral Activities''** is my own original work under the supervision of Professor Y.S Jung and Dr. Hailamicahel Tesso. It is being submitted for the degree of Doctor of Philosophy in **Medicinal Chemistry** in the University of Adama Science and Tecnology University, Oromia, Ethiopia. It has not been submitted before for award of any degree or diploma or examination in any other University. All sources I have used have been acknowledged by means of completed references.

…… Date ----------

Akalu Terfa

Department of Chemistry

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DEDICATION

This thesis is dedicated to my beloved wife Wudinash Kelamu, our daugther Jitu Akalu and son Segni Akalu whose outstanding support, shielding and cushioning me from major obstacles and challenges, love and encouragement steers me through my darkest two-decade years. Thank you for your great love and for giving me love of life. My love for you is immeasurable.

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ABSTRACT

Cancer and viral diseases are an increasing worldwide health concern. There is an increasing evidence for the potential of natural products-derived compounds in cancer and viral diseases prevention and therapy. Approximately 60% of drugs currently used for cancer treatment have been isolated from natural products and the plant kingdom has been the most significant source. In this regard, plants extracts and activity-monitored isolation of compounds from three endemic medicinal plants of Ethiopia, *Millettia ferrguinea* ssp. *ferrguinea, Aloe pirottea* and *Aloe sinana,* were screened *in-vitro* for anticancer and antiviral activities.

Chromatographic (vacumm liquid chromatography on silica gel, TLC and UV light) separation of the extracts from the chloroform fraction of *Millettia ferrguinea* seed led to the identification of eight isoflavones compounds, of which, one is new **(10CH-05-II]**. The structural elucidation of the compounds was performed using spectroscopic: NMR and ESIMS.

The methanol extract, solvent fractions, isolated compounds and mixtures were evaluated *in vitro* for anticancer activities against human non-small cell lung cancer A549, ovarian cancer A2780, pancreatic cancer MIA-Paca-2 and stomach cancer SNU-638 cell lines using a sulforhodamine B (SRB) assay method to determine the percentage of growth inhibition and IC_{50} values. The extracts/solvent fractions of plants showed cytotoxic effect in the four cancer cell lines tested with different degrees of potency in a dose-dependent manner. The percentage growth inhibition was found to be increasing with increasing concentration of test compounds. The MeOH extract, n-Hxf and CHf of *M. ferrguinea* seed showed potent cytotoxic activity in the four cancer cell lines with IC50 values ranging from -0.07-13.66 µg/ml. All fractions of *A. sinana* root, all fractions except *n*-butanol fractions of *A. pirottea* root and CHF of *A. sinana* latex exhibited strong to moderate cytotoxic activity in A549, A2780 and SNU638 cell lines with IC50 values ranging from 6.32 - 29.87 µg/ml, and weak or no cytotoxic activity in MIA-PaCa-2 cell line. Extract/solvent fractions of *M. ferrguinea* seed were almost 1.5 to 8 times higher than that of reference standard anticancer drug etoposide in killing A549, A2780, MIA-PaCa-2 and SNU-638 cell lines. The cytotoxic activity profile of the plant extracts and etoposide decreased in the following order: *M. ferrguinea* seed > etoposide > *A. sinana* root > *A. pirottea* root > *A. sinana* leaf latex. The sensitivity of cancer cells to the extract/fractions decreases in the following order: SNU-638 > A2780 > A549 > MIP-PaCa-2.

The isolated compounds and mixtures, except 10CH-02, showed high cells growth inhibitory activity with IC_{50} value ranging from < 0.1 to 0.34 µg/ml (0.89 - < 0.12 µM) which are more potent than reference standard anticancer drug etoposide (0.58 µM for A2780 and 1.48 µM for PAN-2) against A2780 and PANC-2. Compound 10CH-03 exhibited strongest cell growth inhibitory activity (IC_{50} = 1.38 µM) against MIA-PaCa-2 which is more potent than etoposide (IC_{50} = 1.48 µM).

The methanol extract and solvent fractions were evaluated *in vitro* for anticancer activities against human influenza A and B viruses, picornaviruses and dengue virus using a viral cytopathic effect (CPE) inhibitory method for influenza A and B viruses and picornaviruses, and an immunofluorescence assay (IFA) for dengue virus. Among the 18 samples, 14 samples from three species showed strong antiviral activity with 50% - complete cell protection against influenza viruses induced CPE CHF of A. pirottea and A. sinana latex, and EAf of A. sinana root exhibited higher anti-influenza A and B activity than reference anti-influenza reagents RBV and AMT. In antipicorna virus assay, 4 samples from *M. ferrguinea* and root of *A. sinana* significantly inhibited EV-68 and EV-71(H) viral induced CPE with 65 % - 109% of cells did not show viral cytopathic effect. In anti-dengue virus assay, 13 from three species exhibited complete inhibition of DENV-2, while 11 exhibited potent inhibition effect based on CPE or cell death from DENV-2 with cell viability ranging from 50 % - 98.0 %. Among the purified compounds; 10CH-01 and 10CH-02, and mixtures; 10CHf, 10CH-06 and 10CH-7 exhibited potent antiviral activity in EV-68 with SI values ranging from 6.3 - > 111, compounds 10CH-03 and 10CH-05 in EV-71 (BrCr) with SI values of 5.3 and 3.6, respectively, and A10CH-02 in HRV-A21 and HRV-A71 with SI values of > 12.5.

In the US NCI plant screening program, *A. sinana* root and leave latex, *A. pirottea* root and *M. ferrgunia* seed extracts and isolates have potent cytotoxic activity in the four cancer cell lines and antiviral activity in influenza A and B viruses, picornaviruses and DENV-2, and our findings suggest that these extracts are promising for continued research and containing significant components that may be effectively utilized as broad spectrum anticancer and antiviral agents, and applied to development of a novel herbal medicine.

Keywords: Millettia *ferrguinea* seed, *Aloe pirottea* root, *Aloe sinana* root and leaf latex Anticancer activity, Antiviral activity, Growth inhibition, Viral inhibition, Cell protection, Cell viability, Virus-induced cytopathic effect, Etoposide, Rupintrivir.

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LIST OF ABBREVIATIONS, ACRONYMS AND SYMBOLS

LC-MS	Liquid Chromatography-MassSpectroscopy
VLC	Vacuum Liquid Chromatography
MHz	Mega Hertzs
ACC	Acetyl-CoA carboxylase
OSV-C	Oseltamivir Carboxylate
J	Coupling constant
t	Triplet
d	Doublelet
SI	Selectivity Index
DMSO	Dimethyl sulphoxide
s	Singlet
Dd	doublet of a doublet
ESIMS	Electron Spray Ionization Mass Spectroscopy
CHI	chalcone isomerase
NMR	Nuclear Magnatic Resonance
CC_{50}	50% Cytotoxic Concentration
1D NMR	One Dimensional Nuclear Magnetic Resonance
TLC	Thin Layer Chromatography
IC_{50}	50% Inhibitory Concentration
WHO	World Health Organization

CHAPTER ONE

INTRODUCTION

1.1 General Background

Medicinal plants have been the significant sources of traditional herbal medicine amongst rural dwellers worldwide for both human and animal since antiquity to date. Traditional medicine systems have formed the basis of plant-based drugs that have been used for centuries in many countries such as Egypt, China and India [1]. The earliest record on the use of plants was described on a clay tablet in cuneiform from Mesopotamia, 2600 B.C, which documented about 1,000 plant products including *Cupressus sempervirens* (Cypress), *Commiphora* species (myrrh), *Cedrus* species (cedar), *Glycyrrhiza glabra* (licorice), and *Papaver somniferum* (poppy juice) among others; these were used for the treatment of cough, cold, parasitic infections and inflammation and are still used nowadays [2]. The Ebers Papyrus (2900 B.C.), an Egyptian pharmaceutical record, documented over 700 plant-based drugs ranging from gargles, pills, infusions, to ointments. The Chinese Materia Medica (1100 B.C., Wu Shi Er Bing Fang) contains 52 prescriptions, Shennong Herbal (100 B.C. with 365 drugs list) and the Tang Herbal (659 A.D with 850 drugs list) are among the old records of the historical uses of plants [2, 3]. Although the use of bioactive natural products as herbal drug preparations dates back hundreds of years ago, they have assumed a very central stage in modern civilization as natural source of isolated and characterized compounds of chemotherapy. The search for alternative sources of drugs started in the 19th century, the dawn of chemotherapy era [4]. According to the World Health Organization (WHO), a medicinal plant is any plant which, in one or more of its organs, contains substances that can be used for therapeutic purposes or which are precursors for chemo-pharmaceutical semi-synthesis [5].

Traditional medicine has been brought into focus for meeting the goals of a wider coverage of primary healthcare delivery in all countries of the world. It is the first-choice healthcare treatment for at least 80% of Africans who suffer from high fever and other common ailments including cancer and viral infection [6]. Medicinal plants are increasingly gaining acceptance even among the literates in urban settlements, probably due to the increasing inefficacy of many modern drugs used for the control of many infections' diseases such as typhoid fever, gonorrhoea, and tuberculosis as well as increase in resistance by disease causing organisms to various antibiotics

and the increasing cost of prescription drugs, for the maintenance of personal health [7]. A study by Hamilton [8] indicated the dependence on medicinal plants to the low proportion of medical doctors to patients in Africa (Ethiopia 1:33,000; Kenya 1:7142; Tanzania 1: 33,000; Uganda 1: 25,000, Malawi 1: 50,000; Mozambique 1: 50,000; South Africa 1:1639; Swaziland 1: 10,000). The wide spread use of traditional medicine in Ethiopia and other Africa countries could be attributed to cultural acceptability, efficacy against certain type of diseases, physical accessibility and economic affordability as compared to modern medicine [9]. There is still a great potential for plants in the development of new drugs especially from African, because the continent has an immensely rich biodiversity and knowledge in using plants to treat various ailments [6].

Traditional medicine practices have played a key role in the development and advancement of modern studies by serving as a starting point for the development of novelties in drugs. Plants used in such practice have served as the basis from which most of the contemporary medicines are derived through ethnobotanical, clinical, pharmacological and chemical investigations [10]. The uses of 80% of 122 plant-derived drugs are related to their original ethno-pharmacological purposes [5]. The earliest historical examples (in 1803) of natural products used as medicine [11, 12] include morphine **(1)** and heroin **(2)**, the diacetyl derivative of morphine, was obtained (in the 1870s) from *Papaver somniferum* L (opium poppy). Similarly, methylation of the extract containing morphine resulted codeine **(3)**. *Digitalis purpurea* L (foxglove) is another old medicinal plant which was familiar in the 10th century, whilst its active cardiotonic glycoside constituent, digitoxin **(4)**, was not known until the 18lh century as an enhancer of cardiac conduction [12]. Digitoxin and its analogues have long been used in the management of congestive heart failure; however, they are being replaced due to possible long term detrimental effects [12]. The quinine **(5)**, isolated from *Cinchona* species (in 1820), is an old anti-malarial drug which is still used in modem world [13]. Pilocarpine **(6)**, an alkaloid from *Pilocarpus jaborandi* (Rutaceae), is an old medicine (for more than 100 years) used in the treatment of chronic open-angle glaucoma and acute angle-closure glaucoma [14].

Natural products have received increasing attention over the past 30 years for their potential as novel cancer preventive and therapeutic agents [15]. In parallel, there is increasing evidence for the potential of plant-derived compounds as inhibitors of various stages of tumorigenesis and associated inflammatory processes, underlining the importance of these products in cancer prevention and therapy. Pharmaceutical research in natural products represents a major strategy

for discovering and developing new drugs based on bioactivity-guided isolation methods, which, for example, have led to discoveries of the important anticancer agents, paclitaxel **(7)** from *Taxus brevifolia* and camptothecin **(8)** from *Camptotheca acuminata* [16]. Approximately 60% of drugs currently used for cancer treatment have been isolated from natural products and the plant kingdom has been the most significant source [17]. Anticancer agents from plants currently in clinical use can be categorized into vinca (or Catharanthus) alkaloids, epipodophyllotoxins, taxanes, and camptothecins. Vincristine **(9)** and vinblastine **(10)** were isolated from *Catharanthus roseus* (*L.*) *G. Don* (*Apocynaceae*) (formerly *Vinca rosea L.*) and have been used clinically for over 40 years [18]. Podophyllotoxin (**11**) was isolated from the resin of *Podophyllum peltatum L.* (Berberidaceae) but was found to be too toxic in mice and there by derivatives were made with the first clinically approved drug being etoposide (**12**) [19]. Paclitaxel **(7**) was clinically introduced to the U.S. market in the early 1990s [20]. Camptothecin (**8**) was isolated from *Camptotheca acuminata Decne.* (*Nyssaceae*) but originally showed unacceptable myelosuppression [21]. Resently, of 16 new plant-derived compounds being tested in clinical trials, 13 are in phase I or II and three are in phase III [22].

Medicinal plants have also played an important role in the treatment of virus and, indeed, over the last half century they have been applied towards combating virus, cancer, diabet and inflammation [23]. A wide variety of natural compounds isolated from medicinal plants (herbs) have been extensively studied in terms of their antiviral activity. Several hundred natural active compounds have been identified worldwide [26]. For instance, the use of natural products as anti-HIV agents [27], and several natural products, mostly of plant origin, has been shown to possess promising activities that could assist in the prevention and/or amelioration of the disease. Betulinic acid (a pentacyclic triterpene) isolated from the bark of the *White birch* tree, has been demonstrated to inhibit maturation of the HIV-1 [23]. Baicalin (a flavonoid) and calanolides (coumarins) [23] have been shown to inhibit infectivity and replication of HIV. Flavonoids inhibit HIV-1 activation via a novel mechanism, and these agents are potential candidates for therapeutic strategies aimed at maintaining a cellular state of HIV-1 latency. Acute HIV-1 infection has been shown to be suppressed by certain flavonoids in vitro, and evidence for inhibition of HIV-1 protease, integrase, and reverse transcriptase by flavonoids also exists [23]. To date, some of the anti-influenza agents that have been isolated from plants include a variety of polyphenols, flavonoids, and alkaloids [24]:

Polyphenol-rich extract from the medicinal plant *Geranium sanguineum L*. has been reported to show a strong anti-influenza virus activity,a biflavonoids, ginkgetin isolated from *Ginkgo biloba L*. and *Cephalotaxus harringtonia K*. Koch showed a potent inhibitory activity against influenza virus sialidase, and an indole alkaloid from *Uncaria rhynchophylla* and the pavine alkaloid (-)-thalimonie from *Thalictrum simplex* also exhibit potent inhibitory effects against influenza A virus [24].

In the crude extract form, medicinal plants posses many natural active compounds, which contain more characteristics of high chemical diversity and biochemical specificity than standard combinatorial chemistry, offer major opportunities for finding novel lead structures that are active against a wide range of assay targets [25]. In addition, biologically active natural products are generally small molecules with drug-like properties. They are capable of being absorbed and metabolized by the body. Hence, the development costs of producing orally active medicines are likely to be much lower than that of biotechnological products. Therefore, natural products, including traditional medicinal plants (herbs), offer great promise as potentially effective therapeutic agents [26].

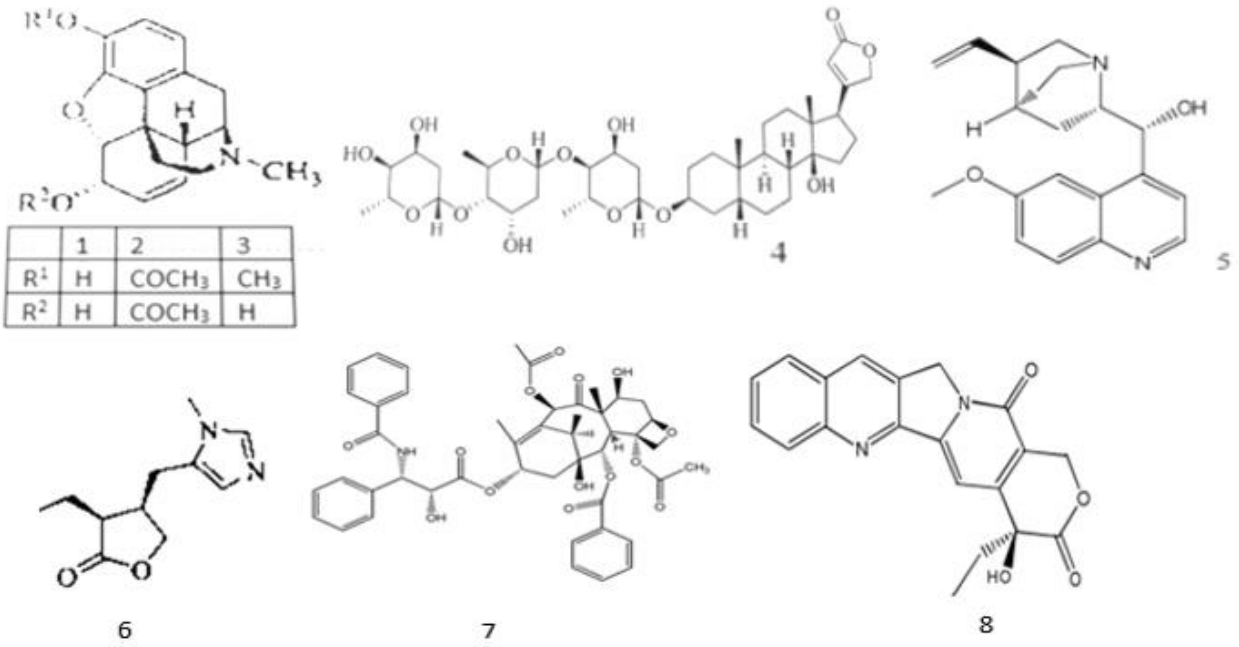


Figure 1.1: Plant-derived drugs

Ethiopia is considered to be the home of most diverse plant species in Africa that serve as sources of many traditionaly used medicinal plants. In Ethiopia, medicinal plants and marine herbs contribute, to about 80% of the traditional medicines used in the country [26]. Most of these plants are obtained from local sources in the wild by knowledgeable traditional practitioners. It has been claimed that approximately 800 species (about 20% to 25%) wild plants are endemic and the rest almost all indigenuous in Ethiopia [27]. These are used for treating about 300 medical conditions. The rich chemical diversity of secondary metabolites of these plants make them, as one of the most important sources of pharmacologically active principles used as drugs for various ailments, including anticancer and antiviral agents. Among the widely known medicinal plants, some are claimed to be prescribed by traditional healers for cancer and viral treatment [26]. Forexample, the genus *Millettia* (Leguminoseae) [26,28] with the major constituents; flavonoids and isoflavonoids (including rotenoids) has an important place in the pharmacopoeias with potential therapeutic uses; it displayed wide variety of activities; such as antitumoral anti-inflammatory**,** cytotoxicity against different cancer cell-lines, antiviral and antiplasmodial activities. *M. ferrguinia* is traditionally used as herbal fish poison, to treat skin infection and for dressing 'mujele' fleas, skin cancer [29], to control leaf-eating caterpillars, used for lice and tick control on pets and as insecticides [30]. It has also been used for treatment of pain, inflammation, ear ache, bacterial infection of nails and viral infection [31]. Most of endemic *Aloe* species [32], with three distinct preparations including *Alo*e latex (Aloe), *Aloe* gel and *Aloe* whole leaf (*Aloe* extract) are mostly used for vareity of treatement in Ethiopian traditional medicine[32]. *Aloe* latex is used for its laxative effect; *Aloe* gel is used topically for skin ailments, such as wound healing, psoriasis, genital herpes and internally by oral administration in diabetic and hyperlipidaemic patients and to heal gastric ulcers. *Aloe* extract is potentially useful for cancer and AIDS ,and *Aloe* gel showed strong antiinflammatory, antifungal, hypoglycemic and gastroprotective properties [32]. *Aloe pirottae* is one of these endemic *Aloe* species and traditionally used as a folk medicine for the treatment of inflammation, viral, bacterial and fungal infection, malaria diseases, tropical ulcer, gastro-intestinal parasites, gallstone, eye diseases, constipation, burns and dermatitis and snake bite. The Gel extract are used for cleaning human colon and leaf latex for insect repellent [33]. *Aloe sinana* is endemic *Aloe* species of Ethiopia and traditionally used for the treatment of viral bacterial and fungal infection, parasitic diseases including malaria, worm infection, amoebiasis, schistomiasis, constipation, burns, dermatitis, snake bite, wound and malaria [34].

1.2 Statement of the Problem

Cancer and viral diseases continue to be a great threat to human life. Despite recent improvements in survival rates due to advances in early detection and the quality of treatment available, cancer remains a major public health hazard in countries all over the world. For example, world wide, about 12.7 million cancers were diagnosed and 7.6 million deaths were reported in 2008 [35]. In 2012, there were an estimated 13.8 million cases around the world, of these 7.4 million cases were in men and 6.4 million women. This number is expected to increase to 24 million by 2035 [36]. Viral diseases are also responsible for considerable morbidity and mortality worldwide. Infectious viral diseases are still major threat to public health and remain a major problem all over the world and the control of viral diseases is the subject of constant scientific endeavor [37]. For instance, enterovirus 71 (EV71) and coxsackievirus 16 (CA16) are the causative agents of hand, foot and mouth disease (HFMD), a common infectious illness in infants and children [38]. EV71 infection can cause severe complications and mortality. Data reported by WHO [39] showed that over two million cases were diagnosed in the year 2013, including China (1,855,457 cases), Japan (300,314 cases), Vietnam (71,627 cases), and Singapore (31,780 cases). EV-A71 outbreaks occur worldwide, especially in the Asia-Pacific region. In 1997 epidemic in Malaysia caused 31 fatalities. In Taiwan, it caused 788 deaths in 1998 and in the 2010 outbreak in China saw over 1 million cases: 15,000 severe, with over 600 fatalities [39].

This projection of growth of cancer and viral cases appears to be due to aging of the global population and also due to rapid development of resistance to chemotherapeutic drugs by cancer cells and viruses. Present day medications show various adverse side effects and associated with high toxicity, which again amplifies the demand for alternative drugs with fewer side effects and greater therapeutic efficacies [36, 37]. Furthermore, the cost of drugs is a sizeable proportion of the total health expenditure in most developing countries. Drug related expenses in these countries account for between 30-50% of the total cost of healthcare needs [40]. Due to the increasing prominence of herbal remedies, additional contributions describing scientific investigations of a rigorous nature are most welcomed. In addition, the medicinal plants contain chemical constituents of therapeutic value that can provide natural products that are effective, chemically balanced and have fewer side effects as compared to synthetic chemicals [41]. Thus, continuous research is desperately needed in order to come up with compounds which can be developed into new, cheap, less toxic and more efficacious anticancer and antiviral drugs for combating these diseases.

1.3. Study Objectives

1.3.1 General Objective

To test anticancer and antiviral activities of extracts and identify active compound from *Millettia ferruginea* seed, *Aloe sinana* root, *Aloe sinana* leaf latex and *Aloe pirottae* root.

1.3.2 Specific Objectives

1. To test *in-vitro* anticancer activities of the methanol extracts and organic solvent fractions of *M. ferruginea* seed, *A. sinana* root and leave latex, and *A. pirottae* root. against four human cancer cell lines.

2. To test *in-vitro* antiviral activities of the methanol extracts and organic solvent fractions of *M. ferruginea* seed, *A. sinana* root and leave latex, and *A. pirottae* root against influenza A and B viruses, picornaviruses (HRV-B14, HRV-A21, and HRV-A71 strains) and entero Viruses (EV-68, EV-71(H), EV-71 Cox-B1, Cox-B3, and PV3 strains), and DENV-2.

3. To isolate and identify bioactive compounds from most active chloroform fraction of *M. ferruginea* seed.

4. To establish the anticancer and antiviral activities of the methanol extracts and organic solvent fractions.

5. To establish the anticancer and antiviral activities of the compounds from chloroform fraction of *M. ferruginea* seed.

1.4 Justification of the Study

Herbs are known to possess anti-viral and anticancer properties as proved by discovery of vinca alkaloids; vincristine **(9)** and vinblastine **(10)** from the Madagascar periwinkle, *Catharanthus roseus* as anticancer drugs. They may help to over come the adverse effects of conventional treatment protocols. Herbal compounds may interact with pharmaceutical drugs when used in conjunction to improve the efficacy and lower the adverse effects of the drug. Several plant derived molecules [42] like curcumin, ginsenosides, piperine, catechins, silymarin, genistein, resveratrol, isothiocyanates etc are reported to increase the effectiveness of conventional therapeutic drugs. They also aid in reduction of drug resistance. P-glycoprotein (P-gp), a multi drug resistance protein is found to be inhibited by various phytochemicals [42]**.** Presently, various plants like *Millettia*

pinnata, *Millettia pachcarpa*, *Aloe vera*, and so on are being explored for their anti-cancer properties [43]. Flavonoids from the seeds of *Millettia pachcarpa* showed a strong cytotoxicity and apoptotic effect against different cell-lines such as HepG2, C26, LL2 and B16 [44]. Similarly, two prenylated isoflavanone; pervilleanone **(11)** and 3'-3'-O-9-dimethyl pervilleanone **(12)**, isolated from *Millettia pervilleana* showed mild growth inhibition on different human cancer cell-lines of lung, breast and central nervous system (CNS) [45]. Similarly, *Aloe* species [32] have potentially active components that have been identified include anthraquinone or anthrone derivative: daunomycin **(13)** and doxorubicin **(14)** and are known for the treatment different solid tumor types as well as acute leukemia, the polysaccharides glucomanna and acemannan bradykininase, lignins, monosaccharide, polysaccharides, saponins, and sterols. These chemical composition posses many therapeutic properties: for skin ailments, such as wound healing, psoriasis, genital herpes and internally by oral administration in diabetic and hyperlipidaemic patients and to heal gastric ulcers; and for cancer and AIDS. It has antiviral antiinflammatory, antifungal, hypoglycemic and gastroprotective properties [32]**.** These three wild endemic plant species to Ethiopia have a broad spectrum uses in the traditional medicine of Ethiopia such as anti-tumoral, anti-cancer, anti-viral, anti-inflammatory, batericidal, insecticidal and pest-destroying. Eventhough, the mutiplicity of these activities well known in traditional medicine of Ethiopia, the phytochemical and biological investigation of these and others endemic plant species of Ethiopia is only limited to small percentage which has led to inadequate information on the chemistry of these plants and those compounds responsible for certain biological activity. Thus from research point of view, there is no single report on investigation these plants extracts/fractions and isolated active compounds for anticancer and antiviral activities. These local ethnomedical preparations and prescriptions of plant sources should be scientifically evaluated and then disseminated properly. It is concluded that once the local ethnomedical preparations of traditional sources are scientifically evaluated before dispensing, they should replace existing drugs commonly used for the therapeutic treatment of cancer, viral infection and others. These can be extended for future investigation into the field of pharmacology, phytochemistry, ethnobotany and other biological actions for drug discovery. Differently, this study targets to use exhaustive extraction technique and well-designed bioassay-guided isolation, and to characterize the different components of their active compounds and anticipating the future potential feedstock for the production of drugs and the uses of this plant in view of solving the medicinal problems.

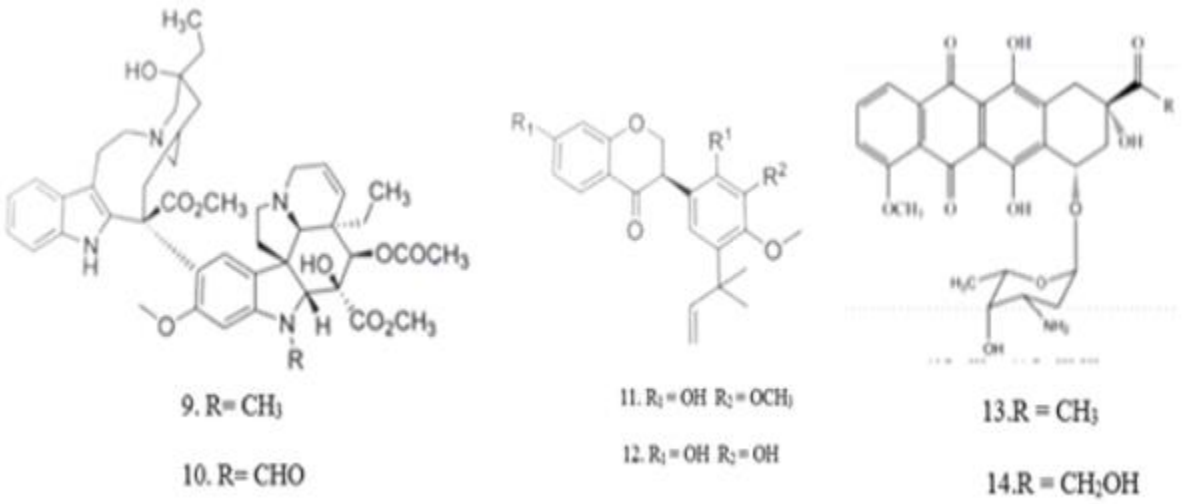


Figure 1.2: Plant drived anticancer drug

Thus, in this research, crude methanol extracts and its organic solvent fractions*: n*-hexane (HxF), chloroform (CHF), ethylacetate (EAF), and *n*-butanol (BuF) of three wild endemic medicinal plant species of Ethiopia namely; *Aloe sinana* root and leaf latex, *Millettia ferrgueana* seed and *Aloe pirrotea* root, were evaluated *in vitro* on four types of human cancer cell lines (human non-small cell lung cancer cell lines A549, ovarian cancer cell line A2780, pancreatic cancer cell line MIA-Paca-2 and stomach cancer cell line SNU-638), and three distinct genus viruses including influenza A and B viruses (PR8 , HK , and Lee strains), picornavirade family of human rhino viruses (HRV B14, HRV A21 ,and HRV A71 strains) and entero viruses (EV68 , EV71(H), EV71 CoxB1, CoxB3 , and PV3 strains), and Dengue virus (DENV-2 strain). The cytotoxicity of compounds from chloroform fraction of *Millettia ferrgueana* seed were evaluated *in vitro* on three types of human cancer cell lines including A2780, MIA-Paca-2 and PANC-1 and on picorna viruses (HRV-B14, HRV-A21, HRV-A71, Cox-B1, Cox-B3, PV3, EV-68, EV-71(H), and EV-71 strains).

CHAPTER TWO

2. LITERATURE REVIEW

2.1 Cancers

2.1.1 Definitions of cancer

The word cancer has originated from a Greek term, carcinos, for a crab to describe the physical similarity of malignant tumors that spread over the human body. Cancers are a large family of diseases that involve abnormal cell growth with the potential to invade or spread to other parts of the body [46]. They form a subset of neoplasms. A neoplasm or tumor is a group of cells that have undergone unregulated growth and will often form a mass or lump, but may be distributed diffusely [47].

All tumor cells show the six hallmarks of cancer [48]. These characteristics are required to produce a malignant tumor. They include: cell growth and division absent the proper signals, continuous growth and division even given contrary signals, avoidance of programmed cell death, limitless number of cell divisions, promoting blood vessel construction and tissue formation of metastases. The progression from normal cells to cells that can form a detectable mass to outright cancer involves multiple steps known as malignant progression [48].

2.1.2 The Cancer Problem

World wide greater than ten million cancer cases were occurred each year and more than half of the patients die from it. For instance, in 2008, approximately 12.7 million cancers were diagnosed (excluding non-melanoma skin cancers and other non-invasive cancers [49] and in 2010 nearly 7.98 million people died [50]. Cancers account for approximately 13% of deaths. The most common are lung cancer (1.4million deaths), stomach cancer (740,000), liver cancer (700,000) and colorectal cancer (610,000) [51]. This makes invasive cancer the leading cause of death in the developed world and the second leading in the developing world [49]. Over half of cases occur in the developing world [49]. Deaths have been increasing primarily due to longer life spans and life style changes in the developing world [49]. The most significant risk factor for developing cancer is age [52]. Although it is possible for cancer to strike at any age, most patients with invasive cancer are over 65 [52]. According to cancer researcher Robert A. Weinberg [53], "If we lived long enough, sooner or later we all would get cancer. Some of the association between aging and cancer is attributed to immunosenescence [54] errors accumulated in DNA over a lifetime [55]

and age-related changes in the endocrine system [56]. Aging's effect on cancer is complicated by factors such as DNA damage and inflammation promoting it and factors such as vascular aging and endocrine changes inhibiting it [57].

Until recently, cancer was regarded as a disease of the Western and industrialized countries, however, more than half of the new cancer cases (56% of 12.7 million) and deaths (63% of 7.6 million) in the year 2008 were reported in developing countries [58]. This is partly due to limited or non-existent resources for prevention, diagnosis and treatment of cancer. In Africa, as in the rest of the world, the burden of cancer is increasing due to growing of the aging population, urbanization as well as increased prevalence of risk factors associated with economic transition. The overall population of Africa is projected to increase by about 50% between 2010 and 2030 [49], and approximately by 90% for those aged above 60 years, the age at which cancer most frequently arises. Due to limited resources coupled with other imperative public health problems, including HIV/AIDS, malaria and tuberculosis; cancer has been under-recognized in Africa and consequently received low health priority, despite its growing burden [60]**.**

2.1.3 Types of Cancer

There are many different kinds of cancers. Some of the most common are: breast cancer, brain cancer, leukemia (a blood cancer), testicular cancer, stomach cancer, lung cancer and pancreatic cancer. Here four types of cancer used in this research will be discussed.

2.1.3.1 Lung Cancer and its Classification

Lung cancer, also known as lung carcinoma, is a malignant lung tumor characterized by uncontrolled cell growth in tissues of the lung [61]. This growth can spread beyond the lung by the process of metastasis into nearby tissue or other parts of the body [62]. Two broad classes are distinguished: non-small-cell lung carcinoma and small-cell lung carcinoma [62].

Worldwide, lung cancer is the most common cancer among men in terms of both incidence and mortality, and among women has the third highest incidence, and is second after breast cancer in mortality. In 2012, there were 1.82 million new cases globally, and 1.56 million deaths due to lung cancer, representing 19.4% of all deaths from cancer [63]. The highest rates are in North America, Europe and East Asia, with over a third of new cases in 2012 in China. Rates in Africa and South Asia are much lower [63].

2.1.4.1.1 Non-small-cell Lung Carcinoma (NSCLC)

NSCLC is the most common type of lung cancer, accounting for 85-90% of all lung cancer cases, and is classified into three main subtypes based on histology or what it looks like under a microscope: adenocarcinoma, squamous cell carcinoma and large-cell carcinoma [65]. Adenocarcinoma represents the most common subtype of NSCLC, which usually originates in peripheral lung tissue and accounts for 40% of cases [63]. Although most cases of adenocarcinoma are associated with smoking, adenocarcinoma is also the most common form of lung cancer among people who have smoked fewer than 100 cigarettes in their lifetimes ("never-smokers") and ex-smokers with a modest smoking history [64]. A subtype of adenocarcinoma, the bronchioloalveolar carcinoma, is more common in female never-smokers, and may have a better long-term survival [64]. In addition, NSCLC can also be characterized based on different underlying genetic abnormalities [64]. Researchers have identified over 12 unique mutations and biomarkers that are responsible for tumor growth including anaplastic lymphoma kinase (ALK), epidermal growth factor receptor (EGFR), human epidermal growth factor (HER) family of receptors, MET and ROS-1 [66]. Squamous-cell carcinoma accounts for about 30% of lung cancers. They typically occur close to large airways. A hollow cavity and associated cell death are commonly found at the center of the tumor [62]. About 9% of lung cancers are large-cell carcinoma. These are so named because the cancer cells are large, with excess cytoplasm, large nuclei and conspicuous nucleoli [62].

2.1.3.1.2 Small-cell Lung Carcinoma (SCLC)

In small-cell lung carcinoma (SCLC), the cells contain dense neurosecretory granules (vesicles containing neuroendocrine hormones), which give this tumor an endocrine/paraneoplastic syndrome association [67]. Most cases arise in the larger airways (primary and secondary bronchi). Sixty to seventy percent have extensive disease (which cannot be targeted within a single radiation therapy field) at presentation [64].

2.1.3.1.3 Pathogenesis

Similar to many other cancers, lung cancer is initiated by activation of oncogenes or inactivation of tumor suppressor genes [68]. Carcinogens cause mutations in these genes which induce the development of cancer [69]. Mutationsin the K-ras proto-oncogene are responsible for 10–30% of lung adenocarcinomas [69]. About 4% of non-small-cell lung carcinomas involve an EML4-ALK tyrosine kinase fusion gene [69]. Epigenetic changes—such as alteration of DNA methylation,

histone tail modification, or microRNA regulation—may lead to inactivation of tumor suppressor genes [70].

The epidermal growth factor receptor (EGFR) regulates cell proliferation, apoptosis, angiogenesis, and tumor invasion [69]**.** Mutations and amplification of EGFR are common in non-small-cell lung carcinoma and provide the basis for treatment with EGFR-inhibitors. Her2/neu is affected less frequently [69]. Other genes that are often mutated or amplified are c-MET, NKX2-1, LKB1, PIK3CA, and BRAF [69]. The cell lines of origin are not fully understood [64]. The mechanism may involve abnormal activation of stem cells. In the proximal airways, stem cells that express keratin 5 are more likely to be affected, typically leading to squamous-cell lung carcinoma. In the middle airways, implicated stem cells include club cells and neuroepithelial cells that express club cell secretory protein. Small-cell lung carcinoma may be derived from these cell lines [71] or neuroendocrine cells [64]**,** and may express CD44 [71].

2.1.3.2 Stomach Cancer

2.1.3.2.1 Classification and Epidemiology

Stomach cancer, also known as gastric cancer, is cancer developing from the lining of the stomach [72]. The cancer may spread from the stomach to other parts of the body, particularly the liver, lungs, bones, lining of the abdomen and lymph nodes. Gastric adenocarcinoma is a malignant epithelial tumour, originating from glandular epithelium of the gastric mucosa. Stomach cancers are overwhelmingly adenocarcinomas (90%) [72]. Histologically [72], there are two major types of gastric adenocarcinoma (Lauren classification): intestinal type or diffuse type. Adenocarcinomas tend to aggressively invade the gastric wall, infiltrating the muscularis mucosae, the submucosa and then the muscularis propria. Intestinal type adenocarcinoma tumour cells describe irregular tubular structures, harbouring pluristratification, multiple lumens, reduced stroma ("back to back" aspect). Often, it associates intestinal metaplasia in neighbouring mucosa. Depending on glandular architecture, cellular pleomorphism and mucosecretion, adenocarcinoma may present 3 degrees of differentiation: well, moderate and poorly differentiated. Diffuse type adenocarcinoma (mucinous, colloid, linitis plastica or leather-bottle stomach) tumour cells are discohesive and secrete mucus, which is delivered in the interstitium, producing large pools of mucus/colloid (optically "empty" spaces). It is poorly differentiated. If the mucus remains inside the tumour cell, it pushes the nucleus to the periphery: "signet-ring cell" [72].

Worldwide, stomach cancer is the fifth most common cancer with 952,000 cases diagnosed in 2012 [63]. It is more common in men and in developing countries. In 2012, it represented 8.5% of cancer cases in men, making it the fourth most common cancer in men [73]. In 2012 number of deaths were 700,000 having decreased slightly from 774,000 in 1990 making it the third leading cause of cancer death after lung cancer and liver cancer [74].Less than 5% of stomach cancers occur in people under 40 years of age with 81.1% of that 5% in the age-group of 30 to 39 and 18.9% in the age-group of 20 to 29 [74].

2.1.3.2.2 Treatment

The use of chemotherapy to treat stomach cancer has no firmly established standard of care. Unfortunately, stomach cancer has not been particularly sensitive to these drugs, and chemotherapy, if used, has usually served to palliatively reduce the size of the tumor, relieve symptoms of the disease and increase survival time. Some drugs [75] used in stomach cancer treatment have included: 5-FU (fluorouracil) or its analog capecitabine, BCNU (carmustine), methyl-CCNU (semustine) and doxorubicin (Adriamycin), as well as mitomycin C, and more recently cisplatin and taxotere, often using drugs in various combinations. The relative benefits of these different drugs, alone and in combination, are unclear [75]. Recently, treatment with human epidermal growth factor receptor 2 (HER2) inhibitor, trastuzumab, has been demonstrated to increase overall survival in inoperable locally advanced or metastatic gastric carcinoma over-expressing the HER2/neu gen [76]. In particular, HER2 is overexpressed in 13-22% of patients with gastric cancer [77]. HER2 overexpression in gastric neoplasia is heterogeneous and comprises a minority of tumor cells (less than 10% of gastric cancers overexpress HER2 in more than 5% of tumor cells). Hence, this heterogeneous expression should be taken into account for HER2 testing, particularly in small samples such as biopsies, requiring the evaluation of more than one bioptic sample [77].

2.1.3.3 Pancreatic Cancer

2.1.3.3.1 Clsssification and Epidemiology

Pancreatic cancer [78] arises when cells in the pancreas, a glandular organ behind the stomach, begin to multiply out of control and form a mass. These cancerous cells have the ability to invade other parts of the body.

The many types of pancreatic cancer can be divided into two general groups [79]. The vast majority

of cases (about 99%) occur in the part of the pancreas which produces digestive enzymes, known as the exocrine component. There are several sub-types of exocrine pancreatic cancers, but their diagnosis and treatment have much in common. The small minority of cancers that arise in the hormone-producing (endocrine) tissue of the pancreas have different clinical characteristics. Both groups occur mainly (but not exclusively) in people over 40, and are slightly more common in men, but some rare sub-types mainly occur in women or children [79].

2.1.3.3.1.1 Exocrine Cancers

The exocrine group is dominated by pancreatic adenocarcinoma (variations of this name may add "invasive" and "ductal"), which is by far the most common type, representing about 85% of all pancreatic cancers [80]. Nearly all these start in the ducts of the pancreas, and pancreatic ductal adenocarcinoma (PDAC) [81]. This is despite the fact that the tissue from which it arises – the pancreatic ductal epithelium- represents less than 10% of the pancreas by cell volume, because it constitutes only the ducts (an extensive but capillary-like duct-system fanning out) within the pancreas. This cancer originates in the ducts that carry secretions (such as enzymes and bicarbonate) away from the pancreas. About 60–70% of adenocarcinomas occur in the 'head' of the pancreas [80]. The next most common type, acinar cell carcinoma of the pancreas, arises in the clusters of cells that produce these enzymes, and represents 5% of exocrine pancreas cancers [82].

2.1.3.3.1.2 Neuroendocrine

The small minority of tumors that arise elsewhere in the pancreas are mainly pancreatic neuroendocrine tumors (PanNETs) [83]. Neuroendocrine tumors (NETs) are a diverse group of benign or malignant tumors that arise from the body's neuroendocrine cells, which are responsible for integrating the nervous and endocrine systems. NETs can start in most organs of the body, including the pancreas, where the various malignant types are all considered to be rare. PanNETs are grouped into 'functioning' and 'non-functioning' types, depending on the degree to which they produce hormones. The functioning types secrete hormones such as insulin, gastrin, and glucagon into the bloodstream, often in large quantities, giving rise to serious symptoms such as low blood sugar, but also favoring relatively early detection. The most common functioning PanNETs are insulinomas and gastrinomas, named after the hormones they secrete. The non-functioning types do not secrete hormones in a sufficient quantity to give rise to overt clinical symptoms. For this reason, non-functioning PanNETs are often diagnosed only after the cancer has spread to other parts of the body [83].

As of 2012, pancreatic cancer resulted in 330,000 deaths globally [6] up from 310,000 in 2010 and 200,000 in 1990 [83]. In 2014, an estimated 46,000 people in the US are expected to be diagnosed with pancreatic cancer and 40,000 to die of it [83]. Although it accounts for only 2.5% of new cases, pancreatic cancer is responsible for 6% of cancer deaths each year [83]. It is the seventh highest cause of death from cancer worldwide [62]. Globally pancreatic cancer is the 11th most common cancer in women and the 12th most common in men [62]. The majority of recorded cases occur in developed countries [62]. People from the United States [84] have an average lifetime risk of about 1 in 67 (or 1.5%) of developing the diseaseslightly higher than the figure for the UK. The disease is more common in men than women [62,80] though the difference in rates has narrowed over recent decades, probably reflecting earlier increases in female smoking. In the United States the risk for African Americans is over 50% greater than for whites, but the rates in Africa and East Asia are much lower than those in North America or Europe. The United States, Central and Eastern Europe, and Argentina and Uruguay all have high rates [62].

2.1.4.3.2 Treatment

Chemotherapy or chemoradiotherapy may be used in cases that are considered to be "borderline resectable" in order to reduce the cancer to a level where surgery could be beneficial.

Gemcitabine [85] was approved by the United States Food and Drug Administration (FDA) in 1997, after a clinical trial reported improvements in quality of life and a 5-week improvement in median survival duration in people with advanced pancreatic cancer. This was the first chemotherapy drug approved by the FDA primarily for a nonsurvival clinical trial endpoint. Chemotherapy using gemcitabine alone was the standard for about a decade, as a number of trials testing it in combination with other drugs failed to demonstrate significantly better outcomes. However, the combination of gemcitabine with erlotinib was found to increase survival modestly, and erlotinib was licensed by the FDA for use in pancreatic cancer in 2005 [86]. The FOLFIRINOX chemotherapy regimen using four drugs [87] was found more effective than gemcitabine, but with substantial side effects, and is thus only suitable for people with good performance status. This is also true of protein-bound paclitaxel (nab-paclitaxel), which was licensed by the FDA in 2013 for use with gemcitabine in pancreas cancer. By the end of 2013, both FOLFIRINOX and nab-paclitaxel with gemcitabine were regarded as good choices for those able to tolerate the side-effects, and gemcitabine remained an effective option for those who were

not. A head-to-head trial between the two new options is awaited, and trials investigating other variations continue. However, the changes of the last few years have only increased survival times by a few months [85].

Efforts are underway to develop new drugs. Some of these involve targeted therapies against the cancer cells' molecular mechanisms [89]. Others aim to target the highly resistant cancer stem cells [90]. Still others aim to affect the non-neoplastic stroma and microenvironment of the tumor, which is known to influence cell proliferation and metastasis [89,90]. A further approach involves the use of immunotherapy, such as oncolytic viruses [91].

2.1.3.4 Ovarian Cancer

Ovarian cancer [93] is a cancer that forms in or on an ovary. It results in abnormal cells that have the ability to invade or spreadto other parts of the body. Common areas to which the cancer may spread include the lining of the abdomen, lymph nodes, lungs, and liver. Ovarian tumours are a group of neoplasms affecting the ovary and have a diverse spectrum of features according to the particular tumour entity. They include benign, low-malignant potential/borderline and malignant subtypes [93].

2.1.3.4.1 Classification

Ovarian tumoursare subdivided into five main categories according to the World Health Organization's classification system [92]:

- Epithelial tumours, which account for about 75% of all ovarian tumours, and 90-95% of ovarian malignancies.
- Sex cord-stromal tumours, which account for about 5-10 % of all ovarian neoplasms.
- Germ cell tumours, which account for about 15-20 % of all ovarian neoplasms.
- Metastatic tumours, accounting for about 5% of ovarian malignancies, and usually arise from breast, colon, endometrium, gastric cancer and cervical cancers.
- Other, a small number of other types of neoplasms which develop from ovarian soft tissue or non-neoplastic processes.

2.1.4.4.2 Pathophysiology

Ovarian cancer forms when errors in normal ovarian cellgrowth occur. Usually, when cells grow old or get damaged, they die, and new cells take their place. Cancer starts when new cells form unneeded, and old or damaged cells do not die as they should. When an ovary releases an egg, the

egg follicle bursts open and becomes the corpus luteum. This structure needs to be repaired by dividing cells in the ovary [93]. Continuous ovulation for a long time means more repair of the ovary by dividing cells, which can acquire mutations in each division [94].

The most common gene mutations in ovarian cancer occur in NF1, BRCA1, BRCA2, and CDK12 [96]. Type I ovarian cancers, which tend to be less aggressive, tend to have microsatellite instability in several genes, including both oncogenes (most notably BRAF and KRAS) and tumor suppressors (most notably PTEN. The most common mutations in Type I cancers are KRAS, BRAF, ERBB2, PTEN, PIK3CA, and ARID1A [97]. Type II cancers, the more aggressive type [95], have different genes mutated, including p53, BRA1, and BRCA2.Low-grade cancers tend to have mutations in KRAS, whereas cancers of any grade that develop from low malignant potential tumors tend to have mutations [94]. Type I cancers tend to develop from precursor lesions, whereas Type II cancers can develop from a serous tubal intraepithelial carcinoma [96]. Serous cancers that have BRCA mutations also inevitably have p53 mutations, indicating that the removal of both functional genes is important for cancer to develop [94].

2.1.4.4.3 Treatment

Chemotherapy [95] has been a general standard of care for ovarian cancer for decades, although with variable protocols. Chemotherapy is used after surgery to treat any residual disease, if appropriate. In some cases, there may be reason to perform chemotherapy first, followed by surgery. This is called "neoadjuvant chemotherapy", and is common when a tumor cannot be completely removed or optimally debulked via surgery. Chemotherapy in ovarian cancer typically consists of platins, a group of platinum-based drugs, combined with non-platins. Common therapies can include paclitaxel, cisplatin, topotecan, doxorubicin, epirubicin, and gemcitabine. Carboplatin is typically given in combination with either paclitaxel or docetaxel; the typical combination is carboplatin with paclitaxel [95, 96]. Carboplatin is superior to cisplatin in that it is less toxic and has fewer side effects, generally allowing for an improved quality of life in comparison, though both are similarly effective [96]. Three-drug regimens have not been found to be more effective [95] and platins alone or nonplatins alone are less effective than platins and nonplatins in combination [96].

While an active area of research, antigen-specific immunotherapy has been shown to be effective as of 2013 [97]. However, trials of the antibody and VEGF inhibitor bevacizumab, which can slow

the growth of new blood vessels in the cancer, have shown promising results, especially in combination with pazopanib, which also slows the process of blood vessel growth. Bevacizumab has been particularly effective in preliminary studies on stage-III and -IV cancer [95] [and has been cited as having at least a 15% response rate. It is being investigated particularly in mucinous ovarian cancers [96]. mTOR inhibitors were a highly investigated potential treatment in the 2000s and 2010s, but the side effects of these drugs (particularly hyperglycemia and hyperlipidemia) were not well tolerated and the survival benefit not confirmed. PI3 kinase inhibitors have been of interest, but they tend to be highly toxic and cause diarrhea. Another investigated drug is selumetinib, a MAPK inhibitor [95]. It improved survival, but did not correlate with any mutations found in tumors. Trastuzumabo is potential immunotherapy, which is active against tumors positive for Her2/neu mutations [98]. Other angiogenesis inhibitors are also being investigated as potential ovarian cancer treatments. An alternative to BEP chemotherapy, a regimen of 3 cycles of carboplatin and etoposide, is a current topic of research for germ cell malignancies [99]. Intraperitoneal chemotherapy has also been under investigation during the 2000s and 2010s for its potential to deliver higher doses of cytotoxic agent to tumors. Tyrosine kinase inhibitors are another investigational drug class that may have applications in ovarian cancer. Angiogenesis inhibitors in the receptor tyrosine kinase inhibitor group, including pazopanib, cediranib, and nintedanib, have also been shown to increase progression free survival (PFS), but their benefit for overall survival has not been investigated as of 2015 [95].

2.1.3.4.4 Epidemiology

In 2014, the number of new cases that occurred in developed countries was about 9.4 per 100,000, compared to 5.0 per 100,000 in developing countries [95]. Globally, about 160,000 people died from ovarian cancer in 2010. This was an increase from 113,000 in 1990 [100]. The number of new cases per year in Europe is approximately 5–15 per 100,000 women [97]. In Europe, Lithuania, Latvia, Ireland, Slovakia, and the Czech Republic have the highest incidences of ovarian cancer, whereas Portugal and Cyprus have the lowest incidences [96]. The overall lifetime risk in the US is around 1.6% [79, 81]. In the US, ovarian cancer affects 1.3–1.4% and is the cause of death of about 1% of women [79]. In the United States, it is also the fifth-most common cancer in women but the fourth-most common cause of cancer death [79]. This decrease made it the ninth-most common cancer in women. It is the 5th most common cancer in UK women [76, 79]. In the UK, the incidence rate over the whole population is 21.6 per 100,000.

2.1.4 Causes

The majority of cancers, some 90–95% of cases, are due to environmental factors. The remaining 5–10% are due to inherited genetics [101]. Environmental, as used by cancer researchers, is that is not inherited genetically, such as lifestyle, economic and behavioral factors and not merely pollution [60]. Common environmental factors that contribute to cancer death include tobacco (25–30%), diet and obesity (30–35%), infections (15–20%), radiation (both ionizing and non-ionizing, up to 10%), stress, lack of physical activity and pollution [60].

2.1.4.1 Smoking

It is not generally possible to prove what caused a particular cancer because the various causes do not have specific finger prints. For example, if a person who uses tobacco heavily develops lung cancer, then it was probably caused by the tobacco use, but since everyone has a small chance of developing lung cancer as a result of air pollution or radiation, the cancer may have developed for one of those reasons. Tobacco smoke, for example, causes 90% of lung cancer [101]. It also causes cancer in the larynx, head, neck, stomach, bladder, kidney, esophagus and pancreas [102]. Tobacco smoke contains over fifty known carcinogens, including nitrosamines and polycyclic aromatic hydrocarbons [103]. Exposure to particular substances have been linked to specific types of cancer. These substances are called carcinogens.

Tobacco is responsible for about one in five cancer deaths worldwideand about one in three in the developed world [103]. Lung cancer death rates in the United States have mirrored smoking patterns, with increases in smoking followed by dramatic increases in lung cancer death rates and, more recently, decreases in smoking rates since the 1950s followed by decreases in lung cancer death rates in men since 1990 [104].

2.1.4.2 Alcohol

The International Agency for Research on Cancer (IARC) has established the carcinogenicity of ethanol in animals, and classified alcohol consumption as carcinogenic to humans [105]. it is associated to the risks of oral cavity, pharynx, larynx, oesophagus, colorectum, liver, pancreas and breast cancers. Heavy alcohol consumption accounts for 3.5% of all cancer deaths [106].

2.1.4.3 Diet and Exercise

Diet, physical inactivity and obesity are related to up to 30–35% of cancer deaths [107]. In the United States excess body weight is associated with the development of many types of cancer and is a factor in 14–20% of cancer deaths [107]. A UK study including data on over 5 million people

showed higher body mass index to be related to at least 10 types of cancer and responsible for around 12,000 cases each year in that country [108]. Physical inactivity is believed to contribute to cancer risk, not only through its effect on body weight but also through negative effects on the immune system and endocrine system [107]. More than half of the effect from diet is due to overnutrition (eating too much), rather than from eating too few vegetables or other healthful foods.

Some specific foods are linked to specific cancers. A high-salt diet is linked to gastric cancer [109]. Aflatoxin B1, a frequent food contaminant, causes liver cancer [109]. Betel nut chewing can cause oral cancer [109]. National differences in dietary practices may partly explain differences in cancer incidence. For example, gastric cancer is more common in Japan due to its high-salt diet [110]. While colon cancer is more common in the United States.

2.1.4.4 Infection

Worldwide approximately 18% of cancer deaths are related to infectious diseases [46]. This proportion ranges from a high of 25% in Africa to less than 10% in the developed world [46]. Viruses are the usual infectious agents that cause cancer but cancer bacteria and parasites may also play a role. Oncoviruses (viruses that can cause cancer) include human papillomavirus (cervical cancer), Epstein–Barr virus (B-cell lymphoproliferative disease and nasopharyngeal carcinoma), Kaposi's sarcoma herpesvirus (Kaposi's sarcoma and primary effusion lymphomas), hepatitis B and hepatitis C viruses (hepatocellular carcinoma) and human T-cell leukemia virus-1 (T-cell leukemias). Bacterial infection may also increase the risk of cancer, as seen in Helicobacter pylori-induced gastric carcinoma [111]. Parasitic infections associated with cancer include Schistosoma haematobium (squamous cell carcinoma of the bladder) and the liver flukes, Opisthorchis viverrini and Clonorchis sinensis (cholangiocarcinoma) [112].

2.1.4.5 Radiation

Up to 10% of invasive cancers are related to radiation exposure, including both ionizing radiation and non-ionizing ultraviolet radiation. Additionally, the majority of non-invasive cancers are non-melanoma skin cancers caused by non-ionizing ultraviolet radiation, mostly from sunlight. Sources of ionizing radiation include medical imaging and radon gas.

Ionizing radiation is not a particularly strong mutagen [113]. Residential exposure to radon gas, for example, has similar cancer risks as passive smoking [113]. Radiation is a more potent source of cancer when combined with other cancer-causing agents, such as radon plus tobacco smoke

[113]. Radiation can cause cancer in most parts of the body, in all animals and at any age. Children and adolescents are twice as likely to develop radiation-induced leukemia as adults; radiation exposure before birth has ten times the effect [113]. Medical use of ionizing radiation is a small but growing source of radiation-induced cancers. Ionizing radiation may be used to treat other cancers, but this may, in some cases, induce a second form of cancer [113]. Prolonged exposure to ultraviolet radiation from the sun can lead to melanoma and other skin malignancies [114]. Clear evidence establishes ultraviolet radiation, especially the non-ionizing medium wave UVB, as the cause of most non-melanoma skin cancers, which are the most common forms of cancer in the world [114].

2.1.4.6 Heredity

The vast majority of cancers are non-hereditary (sporadic). Hereditary cancers are primarily caused by an inherited genetic defect. Less than 0.3% of the population are carriers of a genetic mutation that has a large effect on cancer risk and these cause less than 3–10% of cancer [115]. Some of these syndromes include: certain inherited mutations in the genes BRCA1 and BRCA2 with a more than 75% risk of breast cancer and ovarian cancer and hereditary nonpolyposis colorectal cancer (HNPCC or Lynch syndrome), which is present in about 3% of people with colorectal canceramong others [115].

2.1.4.7 Physical Agents

Some substances cause cancer primarily through their physical, rather than chemical, effects [114]. A prominent example of this is prolonged exposure to asbestos, naturally occurring mineral fibers that are a major cause of mesothelioma (cancer of the serous membrane) usually the serous membrane surrounding the lungs [114].Other substances in this category, including both naturally occurring and synthetic asbestos-like fibers, such as wollastonite, attapulgite, glass wool and rock wool, are believed to have similar effects [114].Non-fibrous particulate materials that because cancer include powdered metallic cobalt and nickel and crystalline silica (quartz, cristobalite and tridymite) [74].Usually, physical carcinogens must get inside the body (such as through inhalation) and require years of exposure to produce cancer [114].

Physical trauma resulting in cancer is relatively rare [114]. Claims that breaking bones resulted in bone cancer, for example, have not been proven [114]. Similarly, physical trauma is not accepted as a cause for cervical cancer, breast cancer or brain cancer [114]. One accepted source is frequent,

long-term application of hot objects to the body. It is possible that repeated burns on the same part of the body, such as those produced by kanger and kairo heaters (charcoal hand warmers), may produce skin cancer, especially ifs carcinogenic chemicals are also present [114]. Frequent consumption of scalding hot tea may produce esophageal cancer [114]. Generally, it is believed that cancer arises, or a pre-existing cancer is encouraged, during the process of healing, rather than directly by the trauma [114]. However, repeated injuries to the same tissues might promote excessive cell proliferation, which could then increase the odds of a cancerous mutation. Chronic inflammation has been hypothesized to directly cause mutation [117]. Inflammation can contribute to proliferation, survival, angiogenesis and migration of cancer cells by influencing the tumor microenvironment. Oncogenes build up an inflammatory pro-tumorigenic microenvironment [117].

2.1.4.8 Hormones

Some hormones play a role in the development of cancer by promoting cell proliferation [114]. Insulin-like growth factors and their binding proteins play a key role in cancer cell proliferation, differentiation and apoptosis, suggesting possible involvement in carcinogenesis [104].

Hormones are important agents in sex-related cancers, such as cancer of the breast, endometrium, prostate, ovary and testis and also of thyroid cancer and bone cancer [114]. For example, the daughters of women who have breast cancer have significantly higher levels of estrogen and progesterone than the daughters of women without breast cancer. These higher hormone levels may explain their higher risk of breast cancer, even in the absence of a breast-cancer gene [114]. Similarly, men of African ancestry have significantly higher levels of testosterone than men of European ancestry and have a correspondingly higher level of prostate cancer [114]. Men of Asian ancestry, with the lowest levels of testosterone-activating androstanediol glucuronide, have the lowest levels of prostate cancer [114]. obese people have higher levels of some hormones associated with cancer and a higher rate of those cancers [116]. Women who take hormone replacement therapy have a higher risk of developing cancers associated with those hormones [116]**.**

2.1.4.9 Autoimmune Diseases

There is an association between celiac disease [117] and an increased risk of all cancers. People with untreated celiac disease have a higher risk, but this risk decreases with time after diagnosis

and strict treatment, probably due to the adoption of a gluten-free diet, which seems to have a protective role against development of malignancy in people with celiac disease. However, the delay in diagnosis and initiation of a gluten-free diet seems to increase the risk of malignancies [105]. Rates of gastrointestinal cancers are increased in people with Crohn's disease and ulcerative colitis, due to chronic inflammation. Also, immunomodulators and biologic agents used to treat these diseases may promote developing extra-intestinal malignancies [117].

2.2 Viruses

2.2.1 Definition

Virus [118] is a small infectious organism—much smaller than a fungus or bacterium—that must invade a living cell to reproduce (replicate). A viral infection is any type of illness or disease caused by a virus, a type of microbe. Viruses exist as a protein coat or capsid, sometimes enclosed within a membrane, when found outside of host cells. The capsid encloses either DNA or RNA which codes for the virus elements. While in this form outside the cell, the virus is metabollically inert. When it attaches to a cell (called the host cell), enters it, and releases its DNA or RNA inside the cell. The virus's DNA or RNA is the genetic material containing the information needed to make copies of (replicate) the virus. The virus's genetic material takes control of the cell and forces it to replicate the virus. The infected cell usually dies because the virus keeps it from performing its normal functions. When it dies, the cell releases new viruses, which go on to infect other cells [118]. VIruses are classified as DNA viruses or RNA viruses, depending on whether they use DNA or RNA to replicate. There are seven families of DNA viruses that are pathogenic for humans. These pathogens come from the Adenoviridae, Hepadnaviridae, Herpesviridae, Polyomaviridae, Papillomaviridae, Parvoviridae, and Poxviridae families. RNA viruses include retroviruses, such as HIV (human immunodeficiency virus). RNA viruses, particularly retroviruses, are prone to mutate [118].

2.2.2 Pathogenesis of Viral Infections

Viruses cause a number of diseases in eukaryotes. In humans, smallpox, the common cold, chickenpox, influenza, shingles, herpes, polio, rabies, Ebola, hanta fever, and AIDS are examples of viral diseases. Even some types of cancer -- though definitely not all -- have been linked to viruses [119]. In order to cause disease, viruses must first infect their host, spread to and within target tissue and cause damage. Transmission to other susceptible individuals is necessary to ensure their perpetuation, thus viruses developed panoply of remarkable strategies for survival

[120]. As viruses are obligate intracellular parasites that are transmitted as inert particles, they must attach to and infect cells at one of the body surfaces to infect the host cell. Viruses usually infect one particular type of cell. For example, common cold viruses infect only cells of the upper respiratory tract. Additionally, most viruses infect only a few species of plants or animals. Some infect only people. Many viruses commonly infect infants and children. The propensity of viruses to infect cells selectively in particular organs is referred to as tropism (either on cellular or organ level), which is dependent on both viral and host factors. Factors that determine whether the cell may become infected are the presence of critical receptors and presence of required intracellular factors [120].

Once inside the cell, viruses use the host cell metabolism to replicate [121]. The mechanism of viral replication varies depending on the type of the virus, but the end-goal is always the same – to produce messenger RNA molecules from which viral proteins are translated by the host cell.Viral life cycles vary in their precise details depending on the type of virus, but they all share a general pattern: Attachment to a host cell, release of viral genes and possibly enzymes into the host cell, replication of viral components using host-cell machinery, assembly of viral components into complete viral particles and release of viral particles to infect new host cells. This unique feature of viruses makes it difficult to design a treatment to attack the virus or its replication directly without any adverse effects on the infected cells. However, viruses share a common stage in their replication cycle, which includes attachment and entry to the host cell, transcription of viral mRNA, replication of viral genome, assembly and budding as progeny virus particles, regardless of different genetic materials (DNA or RNA), or whether has a different invasion strategy of which enveloped with a lipid-containing membrane (enveloped virus) or not. Whereas, viruses with an RNA genome, such as HIV, HCV, and influenza, are genetically highly variable, due to the fact that viral reverse transcriptase or RNA-dependent RNA polymerase lack a proofreading mechanism. Accumulated mutations in viral RNA genome have been proven to be associated with the emergence of drug-resistant viruses [121]. An understanding of the molecular mechanisms of viral invasion and replication enables us to design antiviral drugs targeting the different stages of the viral replication cycle. Although in theory, any viral molecule that is essential for viral replication is a potential drug target, most of the clinically useful antiviral drugs are the molecules that can specifically target a single viral enzyme, which is crucial in viral

replication [122]. Targeting virus molecules is likely more specific, and less toxic. However, there is a narrow spectrum of viruses and a higher risk of creating resistant viruses. Whereas drugs which target cellular molecules may possess a broader antiviral activity spectrum and less risk of developing virus resistance, but may be more toxic to the host cell. Ideally, effective therapeutic agents that target multiple stages in the viral replication cycle with combined approaches but with little or no toxicity are desirable.

2.2.3 Types of Viral Infections

There are many types of viruses that cause a wide variety of viral infections or viral diseases. Because a large number of virus genera and species exist, they are usually considered in groups. Probably the most common viral infections are [118, 119]:

Respiratory infections: Infections of the nose, throat, upper airways, and lungs. The most common respiratory infections are upper respiratory infections, which include sore throat, sinusitis, and the common cold. Virus groups responsible for respiratory infections include influenza virus, respiratory syncytial virus (RSV), rhinovirus and the severe acute respiratory syndrome-associated coronavirus (SARS-Cov).In small children, viruses also commonly cause croup (which is inflammation of the upper and lower airways, called laryngotracheobronchitis) or lower airways (bronchiolitis).Respiratory infections are more likely to cause severe symptoms in infants, older people, and people with a lung or heart disorder.

Gastrointestinal tract: Infections of the gastrointestinal tract, such as gastroenteritis, are commonly caused by viruses, such as noroviruses, adenoviruses, caliciviruses, astroviruses, coronaviruses and rotaviruses. In infants and young children, rotaviruses represent the single most important etiological agents of acute gastroenteritis in terms of numbers of cases and also of patients requiring admission to hospital.

Nervous system: Some viruses, such as the rabies virus and the West Nile virus, infect the brain, causing encephalitis. Others infect the layers of tissue that cover the brain and spinal cord (meninges), causing meningitis or polio.

Human viruses: Human viruses known to be spread by sexual contact include herpes simplex viruses (HSV), human papillomaviruses (HPV), human immunodeficiency virus (HIV), hepatitis B virus, as well as a plethora of other viruses. More than 80 million people have been infected

with HIV since the start of the epidemic in the early 1980s.

Many body infections: Some viruses typically affect many body systems. Such viruses include enteroviruses (such as coxsackieviruses and echoviruses), Epstein-Barr virus and cytomegaloviruses.

2.2.4 Types of Virus

2.2.4.1 Picornavirus

There are many different kinds of viruses. However, here three viruses of interest (Picornavirus, influean a virus and dengue virus) will be discussed.

2.2.4.1.1 Classification

Classification [123] is based on physical properties, the particle density and pH-sensitivity, and serological relatedness, more recently based on nucleotide sequence. The most recent revision of virus taxonomy has recognized nine genera within the family: Picornaviruses are classed under Baltimore's viral classification system as group IV viruses as they contain a single stranded, positive sense RNA genome of between 7.2 and 9.0 kb in length. Like most positive sense RNA genomes, the genetic material alone is infectious; although substantially less virulent than if contained within the viral particle, the RNA can have increased infectivity when transfected into cells. The genome itself is the same sense as mammalian mRNA, being read 5' to 3'. Unlike mammalian mRNA Picornaviruses do not have a 5' CAP but a virally encoded protein known as VPg, however like mammalian mRNA the genome does have a poly A tail at the 3' end. There is an un-translated region (UTR) at both ends of the Picornavirus genome. The 5' UTR is longer, being around 600-1200 BP in length, compared to that of the 3' UTR, which is around 50-100bp. It is thought that the 5' UTR is important in translation and the 3' in negative strand synthesis; however, the 5' end may also have a role to play in virulence of the virus. The rest of the genome encodes structural proteins at the 5' end and non-structural proteins at the 3' end in a single polyprotein.

2.2.4.1.2 Structure

Picornaviruses [119] are non-enveloped, with an icosahedral capsid (Fig. 2.1). The capsid is an arrangement of 60 protomers in a tightly packed icosahedral structure. Each protomer consists of 4 polypeptides known as VP (viral protein) 1, 2, 3 and 4. VP2 and VP4 polypeptides originate from one protomer known as VP that is cleaved to give the different capsid components. The

icosahedral is said to have a triangulation number of 3, this means that in the icosahedral structure each of the 60 triangles that make up the capsid are split into 3 little triangles with a subunit on the corner. In many picornaviruses have a deep cleft formed by around each of the 12 vertices of icosahedrons. The outer surface of the capsid is composed of regions of VP1, VP2 and VP3. Around each of the vertices is a canyon lined with the C termini of VP1 and VP3. The interior surface of the capsid is composed of VP4 and the N termini of VP1. J. Esposito and Professor Freederick A. Murphy demonstrate cleft structure referred to as canyons, using X-ray crystallography and cryo-electron microscopy. Depending on the type and degree of dehydration the viral particle is around 27–30 nm in diameter. The viral genome is around 2500 nm in length so we can therefore conclude that it must be tightly packaged within the capsid along with substances such as sodium ions in order to cancel out the negative charges on the RNA caused by the phosphate groups [119].

Picornavirus virions consist of a non-enveloped, icosahedrally symmetric capsid. The capsid consists of 12 capsomers and has a diameter of 27-30 nm, which makes it one of the smallest of all viruses (thus the name "picornavirus"). The genome is tightly packed into the capsid. The capsid has four unique proteins: VP1, 2, 3, and 4 Source [124].

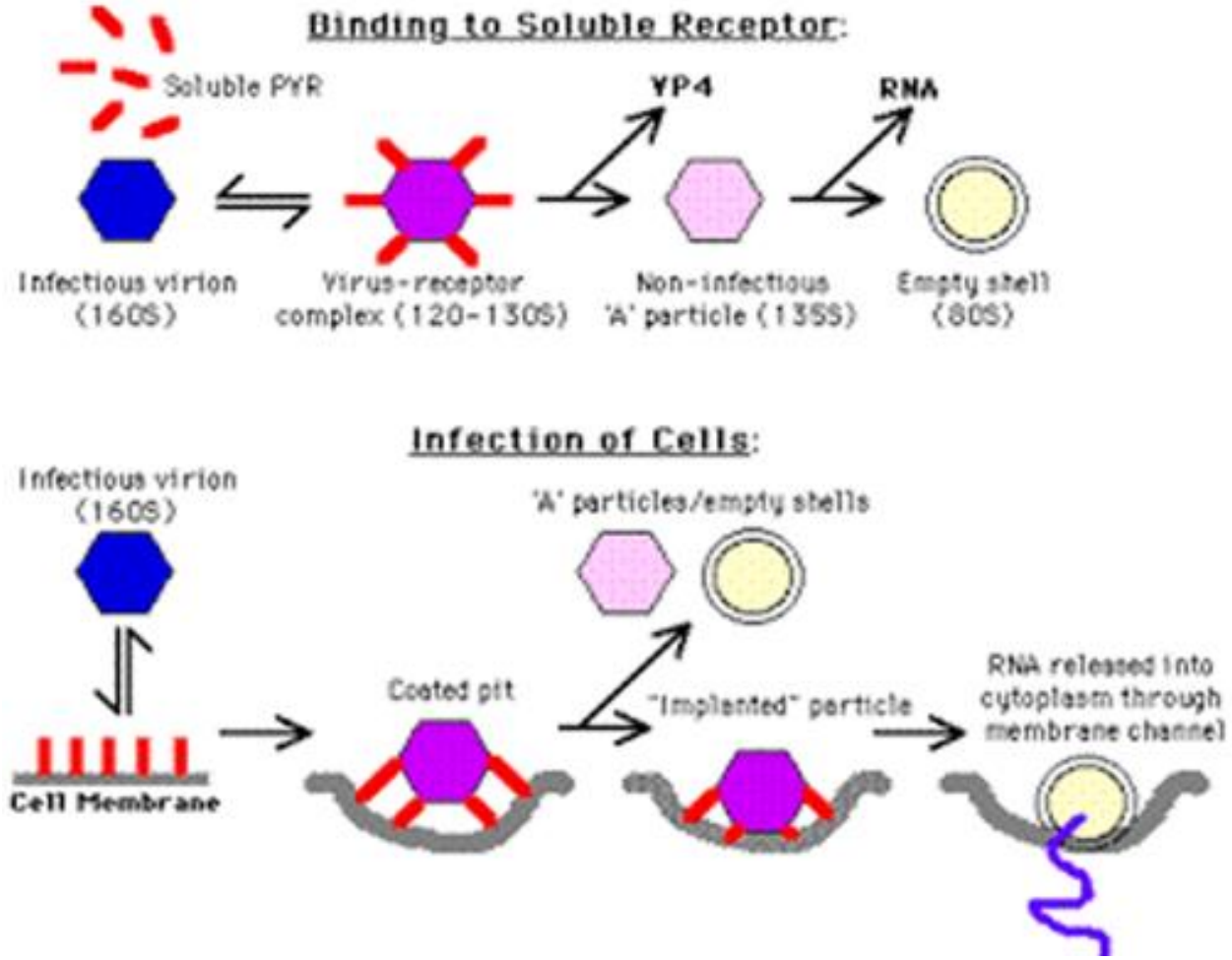


Figure 2.1: Virion structure of a picornaviridae

2.2.4.1.3 Replication

Genomic RNAs of picornaviruses possess multiple RNA elements and they are required for both negative and plus strand RNA synthesis (Fig. 2.2). The cis acting replication (cre) element is required for replication. The stem-loop-structure that contains the cre is independent of position but changes with location between virus types when it has been identified. Also, the 3' end elements of viral RNA are significant and efficient for RNA replication of picornaviruses. The 3' end of picornavirus contains poly(A) tract which be required for infectivity. On the other hand, RNA synthesis is hypothesized to occur in this region.3' end NCR of poliovirus is not necessary for negative-strands synthesis. However, it is important element for positive—strand synthesis. Additionally,5' end NCR that contain secondary structural elements is required for RNA replication and poliovirus translation initiation (IRES). Internal Ribosome Entry Site (IRES) are

RNA structures that allow cap independent initiation of translation, and are able to initiate translation in the middle of a messenger RNA [125].

The viral particle binds to cell surface receptors. Cell surface receptors are characterized for each serotype of picornaviruses. For example, poliovirus receptor is glycoprotein CD155 which is special receptor for human and some other primate species. For this reason, poliovirus couldn't be made in many laboratories until transgenic mice having a CD155 receptor on their cell surface were developed in the 1990s. These animals can be infected and used for studies of replication and pathogenesis [119]. Binding causes a conformational change in the viral capsid proteins, and myristic acid is released. These acids form a pore in the cell membrane through which RNA is injected. Once inside the cell, the RNA un-coats and the (+) strand RNA genome is replicated through a double-stranded RNA intermediate that is formed using viral RDRP (RNA-Dependent RNA polymerase). Translation by host cell ribosomes is not initiated by a 5' G cap as usual, but rather is initiated by IRES (Internal Ribosome Entry Site). The viral lifecycle is very rapid with the whole process of replication being completed on average within 8 hours. However, as little as 30 minutes after initial infection, cell protein synthesis declines to almost zero output – essentially the macromolecular synthesis of cell proteins is shut off. Over the next 1–2 hours there is a loss of margination of chromatin and homogeneity in the nucleus, before the viral proteins start to be synthesized and a vacuole appears in the cytoplasm close to the nucleus that gradually starts to spread as the time after infection reaches around 3 hours. After this time the cell plasma membrane becomes permeable, at 4–6 hours the virus particles assemble, and can sometimes be seen in the cytoplasm. At around 8 hours the cell is effectively dead and lyses to release the viral particles [119].

Using different cellular receptors (depending on the picornavirus), a picornavirus virion attaches to a host cell. Uncoating occurs, and the virus' RNA is released into the cytoplasm of the host cell through a membrane channel. Virus replication occurs entirely in the cytoplasm. The host cell's transcription processes are shut off to a degree that varies with different picornaviruses, while the IRES helps to make sure the virus' transcription is left untouched. Replication occurs. RNA is packaged into preformed capsids. Release of the virus occurs when cell lysis occurs (with the exception of Hepatits A, which is non-lytic and thus creates a more persistent infection. Source [125].

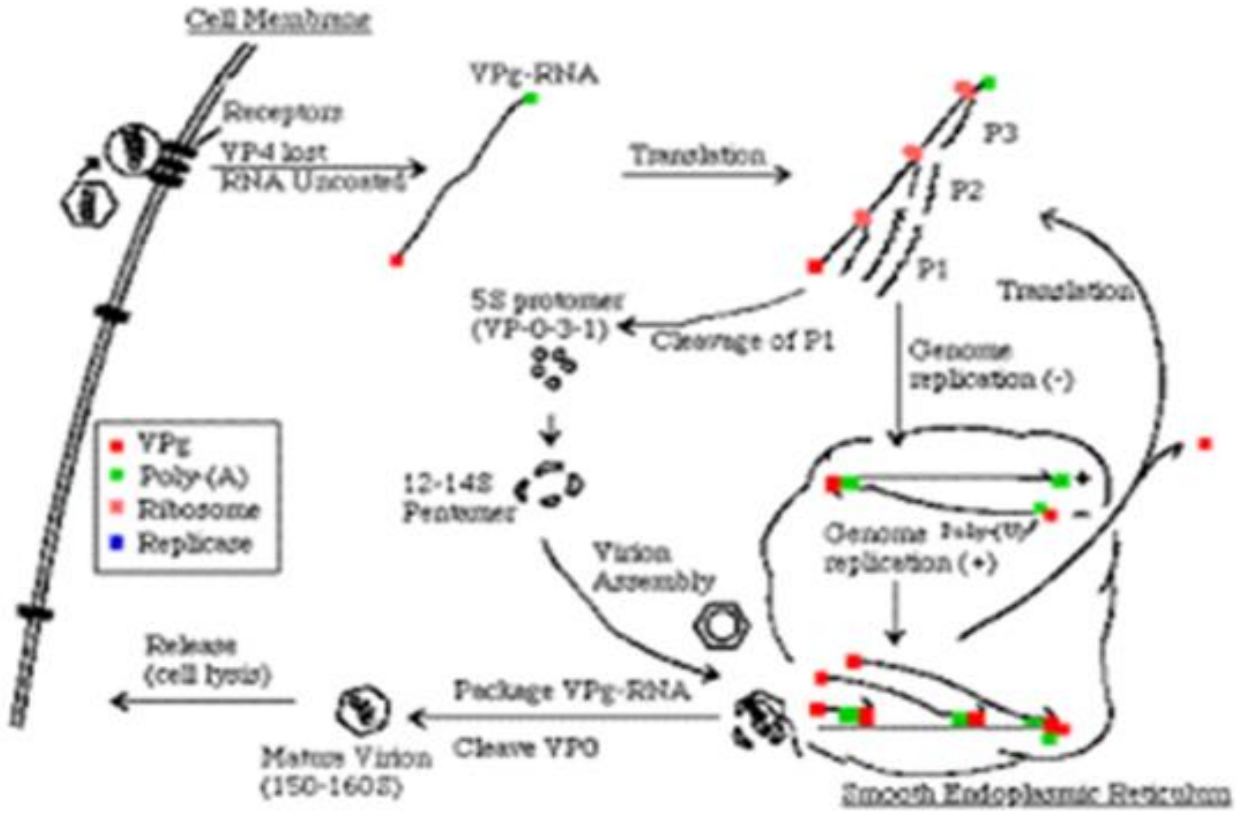


Figure 2.2: Reproduction cycle of Picornaviridae

2.2.4.2 Influeanza Virus

Influenza is considered to be one of the lifethreatening infectious diseases. In some countries seasonal influenza affects annually up to 40% of the population and 500 million people die from it worldwide every year. New highly-virulent influenza strains can arise unexpectedly to cause world-wide pandemics with markedly increased morbidity and mortality, such as the “avian flu” in 1997 and “swine flu” in 2009 and commonly occur during winter, or the rainy season in tropical countries [126].

Influenza virus is an enveloped virus with negativestrand, eight-segmented RNA genomes, belonging to the Orthomyxoviridae family. Each viral segment is encapsidated by a virus-encoded nucleoprotein (NP), called viral ribonucleoprotein (vRNP)[127]. Influenza virions are pleomorphic, roughly spheroidal and approximately 100 nm in diameter [128]. The viral envelope is distinguished by a lipid bilayer containing three transmembrane proteins—hemagglutinin (HA),

neuraminidase (NA), and matrix protein 2 (M2, ion channel) on the outside and matrix protein 1 (M1) beneath the membrane.

Pharmaceutical ingredients can be classified into two groups: NA inhibitors, such as oseltamivir and zanamivir, and M2 inhibitors, such as amantadine and rimantadine. These have been approved and used to treat and prevent influenza infections. The NA inhibitors are effective against both influenzas A and B viruses, while the M2 inhibitors are effective only against influenza A virus [129]. Drugs derived from sialic acid moieties that target NA protein are preferable to M2 inhibitors because of their high efficiency and stringent specificity, as well as their desirable safety profiles. However, the incidence of oseltamivir-resistant viruses is increasing, primarily because of the mutation of a single NA amino acid (H275Y according to the N1 numbering system or H274Y according to the N2 numbering system). This mutation has been reported in patients treated with oseltamivir but also in patients who have not received this drug [126]. The emergence of drug-resistant strains highlights the need to develop novel antiviral drugs that effectively target other viral proteins or cellular factors involved in the influenza virus life cycle, or the identification of novel NA inhibitors that do not recognize the same amino acids binding to the existing sialic acid derivatives. Antiviral drugs can overcome the limitations of vaccination strategies, such as the time-consuming vaccine design, insufficient protection for immunocompromised patients and the unpredictable antigenic changes in influenza strains which render vaccination ineffective. In general, the anti-influenza agents can be divided into two basic groups, i.e., synthetic analogues of biomolecules required during virus infection and substances derived from natural plant extracts. With regard to the considerable genetic and antigenic variability of the influenza virus, research has been predominantly focused on broad-spectrum antiviral drugs, which are effective against a large variety of influenza strains.

The anti-influenza drugs are usually classified according to their target in the viral life-cycle, which is schematically depicted in Figure 2.3. Such antiviral molecules are particularly used as inhibitors of the following processes: attachment of the virus to host cell receptors, endocytosis and fusion of viral and cell membranes, replication and transcription of the viral genome, synthesis of viral proteins, assembly of the viral progeny and release of the new virions into the outside environment.

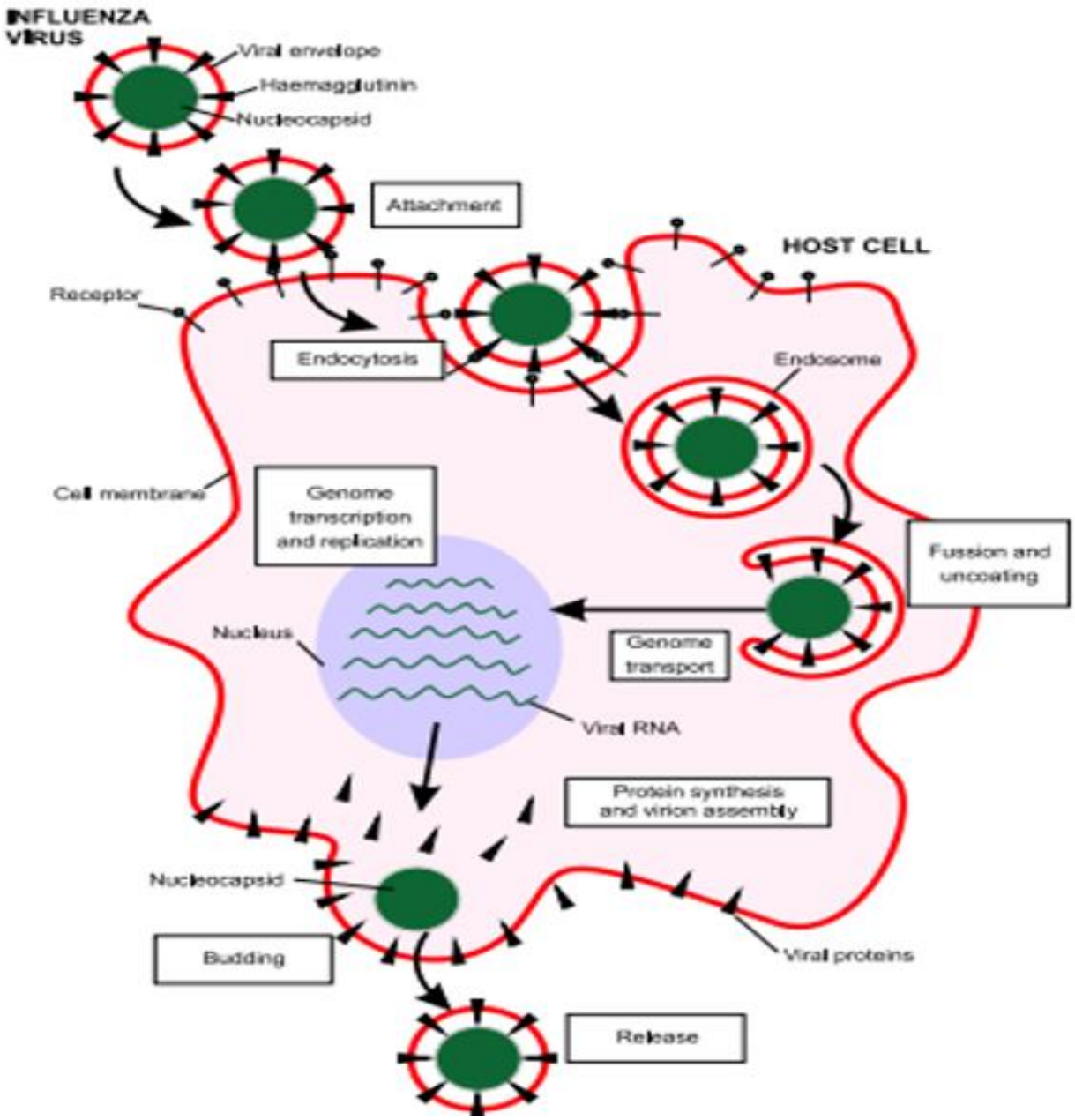


Figure 2.3: Life cycle of influenza virus [130]

2.2.4.3 Dengu Virus

Dengue fever (DF) and dengue hemorrhagic fever (DHF) are acute febrile diseases transmitted by mosquitoes. Nowadays, they are the most rapidly spreading mosquito-borne diseases in the world. About 2.5 billion people, two-fifths of the world's population, are now at risk of infection and 50 million cases of DF are reported worldwide every year [131]. The World Health Organization (WHO) estimates that the annual global incidence of dengue is close to 390 million, a number

nearly three times higher than the number of cases estimated by the same organization for 2009 [131].

2.2.4.3.1 Genome Organization

The genome organisation [132**]** is that of single positive strand RNA genome virus, ie., it is similar to a large cellular mRNA molecule (Fig. 2.4). The genome is approximately 11 kb in size or about 50 nm in diameter, bears a type I cap structure at its 5'-end, and lacks a 3'-polyadenylate tail. The long open reading frame encoding a large polyprotein is flanked in 5' and 3' by untranslated regions (UTRs). The latter carry a number of cis-acting signals (stem loops, conserved sequences …) required for viral replication, and possibly RNA capping. There are complementary sequences in these UTRs that are thought to be responsible for cyclization of the genome, which is essential for replication.

2.2.4.3.2 DV Particle and DV Proteins as Targets for Drugss

Flavivirus [132] are enveloped viruses having two outer membrane proteins, the envelope (E) and the membrane (M) processed from the precursor prM. The genome is thought to be wrapped/associated with the capsid protein C. A single polyprotein is translated from the genome, and the former is cleaved by a combination of cellular proteases and a viral serine protease made of NS2B and NS3 (protease N-terminus domain). Proteolysis yields ten proteins, the three structural proteins (C, prM, and E) and the seven nonstructural (NS) proteins involved in genome replication and capping (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). Some of these NS proteins also participate in pathogenesis and counteract the innate immunity of the host cell.

Replication of the viral genome does not occur freely in the cytoplasm. Instead, there is an extensive intracellular membrane re-arrangement in the infected cell, with various observable cell substructures containing most NS proteins organized along the virus replication cycle.

2.2.4.3.3 The Structural Proteins

The M and E proteins [133] have been considered so far as drug targets. The E protein is endowed with a dual function: to recognize cellular receptor, and to fuse the viral membrane to cellular endosomic membranes. Five receptors have been found to be involved in binding to E: DC-SIGN, L-SIGN, the high affinity laminin receptor, the mannose receptor, and GRP78. E is an attractive target because theoretically, an antiviral molecule binding and impeding attachement would act before infection or spreading the virus to yet-uninfected cells. In the RNA virus antiviral world,

there are several examples of such inhibitors targeting early phase of the viral life cycle: that of T20 (a fusion inhibitor corresponding to the C terminus ectodomain of gp41) in anti-HIV therapy and that of Pleconaryl, a small molecule enterovirus capsid binder.

The dengue E protein crystal structure is known. E belongs to class II fusion proteins. Upon binding to a receptor and endocytosis under a trimeric form, the acidic environment of the endosome induces a structural re-arrangement yielding fusion of cellular and viral membranes. Theoretically, a similar strategy as that of HIV and T20 could be followed. Crystal structure study of a E fragment has revealed a pocket that could be used for antiviral drug-design. The crystal structure of the prM protein bound to E (prM-E heterodimer) is known, opening avenues for drug design. The structure of the capsid protein C has bee elucidated in solution. The E protein is the most obvious target for therapeutic monoclonal antibodies.

2.2.4.3.4 The Non-Structural Proteins

Out of the seven NS1-to-NS7, only NS3 and NS5 [137] have been considered so far as drug targets, not only because they are essential to virus growth but also because they exhibit enzyme activity, which is a plus regarding drug screening. The role and structure of NS1 is unknown. It is a soluble protein detected very early during infection, but has received little attention so far as an antiviral target. NS2a, NS2b, NS4a, and NS4B are membrane –associated proteins believed to anchor and regulate the replication complex during the virus life-cycle.

2.2.4.3.5 The Dengue Validated Targets

Presently, there are no drugs against dengue in the clinic. The validated target [133] is only derived by analogy to other viruses for which drugs have proven to be effective. Herpes and HIV have been the drug-design founding viruses. Now for DENV, HCV is fulfilling this role. Recent data presented regarding a nucleoside analogue, although toxic, have shown that the NS5 protein is a validated target (see compound NITD008). The validated targets are thus the RNA-dependent RNA polymerase and, by analogy to HCV, the protease. More recently, the HCV NS5A protein seems to emerge as a very interesting target, but NS5A does not have an equivalent in dengue, so far. Conversely, the dengue RNA genome is capped, and the DENV genome encodes most of its own RNA capping machinery. This is not the case for HCV, which does not rely on RNA capping for gene expression. The RNA capping enzymes of dengue so far await validation in an animal model both at the level of efficiency, and toxicity. Indeed, it is not known if the abundance of

cellular MTases will cause a specificity problem, ie., if it will be possible to design an anti-dengue MTase inhibitor having non-significant toxicity effect through co-lateral inhibition of host cell MTases.

Different approaches [131] have been used in the search for dengue antivirals, such as screening of compounds against dengue enzymes and structure-based computational discovery. During the last decades, researchers have turned their attention to nature, trying to identify compounds that can be used as dengue antivirals. Several plants around the world present potential dengue antiviral activity. Recently, Kadir and co-workers reviewed sixty-nine studies from 1997 to 2012 related to plants presenting potential antidengue activity.

Figure 2.3: The DENV (+) RNA genome and its co-linear polyprotein [132].

2.3 Plants as a Source of Cancer Therapeutic Drugs

Medicinal plants constitute a common alternative for cancer treatment in many countries around the world. At this time, more than 3000 plants worldwide have been reported to have anticancer properties. Approximately 60% of drugs currently used for cancer treatment have been isolated from natural products and the plant kingdom has been the most significant source [134]. According to a recent review [135], among the 79 FDA approved anticancer drugs and vaccines from 1983-2002, 9 of them were directly from the isolation of natural products and 21 of them were natural product derivatives. Also among the 39 synthetic anticancer drugs, 13 of them were based on a pharmacophore originated from natural compounds [135].

plant-derived agents, such as VBL and vincristine (VCR), etoposide, paclitaxel (Taxol ®), docetaxel, topotecan, and irinotecan, are among the most effective cancer chemotherapeutics currently available [136]. Nevertheless, many suffer from the liabilities of poor solubility in aqueous media and significant toxic side effects. Thus, there continues to be considerable research devoted to diminishing the impact of these factors, and numerous analogues and prodrugs of these agents have been synthesized, and methods devised for increasing aqueous solubility and targeting specific tumors.

2.3.1 Vinca Alkaloids

The first plant-derived agents to advance into clinical use were the vinca alkaloids, VBL **(9)** and VCR (10) [137], isolated from the Madagascar periwinkle, *Catharanthus roseus* G. Don. (Apocynaceae). Extracts of the plant reduced white blood cell counts and caused bone marrow depression in rats, and subsequently they were found to be active against lymphocytic leukemia in mice. This led to the isolation of VBL (**9**) and VCR (**10)** as the active agents, so their discovery may be indirectly attributed to the observation of an unrelated medicinal use of the source plant. The mechanism of action is to disrupt microtubules, causing the arrest of the cells at metaphase and leading to apoptotic cell death.

The effective semisynthetic analogues that have been developed include vinorelbine and vindesine, with the most recent being vinflunine, a second-generation bifluorinated analogue of vinorelbine. These agents are primarily used in combination with other cancer chemotherapeutic drugs for the treatment of a variety of cancers [138], including leukemias, lymphomas, advanced testicular cancer, breast and lung cancers, and Kaposi's sarcoma. These alkaloids and their semisynthetic derivatives block mitosis at the metaphase arrest by binding specifically to tubulin resulting in its depolymerization [137].

2.3.2 Podophyllotoxins

Podophyllotoxins [139] were isolated from the species *Podophyllum peltatum L.* (commonly known as the American mandrake or mayapple) and *Podophyllum emodii Wallich* from the Indian subcontinent have a long history of medicinal use, including the treatment of skin cancers and warts. Clinical trials of several closely related podophyllotoxin-like lignans, however, failed due to lack of efficacy and unacceptable toxicity. Extensive research led to the development of etoposide **(15)** and teniposide as clinically effective agents. For mechanism of action, while podophyllotoxin reversibly binds to tubulin, etoposide and teniposide inhibit topoisomerase II, inducing topoisomerase II-mediated DNA cleavage. For treatment, etoposide and teniposide are used for lymphomas, and bronchial and testicular cancers [140].

2.3.3 Taxanes

Taxanes are one of the most important classes of cancer chemotherapeutic drugs in clinical use. Currently, the two most clinically effective drugs of this class are paclitaxel (Taxol ® **7**), originally isolated from the bark of the *Pacific yew*, *Taxus brevifolia* Nutt. (Taxaceae), and paclitaxel (**16**), a semisynthetic analogue synthesized from DAB (10-deacetylbaccatin III) isolated from the leaves

of the *European yew*, *Taxus baccata*. DAB has also been semisynthetically converted to paclitaxel, there by providing a sustainable source of the drug [141].

Regarding the mechanism of action, paclitaxel and other taxanes promote the polymerization of tubulin heterodimers to microtubules, suppressing dynamic changes in microtubules resulting in mitotic arrest. Paclitaxel is used in the treatment of breast, ovarian, and non-small cell lung cancer (NSCLC), and has also shown efficacy against Kaposi's sarcoma, while docetaxel is primarily used in the treatment of breast cancer and NSCLC. The many structural analogues of Taxol ® synthesized and new formulations [142] developed to overcome the clinical limitations of paclitaxel and docetaxel, including poor solubility, allergic reactions, dose-limiting toxicities such as myelosuppression or peripheral sensory neuropathy, and the development of drug resistance due to P-glycoprotein-mediated efflux. The analogue docetaxe (**17**) has been approved for the treatment of metastatic prostate cancer (in combination with prednisone and/or prednisolone) [143], and is in clinical trials, either as a single agent or in combination with other agents, for the treatment of a range of cancers including prostate, bladder, brain (glioblastoma multiforme), head-and-neck, ovarian, stomach and urinary tract cancers, and NSCLC.

2.3.4 Combretastatins

The combretastatins are a family of stilbenes originally isolated from the root bark *Combretum caffrum*, also known as the Cape bush willow in southern Africa [144]. They act as vascular disrupting agents, selectively targeting the endothelial cells lining the tumor vasculature. They also disrupt the tubulin cytoskeleton and remodel the actin cytoskeleton, inducing a significant change in the three-dimensional shape of immature endothelial cells, thereby stopping blood flow through the capillaryand starving the tumor of nutrients, causing tumor cell death. This mechanism of action differentiates the combretastatins from angiogenesis inhibitors that are designed to work by preventing the growth of new blood vessels [144].

Combretastatin Prodrugs, combretastatin A4 phosphate (CA4P; fosbretabulin; Zybrestat: **19**) is a phosphate prodrug of CA4 (**18)**. In 2003, CA4P was granted orphan drug designation by the FDA for the treatment of anaplastic thyroid cancer, medullary thyroid cancer, and stage IV papillary or follicular thyroid cancer. Later trials in the treatment of patients with advanced anaplastic thyroid carcinoma, using CA4P as a single agent [145] or in combination with paclitaxel/carboplatin [146]. In 2006, CA4P was granted orphan drug designation by the FDA for the treatment of ovarian

cancer, and treatment of patients with platinum-resistant ovarian cancer with a combination of CA4P, carboplatin, and paclitaxel reportedly produced a higher response rate in this patient population compared to chemotherapy given without CA4P [147]. Combretastatin A1 diphosphate (CA1P; OXI-4503:**21**) is a phosphate prodrug of CA1 (**20**), and it has shown promising efficacy in the treatment of patients with relapsed and refractory acute myelogenous leukemia (AML) and myelodysplastic syndromes [148]. In 2012, orphan drug designation was assigned by the FDA for the treatment of AM [148].

2.3.5 Homoharringtonine

The isolation and structure of homoharringtonine (HHT; omacetaxine mepesuccinate; Synribo: **22**) was first stated in 1970 from the Chinese tree, *Cephalotaxus harringtonia var. drupacea* (*Sieb and Zucc.; Cephalotaxaceae*) [149]. A racemic mixture of HHT and harringtonine has been used in China for the treatment of AML and chronic myelogenous leukemia (CML), and purified HHT has shown efficacy against various leukemias, including some resistant to standard treatment, with complete hematologic remission being reported in the treatment of patients with late chronic phase CML [149].HHT (Synribo) is thought to act as a broad-spectrum protein tyrosine kinase inhibitor and was approved in 2012 by the FDA for the treatment of adult patients with CML or accelerated phase CML [150]. The therapeutic response of patients with CML in myeloid blast crisis (CML-MBC) is generally very poor, but a combination of HHT and cytarabine has been reported to be an effective treatment for CML-MBC [151].

2.3.6 Ingenol Mebutate (Ingenol-3-Angelate)

The common Australian Plant *Euphorbia peplus* (Euphorbiaceae) is widely used as a home remedy for the treatment of various skin conditions, and clinical studies with crude *E. peplus* sap in the 1970s provided compelling evidence of its efficacy. The active agent was identified as the hydrophobic diterpene ester, ingenol-3-angelate (PEP005; ingenol-3-mebutate; Picato:**23**), and it was shown to act through activation of protein kinase C (PKC) [152].

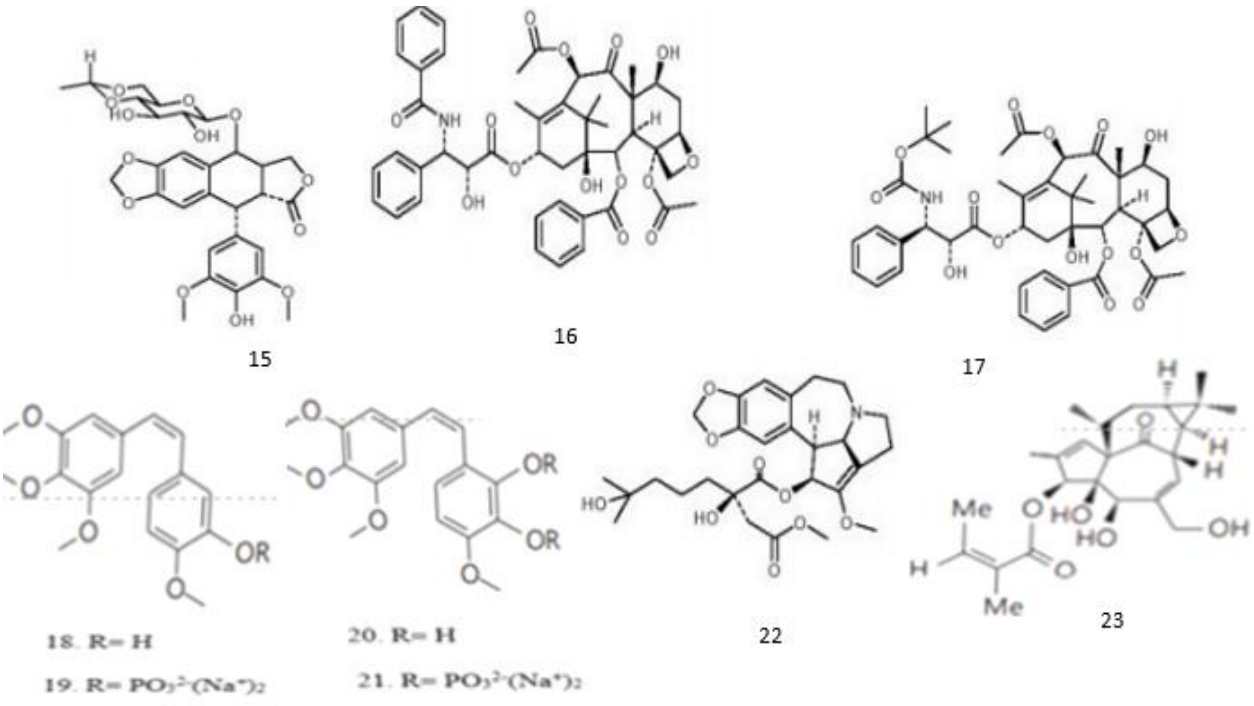


Figure 2.4; List of some of plant-derived anticancer compounds.

2.4 Cancer Chemopreventive Agents of Plant Origin

Plant secondary metabolites also show promise for the cancer chemoprevention, which has been defined as "the use of non-cytotoxic nutrients or pharmacological agents to enhance physiological mechanisms that protect the organism against mutant clones of malignant cells" [153]. Chemoprevention is an approach designed to reduce the morbidity and mortality due to cancer through slowing, blocking, or reversing the process of carcinogenesis before malignancy by using natural or synthetic compounds [154]. Chemoprevention strategies target each of these steps including anti-initiation strategies (e.g., DNA repair, detoxification, free-radical scavenging, and carcinogen metabolism) and anti-promotion/anti-progression strategies (e.g., free-radical scavenging, proliferation suppression, differentiation induction, immunity enhancement, inflammation reduction, increase in apoptosis, altered gene expression, and decrease in angiogenesis) [154]. There has been considerable prior work on the cancer chemopreventive effects of extracts and purified constituents of certain culinary herbs, fruits, spices, teas, and vegetables, which have shown the ability to inhibit the development of cancer in laboratory animal models [155,156].

Several promising plant-derived compounds are in clinical use and clinical trials through the auspices of the U.S. National Cancer Institute as potential cancer chemopreventive agents.

2.4.1 Natural Product-Derived Agents Currently in Clinical Use

Tamoxifen (**24**) and related compounds, such as raloxifene (**25**) and letrozole (**26**), are well-established agents that are currently in use for the prevention of cancer in high-risk breast cancer patients. The key mechanism appears to involve estrogen receptor antagonism [157]. More generally, aspirin (**27**) is considered as useful for the reduction of colon cancer due to anti-inflammatory activity [158].

2.4.2 Natural Product-Derived Agents Currently in Clinical Trials

There are nearly 300 clinical trials are currently on going [159], most of which are investigating fairly generic agents or structural derivatives of known agents believed to have some activity. Cancer chemoprevention has been associated with aspirin, and 13 clinical trials are currently underway with this substance [158]. Other examples of natural product-derived agents in clinical trials include metformin (**28**; 7 trials), Polyphenon E (**29**; 9 trials), retinoids (**30**; 20 trials), soy isoflavones (**31**; 16 trials) and vitamin D (**32**; 20 trials). In addition, curcumin has not done well in clinical trials (ostensibly due to problems with absorption and metabolism), but chemical derivatives (compound **33**, and compound **34**) are currently being investigated (12 trials) [159].

Some known natural products with hitherto unknown mechanisms of action have been known as chemopreventive agents, for example, quassinoid bruceantin (**35**) and the close structural relative brusatol (**36**). Bruceantin was originally discovered as a cancer chemotherapeutic agent and dropped due to poor efficacy in advanced-stage cancer patients [160]. It was ‘rediscovered’ as a potent inducer of cell differentiation and subsequently found to inhibit tumor growth at low doses in the absence of toxicity [161]. Resveratrol (**37**) was isolated from a non-edible legume following bioassay-guided fractionation with inhibition of cyclooxygenase.

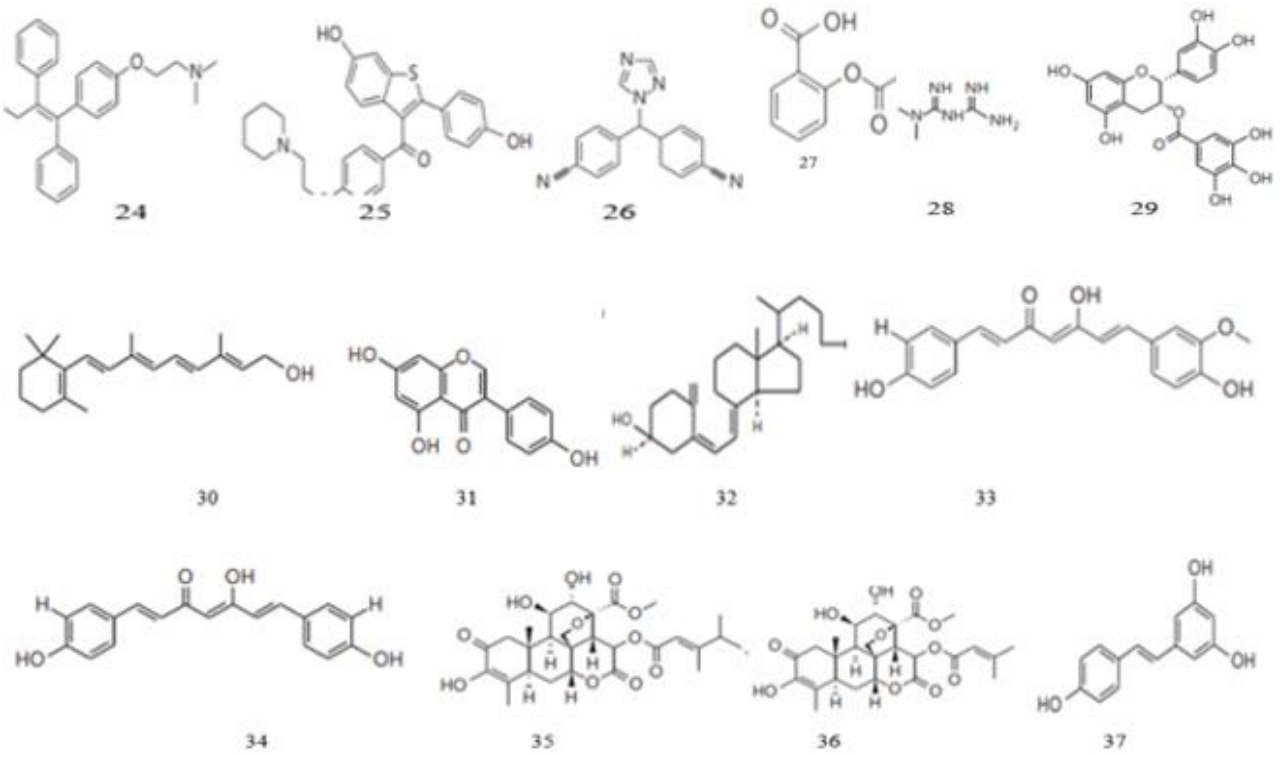


Figure 2.5: List of some plant-derived chemopreventive agents in clinical use and trials**.**

2.5 Plants Compounds as Antiviral Activities

Viruses are one of the main hazards for humans and animals, they enter in human body and redirect body's metabolism to produce large copies of their genome and proteins [120]. It is difficult to control viral infections with currently available drugs like moroxydine, valaciclovir, ganciclovir and valganciclovir and they act by inhibiting the virus replication via different mechanisms. The difficulty in drug treatment arises due to their low efficiencies, cytotoxic effect and development of viral resistance against them as viruses are smart organisms which mutate themselves and show resistance to drug therapy [121]. So demand for plant-based medicines is growing as they are generally considered to be safer, cheaper, non-toxic and less harmful than synthetic drugs. A number of natural compounds reported in traditional medicinal plants to have anti-viral properties were studied and were also screened for anti-viral compounds structure. Chattopadhyay et al, have generated the important information on wide array of ethno- medicinal plants showing high level of antiviral activities with various mechanisms, complementary and overlapping mechanism of action, either inhibiting viral replication or viral genome synthesis. Till date number of compounds

with antiviral activity is scarce [162]. Natural compounds, because of their structural diversity, provide a large opportunity for screening anti-viral agents with novel structure and distinct mechanism of action.

A wide variety of active phytochemicals, including the flavonoids, terpenoids, lignans, sulphides, polyphenolics, coumarins, saponins, furyl compounds, alkaloids, polyines, thiophenes, proteins and peptides have been identified having potential as a novel antiviral agent.

2.5.1 Flavonoids

The antiviral activity of various flavonoids against several viruses in cell cultures and in animal models has been demonstrated. Apigenin **(38)** is widely distributed in a variety of plants, such as *Ocimum basilicum* (Luo Le), *Milelttia* species and *Kalanchoe* species. Apigenin showed anti-EV71 activity at approximately 25 μM, and inhibited viral protein expression, reactive oxygen species (ROS) generation, and cytokine upregulation. Apigenin also interfered with viral internal ribosome entry site (IRES) activity and JNK activation [163]. The study also suggested that apigenin inhibited the association of EV71 RNA with RNA editing-related hnRNP proteins [163].

Chrysosplenetin (**39**) and penduletin (**40**) are two flavonols isolated from the leaves of *Laggera pterodonta* (Chou Ling Dan), which is used for clearing heat and detoxification [164]. These flavonols exhibited potent anti-EV71 activity with low EC_{50} values of 0.20 μM for chrysosplenetin and 0.17 μM for penduletin, and had SI values of > 100 [164]. The flavonols showed strong antiviral potency by targeting the viral post-attachment stage. Flavonoids with 3-methoxy, 5-hydroxy, and 4′-hydroxyl groups showed antipicornavirus activity by targeting the phosphatidylinositol 4-kinase III β (PI4KB) pathway, and being within the same group, chrysosplenetin and penduletin might inhibit PI4KB, which would contribute to their anti-EV71 activity [165]. The PI4KB/oxysterol binding protein (OSBP) pathway was the major target of the anti-picornavirus activity of enviroxime-like compounds and flavonoids with 3-methoxy, 5-hydroxy, and 4′-hydroxyl groups [165]. 7,8-dihydroxyflavone, kaempferol, quercetin, hesperetin, and hesperidin are polyphenolic flavones with inhibitory effects on EV71 infection at a concentration of 50 μM [166]. Among them, 7,8-dihydroxyflavone, kaempferol, and hesperetin inhibited 40 % of viral IRES activity. Kaempferol also significantly reduced the viral yield by its regulatory effects on IRES function and EV71 replication.

Chrysin (**41**) is a flavone extracted from the seeds of *Oroxylum indicum* (L.) Vent. (Mu Hu Die) and other plants, and exhibited antitumor and antidiabetic bioactivities [167]**.** Chrysin was indicated to show possible binding to EV71 protease 3C in Autodock 4.0 simulations. Chrysin exhibited strong anti-EV71 activity with an EC_{50} of 10 μM in CPE inhibition assays, while its phosphate ester (CPI) showed a more potent effect with a lower EC_{50} of 6 μM. Luteolin (**42**) can be found in many plants, such as *Lonicera japonica* (Jin Yin Hua) and *Perilla frutescens* (L.) Britt (Bai Su). This flavonoid exhibited various pharmacological activities, including inhibition of EV71 and CVA16 with EC_{50} values of approximately 10 μM [168]. This reported that luteolin targeted the post-attachment stage of EV71 and CVA16 infection by inhibiting viral RNA replication reported that it might act on viral polyprotein expression after viral entry of EV71, and prevent EV71-induced cell apoptosis, intracellular ROS generation, and cytokine upregulation. Formononetin can be extracted from many herbs and plants, such as leguminous plants. It exhibited various bioactivities including anti-inflammatory, antioxidative, and anticancer effects [169]. In a large-scale screening, formononetin demonstrated significant anti-EV71 activity [169**].** Specifically, it inhibited the EV71-induced CPE with an EC_{50} of 3.98 μM, reduced virus RNA replication and protein expression in a dose-dependent manner, and exerted antiviral activity by application before and after EV71 infection. The mechanism of the formononetin activity involved the suppression of ERK, p38 MAPK, and JNK activation as well as the suppression of EV71-induced COX-2/PGE2 expression.

Prenylated flavonoids, 6,8-diprenylaromadendrin (**43**) and 6,8-diprenylkaempferol (**44**) isolated from the extract of *Monotes africanus* exhibited HIV-inhibitory activity in the XTT-based, whole-cell screen [170]. Quercetin 3-O-(2¢-galloyl) a-L-arbinopyranose (**45**) and flavonoid gallate ester isolated from ethanolic extract of *Acer okamotoanum* (family Aceraceae), possessed anti-HIV-1 integrase activity with IC_{50} values of 18.1 ± 1.3 and 24.2 ± 6.6 mg/ml respectively [171]. Biflavonoids, robustaflavone (**46**) and hinokiflavone (**47**) isolated from methanolic extracts of twigs and leaves of *Rhus succedanea* (family Anacardiaceae), showed strong inhibition of the polymerase of HIV-1 RTase in in vitro assay [172]. Another biflavonoid, wikstrol B (**48**) obtained from extracts of roots of *Wikstroemia indica* (family Thymelaeaceae), showed good activity against HIV-1 in in vitro studies [173]. A biflavonoids, ginkgetin isolated from *Ginkgo biloba* L. and tetrahydroxyflavone isolated from Scutellarias how a potent inhibitory activity against

influenza virus sialidase [174]. A combination of NA inhibitors and protease inhibitors could be potentially used as a potent anti-influenza therapy in order to minimize the emergence of drug-resistant mutant viruses. The flavonoid chrysosplenol C is one of a group of compounds known to be a potent and specific inhibitor of picornaviruses and rhinoviruses, the most frequent causative agents of the common cold [175]. The *Dianella longifolia* and *Pterocaulon sphacelatum* were found to contain flavonoid chrysosplenol C and anthraquinone chrysophanic acid, respectively, which inhibit the replication of poliovirus types 2 and 3 (Picornaviridae) in vitro [175]. Recently, new flavonol glycoside the iridoid glycosides and three phenylpropanoid glycosides, named luteoside A, luteoside B and luteoside C were isolated from *Barleria prionitis* and from the roots of the medicinal plant *Markhamia lutea*, respectively, and shown to have potent in vitro activity against RSV [176]. In another study, five groups of biflavonoids (amentoflavone, agathisflavone, robustaflavone, rhusflavanone and succedanea flavanone) were isolated from medicinal plants of *Rhus succedanea* and *Garcinia multiflora*, and exhibited various antiviral effects against a number of viruses including respiratory viruses (influenza A, influenza B, parainfluenza type 3, RSV, adenovirus type 5 and measles) and herpes viruses (HSV-1, HSV- 2, HCMV and varicella zoster virus, VZV) [188]. Amentoflavone and robustaflavone demonstrated significant activity against anti-HSV-1 and anti-HSV-2 with only moderate anti-HSV-2 from rhusflavanone. A significant anti-influenza A and B activity was achieved by amentoflavone, robustaflavone and agathisflavone. By comparison, rhusflavanone and succedaneflavanone were found to produce a selective anti-influenza type B only [178]. Baicalein (BA), a flavonoid compound purified from the medicinal plant *Scutellaria baicalensis* Georgi, has been shown to possess anti-inflammatory and anti-HIV-1 activities. BA may interfere with the interaction of HIV-1 envelope proteins with chemokine coreceptors and block HIV-1 entry of target CD4 cells and BA could be used as a basis for developing novel anti-HIV-1 agent [178].

2.5.2 Polyphenols

Polyphenols and bioflavanoids [179] are the major classes with antiviral activity against various diverse virus families such as retroviridae, hepadnaviridae, hespervirides, HIV (Human immune deficiency virus) virus, influenza virus, herpes simplex virus, dengue virus, polio virus, diarrhea virus etc. Bioflavanoids are one of the major class of polyphenols has important antiviral activity such as quercetin (**49**), kaempferol (**50**), rutin (**51**). It has been found that flavonols are more active than flavones against herpes simplex virus type-1 and HIV virus. Synergism has also been reported

between flavonoids and other antiviral agents. Quercetin, for example, potentiates the effects of 5-ethyl-2'-dioxyuridine and acyclovir against HSV and pseudorabies infection. Apigenin also enhances the antiviral activity of acyclovir against these viruses [180]. The various polyphenols which have antiviral activity are formononetin (**52**), eupafolin (**53**), epigallocatechin gallate (**54**), gallocatechin gallate (**55**), rutin, quercetin, kaempferol, apigenin, chrysin and various gallates of phenolic acid [180]**.**

In general, polyphenols act by associating with proteins of viral particles and/or host cell surfaces, resulting in reduction or prevention of viral adsorption. Several hydrolysable tannins such as chebulagic acid, punicalin and punicalagin from the fruits of *Terminalia chebula* (He Zi), showed anti-HIV, anti-diarrheal, anti-bacterial, anti-hyperglycemic, and broad-spectrum antiviral activities [181]. Chebulagic acid showed anti-EV71 activity in vitro with an EC50 of 13.1 μM, and reduced the mortality and relieved the symptoms of EV71-infected mice by inhibiting viral replication in vivo [181]. Geraniin derived from *Geranium thunbergii* (Lao Guan Cao) possessed anti-bacterial, anti-diarrheal, antioxidative, and anti-hypertensive effects, and induced cell death. Geraniin reduced the EV71-induced CPE in vitro with an EC50 of 10.5 μM and improved the survival rate and clinical score of EV71-infected mice [182]. Glabranine (58) showed anti-dengue activity [183].

2.5.3. Steroids

From *Anemarrhena asphodeloides*, by applying an isolation method called folding fan mode counter-current chromatography and CPE reduction assays, six anti-EV71 saponins were identified among which timosaponin B-II displayed the best medicinal potential with an EC_{50} of 4.3 μM and the highest SI of 92.9 [184].

Aloe-emodin (**56**) [185] isolated from *Rheum palmatum* (Da Huang) showed antiviral activity against EV71 in HL-CZ and TE-671 cells with EC50 values of 0.5–1.9 μM**.** Aloe-emodin induced the expression of IFNs, and might be involved in the activation of the type I and II IFN signaling pathways against viral replication.

The *in-vitro* antiviral activity was carried out against poliovirus type1 by using variable domains of the heavy chain antibiotics (VHHs) or Nanobodies by Rombaut et al [186]. VHHs are smallest naturally occurring intact antigen-binding domains, abundantly present at the outer surface of polioviruses. VHHs typically exhibit a superior stability as compared with conventional antibodies,

they can be formulated as long shelf-life, ready-to-use solutions and beneficial in various other aspects not mentioned above such as bio-distribution, renal clearance, tissue penetration and target retention. This study demonstrates the proper use of VHHs as a novel class of antiviral drugs and provides, at least two neutralizing VHHs, for the development of a potentially efficacious antiviral drug for the treatment and prophylaxis of poliomyelitis induced by poliovirus type-1 at a low, economic cost per dose. The result of this study can be interpreted as these two VHHs were PVSP6A and PVSP29F, the two VHHs with the most potent antiviral activity [186].

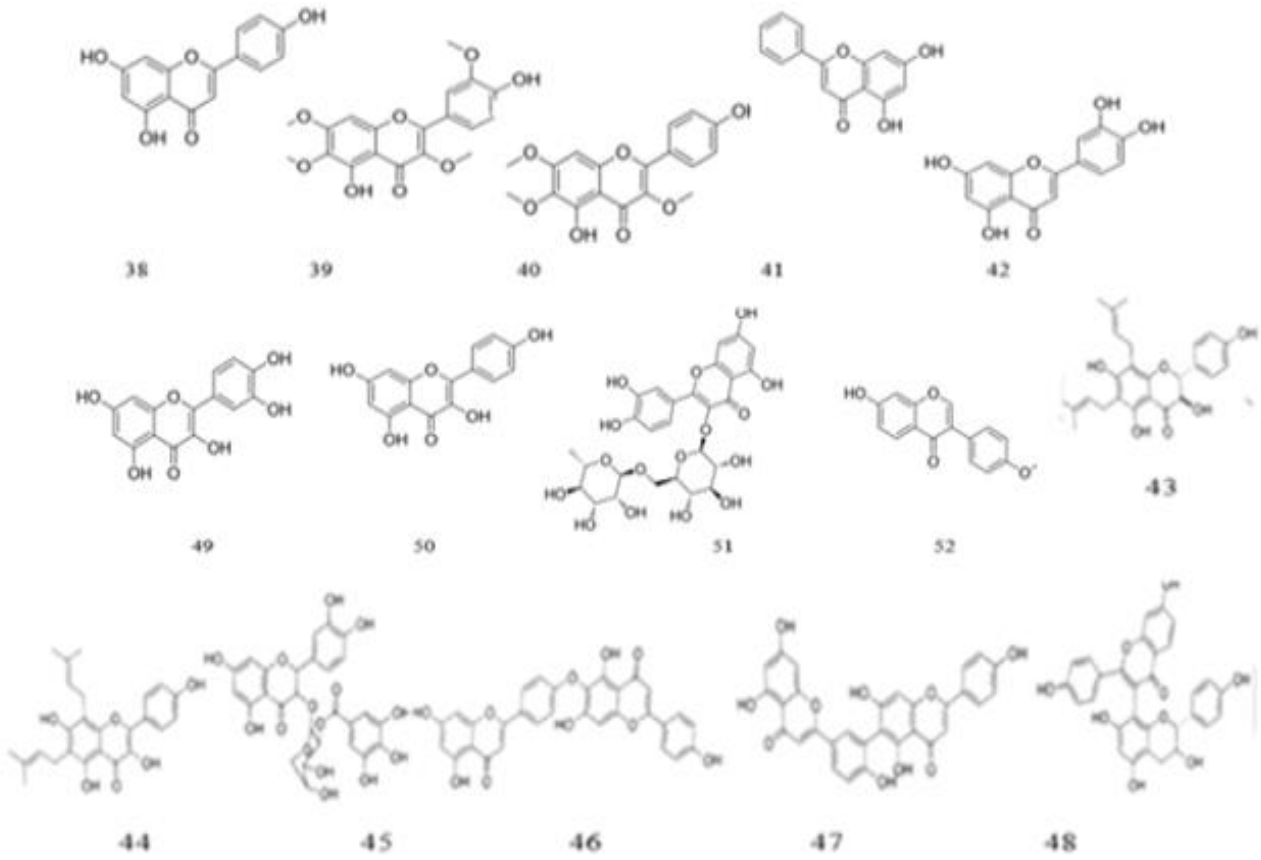


Figure 2.6: Anti-viral compounds from plant

2.6. Millettia ferruginea

2.6.1 Botanical Description

2.6.1.1 The Family Leguminosae

The Leguminosae, also called the Legume family is the third largest family of angiosperms/ flowering plants, exceeded only by Asteraceae (Compositae) and Orchidaceae families. It comprises over 19,000 species classified in more than 700 genera and widely distributed throughout the temperate and tropical regions of the world [187]**.** Plants of this family are usually

trees, shrubs, woody or perennial or annual herbs with the roots producing tinny nodules containing bacteria for nitrogen fixation. It is subdivided into three subfamilies distinguished as Mimosoideae, Caesalpinioideae and Papilionoideae. In terms of economic importance, Legumes are only second to Poaceae (grasses), and some members of this family are cultivated as crops, for oils, fiber, fuel, timber, tannins, gums, fodders, green manures and forages [187]. Copaifera (Leguminoseae) species are also known to yield valuable resins, used in varnishes, paints and lacquers and Indigofera speies are cultivated for the blue dye. More importantly, several species in the genera Lonchocarpus and Derris are known as sources of rotenone, which is used as an insecticide, fish poison or molluscicide.

2.6.1.2 The Subfamily Papilionoideae

The Papilionoideae is the largest and most widespread of the three Legume subfamilies in which more than 50% (ca. 476 genera and 13,860 species) of the species belong to this subfamily. It includes mostly trees, shrubs, herbs and sometimes lianas, often with root nodules containing nitrogen-fixing bacteria and frequently with non-protein amino-acids in the seeds. This sub-family [187] has 32 tribes and It is distinguished from the other two subfamilies by vegetative, floral and fruiting nature including floral development. Papilionoideae species are extremely important and its members yield nutritious food, fiber, shelter, valuable medicines and also potent poisons [187].

2.6.1.3 The Genus Millettia

The genus *Millettia* (family Leguminoseae, subfamily Papilionoideae) contains over 323 species that are distributed in tropical and subtropical regions of Africa (139 species), Asia and Australia [188]. It is a tree, shrub or liana, or rarely semi-herbaceous plants with a woody rootstock. The genus *Millettia* belongs to the tribe Tephrosiae that is known to synthesize prenylated flavonoids and isoflavonoids [188]**.**

2.6.1.4 The Species Ferruginea

Millettia ferruginea (Hochst.) Bak. (Fig.2.9) is locally known as *Sotalloo, Sari, Yego* (in Afan Oromo), *Brbra* (in Amharic), and *Enghedikesho* (in Sidama languge). It is a tree, endemic to Ethiopia [189]. Two subspecies are recognized, the typical subsp, *ferruginea* occurs in northern Ethiopia while subsp, *darassana* (Cuf.) Gillett is restricted to the southern province of Sidamo [189]. From the area between these two clearly defined subspecies, plants show a mixture of characters attributable to both suggesting the possibility of hybridization.

It is umbrella-shaped or flat-topped, and grows up to a height of 25 m to 35 m. It is widely distributed all over Ethiopia andis commonly grown in Tigray, Gondar, Gojam, Shewa, Welega, Hareregе, Bale, Ilubabor, Kefa and Sidamo [190]. The potential growing area ranges agro climatic zones of 1,000–2,500 m (~3,000–7,500 feet) above the sea level. That means most parts of *''Kola*'' and the whole ''*woinadega*'' of Ethiopia are potential areas for growth. Therefore, the natural habitat of *Millettia ferruginea* is rather diverse as it has advanced mechanism of minimizing water loss which enabled it to utilize water in a very conservative. It maintained higher tissue water status under mild, moderate and severe water stresses than the other species due to its ability to reorient its leaves and leaflets during midday, thus avoiding direct solar irradiance [190]. Leaves are compound with the number of leaflets. In most trees, the flour is large with mostly violent on the stalks and golden-brown in colour. The mature pod is flat and pale brown in colour. The shape of the seeds is semi round and nearly golden-brown in colour. Its leaves are up to 40 cm long; leaflets up to 27, oblong-acuminate up to 5-9 x 1-2.5 cm; pubescent present or absent on both sides or sub-glabrous above; lobes ca 1 mm; pod flat up to 27 cm and brown upon maturity; pubescent first and then glabrescent; seeds 5-10; corolla 17-30 mm, dark red to violet, standard sticky outside, disc tubular 1-10 [190]. Flowering period usually extends from the end of February to the end of March. Its fruits are normally abundant from early June to end of December. Mostly from January to February, seeds are usually released by mechanical or explosive mechanisms from the pod to the ground [190]. The seed has a potential of high oil and protein content to satisfy calorie and protein demand of the populations 191]. Oil is clearer brown yellow in colour and less viscous. In addition to its high protein content, birbira seed contains a high concentration of minerals, especially phosphorus, potassium, magnesium sodium and calcium. It has a potential to supply sufficient amount of minerals for consumers and microbial media for microorganisms [192].

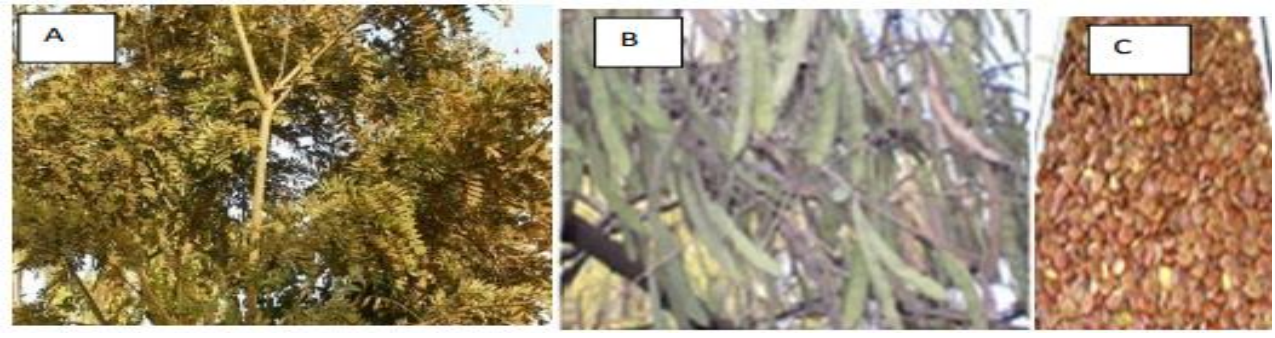


Figure 2.7: *Millettia ferruginea* (A) tree, (B) pod (C) seeds.

2.6.2 Traditional Uses of the Genus *Millettia*

The genus *Millettia* has been used in traditional medicinal practices in alleviating several aliments, with about 60% of the species are known for their medicinal uses for the treatment of different conditions. The insecticidal, piscicidal activity and fish poisoning effects of the seeds of *Millettia* species are among the old practices [188].

In Ethiopia folk medicine [193], *M. ferruginea* bark and mature fruit grounded to powder and spread over the surface of water is used as fish poisoning and bark powder as controlling insect's pests. Fruit ground to powder mixed with butter taken orally is used for the treatment of pain. Leaves sap is used for the treatment of earache and in case of bacterial infection of nails, they are bandaged with a paste of leaves. The Stems decoction is used in gargarism aganist toothaches. The root is used for treating gonorrhea [215]. Seed [194] is used as fish poisoning insecticides, controlling insect's pests and also as a remedy to treat infection caused by chigger flea. The mature seeds are used to treat skin infection (and for dressing 'mujele' an infection caused by an insect present in the soil [194]. The seed extract of this plant [195] has been observed to be effective in controlling storage insect pests as bean beetle, *Callasobruchus chinensis*, maize weevil, *Sitophilus zeamais*, bean bruchid, *Zebrotes subfaciatus* and mosquito larvae. It is a multi-purpose tree and highly valued by farmers [196]. *Millettia ferruginea* in agroforestry systems such as for erosion control, home gardens as shade/cover crop for coffee and enset or false banana (*Ensete ventricosum*) particularly in South and South-West Ethiopia. The leaves of *M. ferruginea* can be used as fodder for ruminants. In addition, its wood can be used as firewood, as local building material, to make tool handles and household utensils, for fencing and keeping traditional bee hives. Its flowers have good agricultural value as bee forage [197]. *M. ferruginea* [197] fixes nitrogen that acts as nutrient pump by adding its leaves and flowers to the soilto fixenitrogen, thus successfully surviving in N-defficient soils and improving soil fertility.

In Chinese folk medicine, for example, *M. nitida var. hirsutissima* and *M. speciosa* are used to treat menstrual conditions (pain and irregularity), rheumatic pain, aching pain, and paralysis [198]. It was also reported [199] that *M. nitida* has been used for the treatments of blood stasis condition such as anemia, inflammation of peripheral blood vessels and thrombotic changes in vessels through promotion of the blood circulation. The roots and leaves of *Millettia leptobotryaDunn*

have been used for the treatment of fracture, traumatic injury and rheumatoid arthritis by Chinese [200].

The juice of the leaves of *M. thonningii* [201] is also reported to be a poison to Bulinus snail, vector for schistosomiasis. The root of *M usaramensis, M. dura*and *M. oblate* [188] is reported to be used as antidote against snake bite, stomachaches, as a remedy for cough, swollen part of the body, bladder problems, treatment of hernias, diarrheas, menstruation complications and for healing wounds.*M. pachycarpa* [44] is widely used as an antihelminthic, a medication capable of expelling parasitic intestinal worms. There is also evidence that a concoction of the root and stem bark of *M. griffoniana* [202] is employed in Cameroon as an oral treatment for boils (also called furuncle), insects bits, inflammatory conditions (such as pneumonia and asthma), amenorrhea (an abnormal absence of menstruation), sterility and menopausal disorders. *M. ovalifolia* [203] is traditionally used as antifungal, insecticidal treatment of pain, fever, rheumatism, diabetes, and a number of other infectious diseases. There is also a report regarding the medicinal properties of *M. aboensis* [204] that its leaf is used for ulcer healing and as laxatives, whereas the roots are used to treatgastro-intestinal disturbances and liver diseases**.** The root of *M. pulchra* [205] is used for postpartum women and people with certain health conditions, apparently to stock up blood to treat postpartum frail and malnutrition whereas its aerial parts is used to eliminate inflammation, alleviate pain, increase blood circulation, and relax and activate tendons in rheumatic arthralgia [205].

2.6.3 Compounds from the Genus *Millettia*

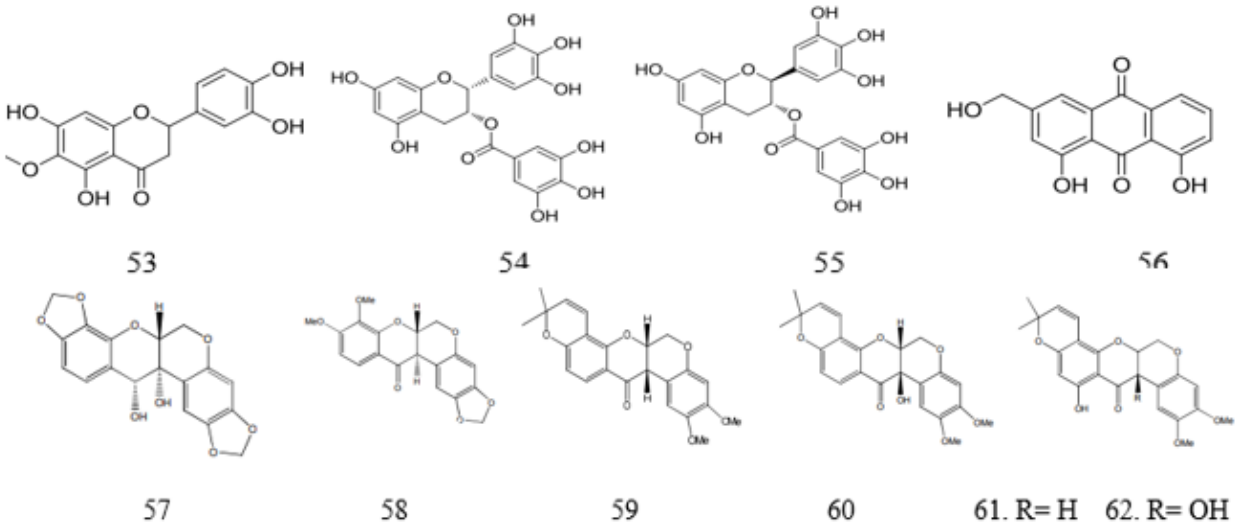
2.6.3.1 Rotenoids

Rotenoids mainly occur in the seeds of *Millettia* as opposed to isoflavones that occur in all plant parts. The rotenoids of *Millettia* species are characterized by regular methoxylation/methylene dioxy formation at the 2,3-positions in ring A. For example, the rotenoids isolated from *M. usaramensis ssp. usaramensis* having methylenedioxy groups at C-2/3 of ring A. A prenyl group at C-8 is also commonly observed either in a cyclized form into five- /six-membered ring to form ring E or as open chain. Most rotenoids previously characterized from this genus have a Cis-B/C ring junction. Nevertheless, rotenoids from the stem bark of *M. usaramensis* have a novel trans-B/C ring junction with a 6aR, 12aS configuration [206]. Table 2.1 and Figure 2.9 lists some rotenoids reported from the genus *Millettia.*

Table 2.1: Some examples of rotenoids from *Millettia* species

No	Compound	Species	Reference
57	(+)-12-Dihydrousararotenoid A	Usaramensis ssp.usaramensis	206
58	(+)-Usararotenoid B	Usaramensis ssp. saramensis	206
59	Deguelin	M. ferrgunea(SD)	207
60	Tephrosin	M. ferrgunea(SD)	207
61	α-Toxicarol (202	brandisiana (LF)	209
62	12a-hydroxyelliptone	M. duchesnei (AP)	208
63	Rotenone	M. ferrgunea(SD)	207
64	6-Methoxy-6a, 12a-dehydrodeguelin	M. duchesnei (AP)	208
65	6-Hydroxy-6a, 12a-dehydrodeguelin	M. duchesnei (AP)	208
66	6-Oxo-6a,12a-dehydrodeguelin	M. duchesnei (AP)	208
67	6a, 12a-Dehydrodeguelin	M. duchesnei (AP)	208
68	12-Deoxo-12a-methoxylelliptone	M. duchesnei (AP)	208
69	Millrtone	*M.dura* (SD)	209
70	6a,12a-Dehydro-α-toxicarol	*M.brandisiana* (LF)	209
71	6-Hydroxy-6a,12a-dehydro-α-toxicarol	*M. brandisiana* (LF)	209
72	Usararotenoid C	*M. brandisiana* (LF)	209

SD = Seed, AP =Aerial part, LF = Leaf



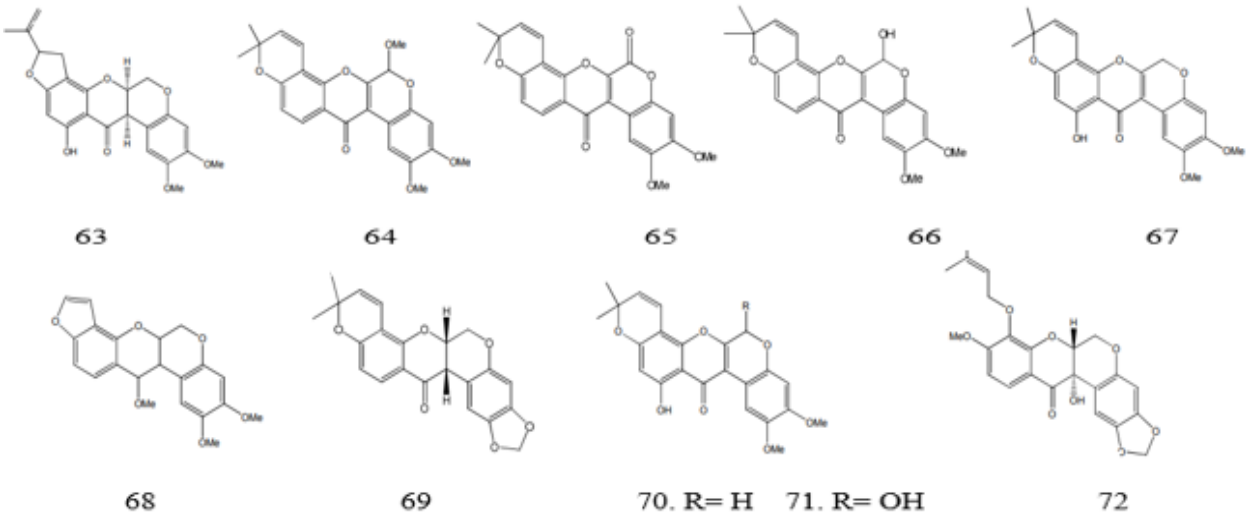


Figure 2.8: Some of rotenoids isolated from Millettia species

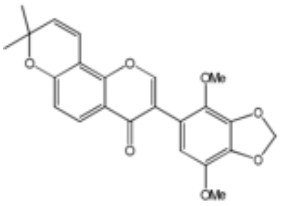
2.6.3.2. Isoflavonoids

The genus *Millettia* is known to be a rich source of isoflavonoids. The majority of these isoflavonoids contain one or two prenyl group(s) as a side chain, either in acyclic or in cyclic forms, or as a combination of the two forms [332]. Structural diversity occurs as the result of different oxidation levels of the isoflavonoid core skeleton, and also from the number and complexity of substituents attached. It has also been noted that additional hydroxylation, geranylation, methoxylation, and methylenedioxy formation are common structural features of the isoflavonoids of this taxa, which has eventually enhanced the structural diversification. Among the flavonoids, the isoflavones are the most common in this genus. Table 2.2 and Fig. 2.10 lists some example of isoflavonoids isolated from *Milleltia* species.

Table 2.2: Some example of Isoflavonoids isolated from *Milleltia* species

	Compound	Species	Ref.
73	Ferrugone	*M. ferruginea .sub. darasana* (SB)	210
74	Jamaicin	*M. ferruginea .sub. darasana* (SB)	210
75	Ichthynone	*M.ferruginea .sub. darasana* ar(SB)	210
76	Calopogonium isoflavone B	*M. ferruginea .sub. darasana* (SB)	210
77	Isojamaicin	*M. ferruginea .sub. darasana* (SB)	210
78	Nordurlettone	*M. ferruginea .sub. darasana* (SB)	210
79	Prebarbigerone	*M. ferruginea .sub. darasana* (SB)	210
80	Predurmillon	*M. ferruginea .sub. darasana* (SB)	210
81	Preferrugone	*M. ferruginea .sub. darasana* (SB)	210
81A	Pre-5-methoxydurmillone	*M. ferruginea .sub. darasana* (SB)	210
82	Calopogonium isoflavone A	*M. ferruginea .sub. darasana* (SB)	211
83	Barbigerone	*M. ferruginea .sub. darasana* (SB)	211
83A	2'-Methoxylupalbigenin	*M. pulchra* (AP)	213
84	4'-O-methylderrone	Millettia pachyloba (SD)	212
85	Pomiferin	Millettia pachyloba(SD)	212
86	6,8-Diprenylorobol	*M. pachyloba*(AP)	212
87	6,8-Diprenylgenistein	*M. pachyloba* (AP)	212
88	6,8-Diprenylpratensin	*M. pachyloba* (SD)	212
89	2'-Hydroxylupalbigenin	*M. pulchra* (AP)	213
90	Durlmillone	*M. ferruginea .sub. darasana* (SB)	210
91	Conrauinone A	*M conraui* (SB)	214
92	Conrauinone B	*M conraui* (SB)	214
93	Conrauinone C	*M conraui* (SB)	214
94	5-Methoxyldurmillone	*M conraui* (SB)	214
95	Afrormosin	*M. reticulata* (SB)	215
96	Biochanin	*M. nitida* (VS)	215
97	Calycosin	*M. dielsiana* (SB)	215
98	Daidzein	*M. dielsiana* (SB)	215
99	Genistein	*M. dielsiana* (SB)	215
100	Millewanin E	*M. taiwaniana (S)*	216
101	Warangalone	*M. taiwaniana* (S)	216

Key S=Seed, SP= Seed Pod, SB=Stem Bark, S= Stem, R= Root, RB=Root Bark RW=Root wood



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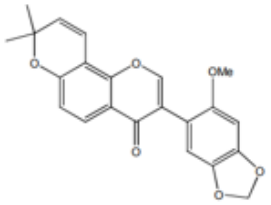

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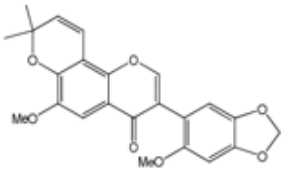

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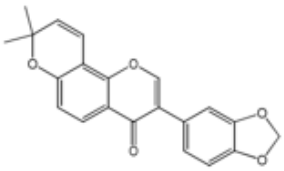

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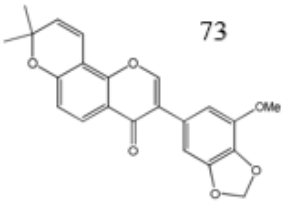

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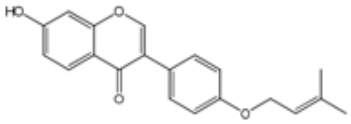

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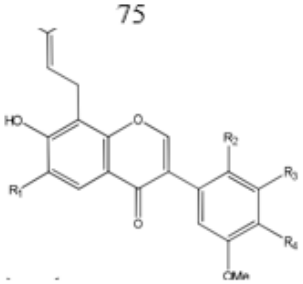
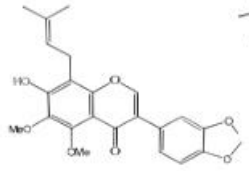
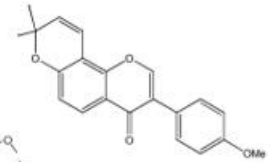


	R1	R2	R3	R4
79	H	OCH_3	H	OCH_3
80	OCH_3	H	--OCH_2O--	
81	H	OCH_3	--OCH_2O--	



81A



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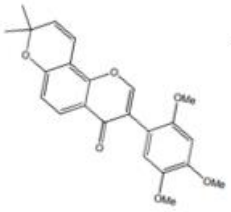

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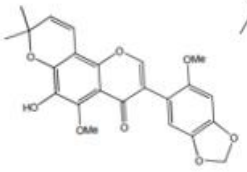

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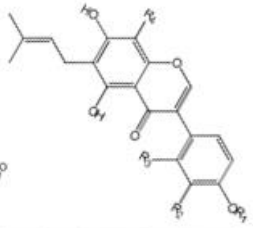
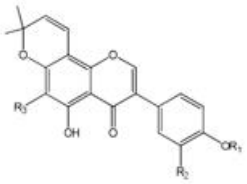
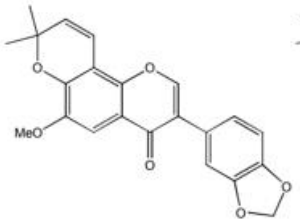


	R_1	R_2	R_3	R_4
86	H	OH	H	prenyl
87	H	H	H	prenyl
88	CH_3	OH	H	prenyl
89	H	prenyl	OH	H



	R1	R2	R3
84	CH_3	H	H
85	H	H	prenyl



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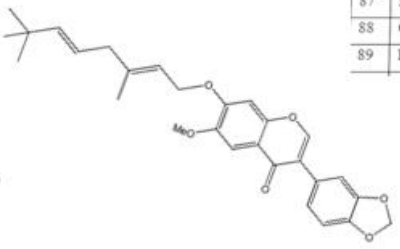

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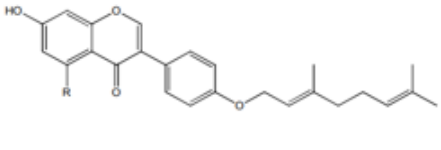
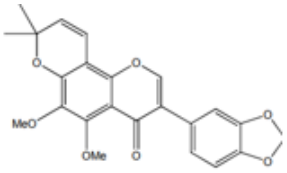


92. R= H 93. R= OH



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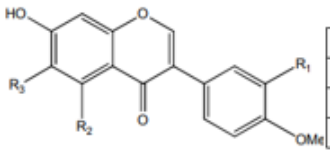
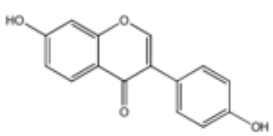


	R_1	R_2	R_3
95	H	H	OCH_3
96	H	OH	H
97	OH	H	H



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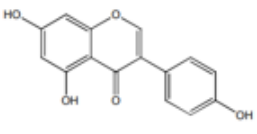

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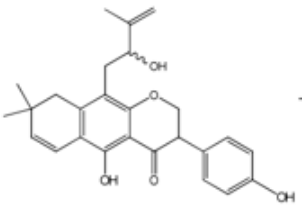

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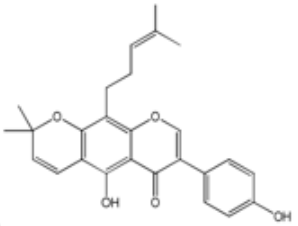


101

Figure 2.9: Some isoflavonoids compounds isolated from *Millettia* species

2.6.3.3 Flavones

The genus is rich in flavones. Some examples of flavones isolated from *Milleltia* species are listed in Table 2.3 and Fig.2.11.

Table 2.3: Some examples of flavones and anthocyanins isolated from *Milleltia* species

.	Compound	Species	Ref.
102	Quercitrin	*M. Zechiana* (AP)	217
103	Isoquercitrin	*M. Zechiana* (AP)	217
104	7-O-β-D-glucoside-8-hydroxyquercetin	*M. Zechiana* (AP)	217
105	3-Methyletherquercetin	*M. Zechiana* (AP)	217
106	Millettocalyxin C	*M. erythrocalyx*(SB)	218
107	Miilletttocalyxins A	*M. erythrocalyx* (SB)	218
108	Millettocalyxin B	*M. erythrocalyx* (SB)	218
109	Pongol methyl ether	*M. erythrocalyx* (SB)	218
110	Ovalifolin	*M. erythrocalyx* (SB)	218
111	3,6-Dimethoxyfuranol[7,8:2'',3''] flavones	*M. ichthyochtona* (LF)	218
112	Laurentinol	*M sanagana* (RB),	219
113	Kanjone	*M sanagana* (RB)	219
114	Pongamol	*M. penguensis* (LF)	219
115	Karanjin	*M. ovalifolia* (SD)	220
116	Lanceolatin	*M. ovalifolia* (SD)	221

Key: SP=seed pod, AP= Ariel Part, FL= Flower, LF= Leaf, VS= Vine stem, SB=Steem bark, RB=root bark, SD=Seed

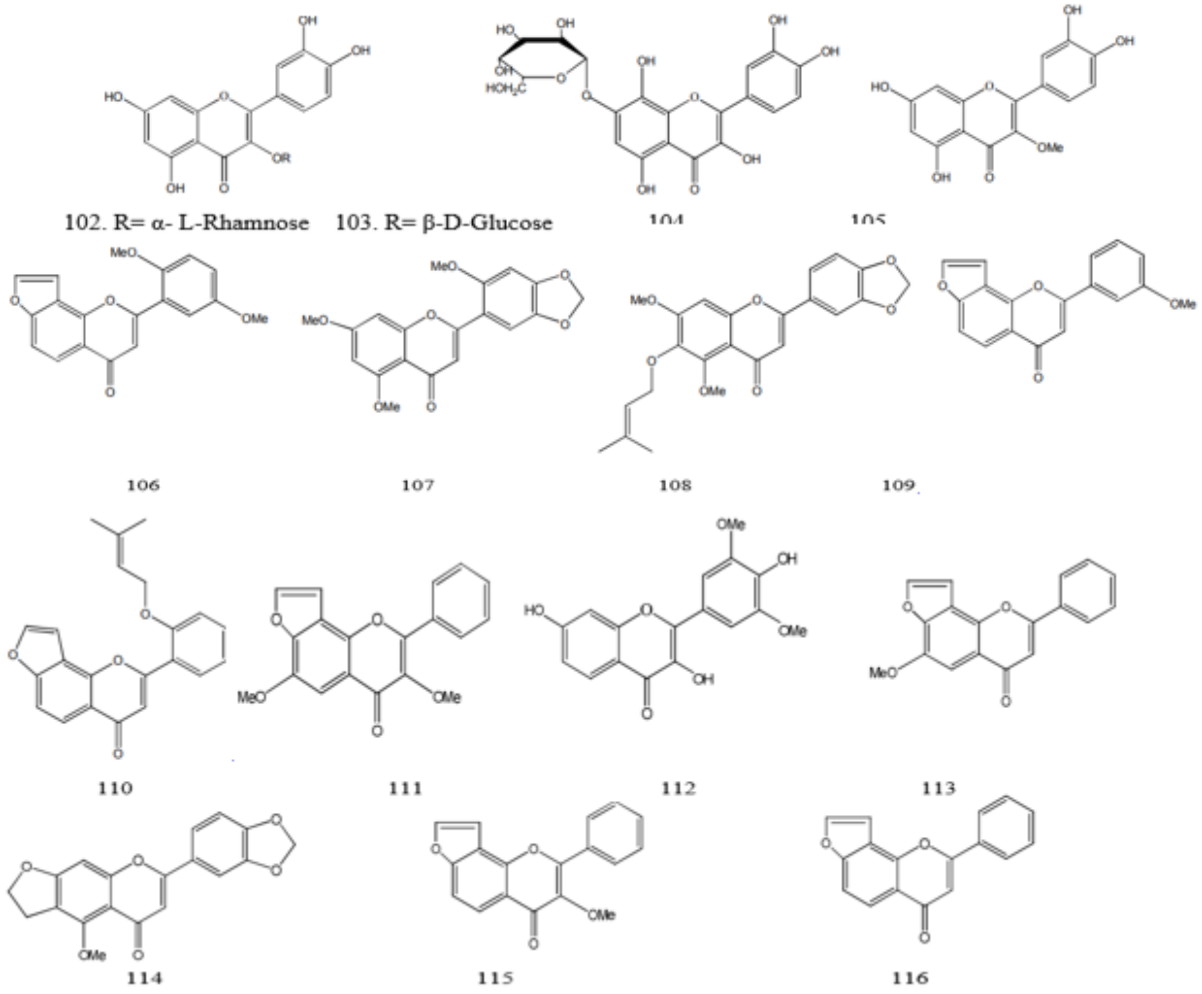


Figure 2.10: Some flavones and anthocyanins isolated from Milleltia species

2.6.3.4 Chalcones

A number of chalcones and dihydrochalcones have been identified from different *Millettia* specie s. Several chalcones of this genus are prenylated and most chalcones isolated from *M. ovalifolia* have a methylenedioxy group incorporated in their structures [306]. Chalcones without oxygenation of ring A are also common to this genus. Table 2.4 and Figure 2.12 lists some chalcones of *Millettia* species.

Table 2. 4: Some examples of chalconoids isolated from Millettia species

	Compound	Species	Ref.
117	4-hydroxylonchocarpin	M. ferruginea sub: daesana (RB)	210
118	4-O-Geranylisoliquiritegenin	M. ferruginea (RB)	210
119	4-Methoxylonchocarpin	*M pachycarpa (seeds)*	44
120	Dihydromilletenone,methyl ether	*M. hemsleyana* (SB)	247
121	Dihydroisomilletenone,methylether	*M. hemsleyana* (SB)	220
122	Ovalichalcone	*M. ovalifolia* (SD)	220
123	Ovalichalcone A	*M. ovalifolia* (SD)	220
124	Ovalitenin A	*M. ovalifolia* (SD)	221
125	Ovalitenin B	*M. ovalifolia* (SD)	221
126	Ovalitenin C	*M. ovalifolia* (SD)	221
127	Pongamol	*M. ovalifolia* (RB)	221
128	4'-Geranyloxy-α,4,2'- trihydroxydihydrochalcone	*M. usaramensis* (SB)	221

Key: SP=seed pod, AP= Ariel Part, FL= Flower, LF= Leaf, VS= Vine stem, SB=Steem bark, RB =root bark, SD=Seed·

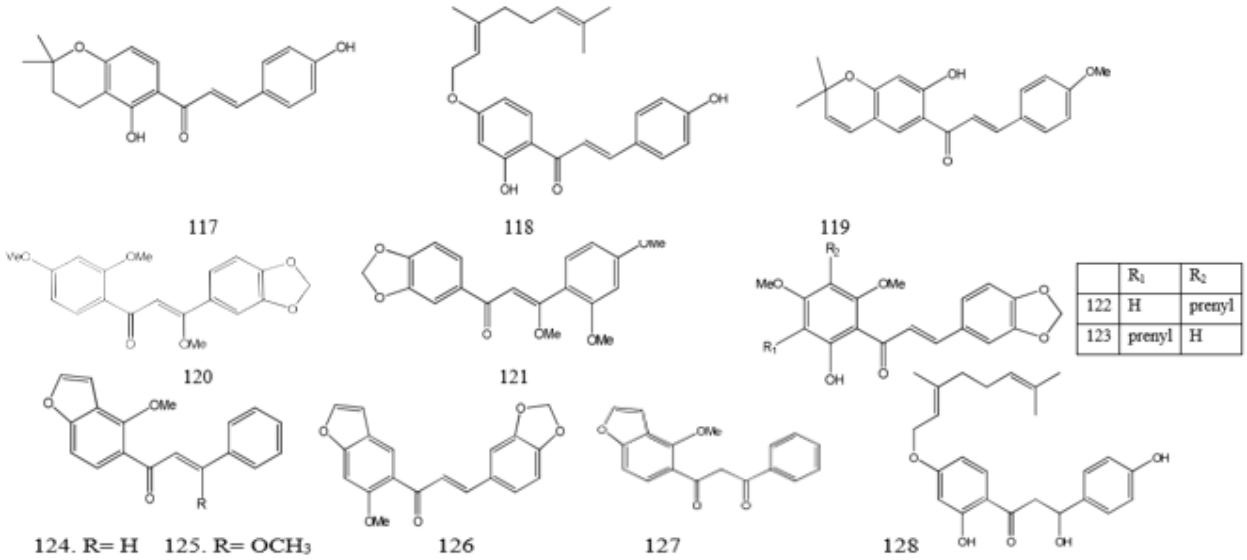


Figure 2.12: Some chalcones of *Millettia* species

2.6.3.5 Flavanones

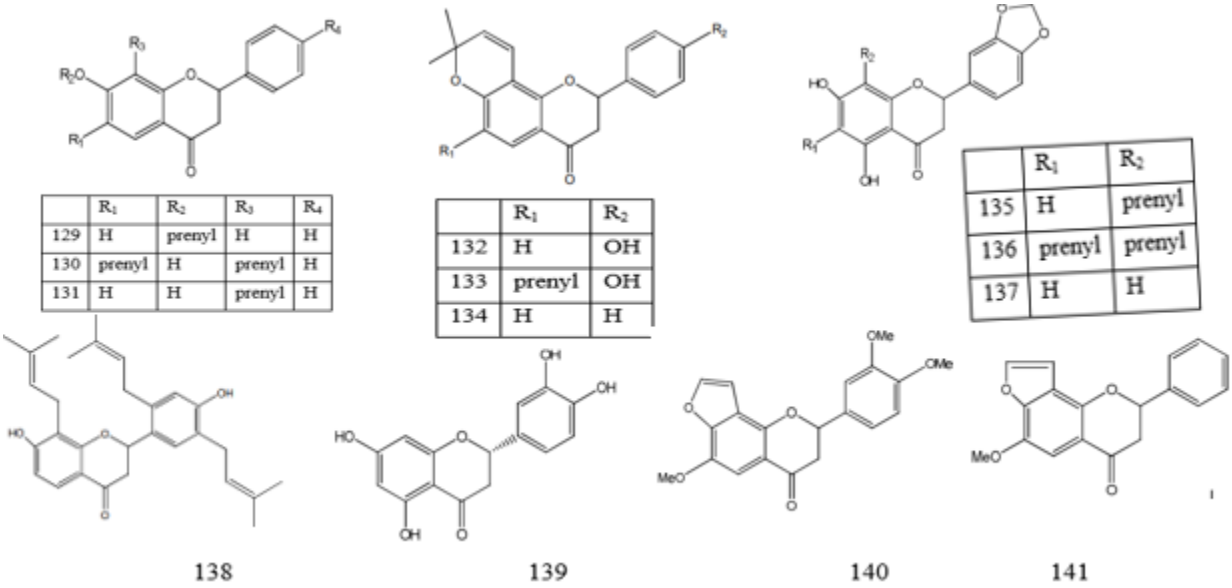
All the flavanones so far characterized from the genus *Millettia* are prenylated and lack oxygenat ion at C-5 position. This is considered to be typical of the flavanonoids of the family *Fabaceae* [254]. The structural variation existes based on the level of oxidation of hetrocyclic Ring-C of

flavonoids. Forexample, the oxidation of Ring-C of isoflavones at C-3 results hydroxyl isoflavan ones and complete loss of oxygenation at C-4 results in the formation of isoflavans (or flavans). There are a number of such compounds identified in the genus *Millettia* and some of them are listed in Table 2.5 and Fig.2.13.

Table 2.4: Some examples of flavanones isolated from *Millettia* species

	Compound	Pecies	Ref.
129	7-Prenyloxyflavanone	*M. erythrocalyx* (RB)	220
130	Ovaliflavanone A	*M. ovalifolia* (SD)	220
131	Ovaliflavanone B	*M. ovalifolia* (SD)	220
132	4'-Hydroxyisolonchocarpin	*M. ferrugineae* (SB)	220
133	ovalichromene	*M. ovalifolia* (SD)	220
134	Isolonchocarpin	*M. ovalifolia* (SD)	220
135	Ovaliflavanone C	*M. ovalifolia* (SD)	222
136	Ovaliflavanone D	*M. ovalifolia* (SD)	222
137	7-Hydroxy-3',4'- methylenedioxyflavanone	*M. ovalifolia* (SD)	222
138	Sophoranone	*M. pulchra* (AP)	213
139	Eriodictyol	*M. duchesnei* (AP)	213
140	(−)-(2S)-6,3′,4′-trimethoxy-[2″,3″7,8]-furanoflavanonne	*M. erythrocalyx* (SD	220
141	6-Methoxy- [7,8:2'',3'']furanoflavanone	M. erythrocalyx (RB)	220

AP = Ariel part, SD= Seeds, LF= Leaf RB = Root Bark SB = Stem Bark



	R_1	R_2	R_3	R_4
129	H	prenyl	H	H
130	prenyl	H	prenyl	H
131	H	H	prenyl	H

	R_1	R_2
132	H	OH
133	prenyl	OH
134	H	H

	R_1	R_2
135	H	prenyl
136	prenyl	prenyl
137	H	H

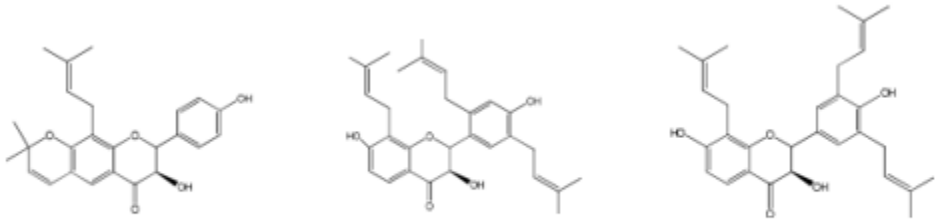
Figure 2.113: Some flavanones of *Millettia* species

2.6.3.6 Flavanonols

Flavonols are reported to be the most ubiquitous flavonoid sub-group present in foods. They do not exist in glycosylated form as the other flavonoids. They can be found in both monomer form (catechins) and polymer form (procyanidins). A number of flavanonols have been identified from different *millettia* species. Table 2.6 and Fig. 2.14 list some of the isolated flavanols from *millettia* species.

Table 2.5: Some examples of flavanols isolated from *Millettia* species

.no	Compound	Species	Ref.
142	Lupinifolol	*M. pachycarpa* (SB)	213
143	7,4'-Dihydroxy-8,2',5'-triprenyldihydroflavanol	*M. pulchra* (SB)	213
144	7,4'-Dihydrox 8, 3', 5'- triprenyldihydroflavanol	*M. pulchra* (SB)	213



142 143 144

Figure 2.12: Some flavanonols of Millettia Species

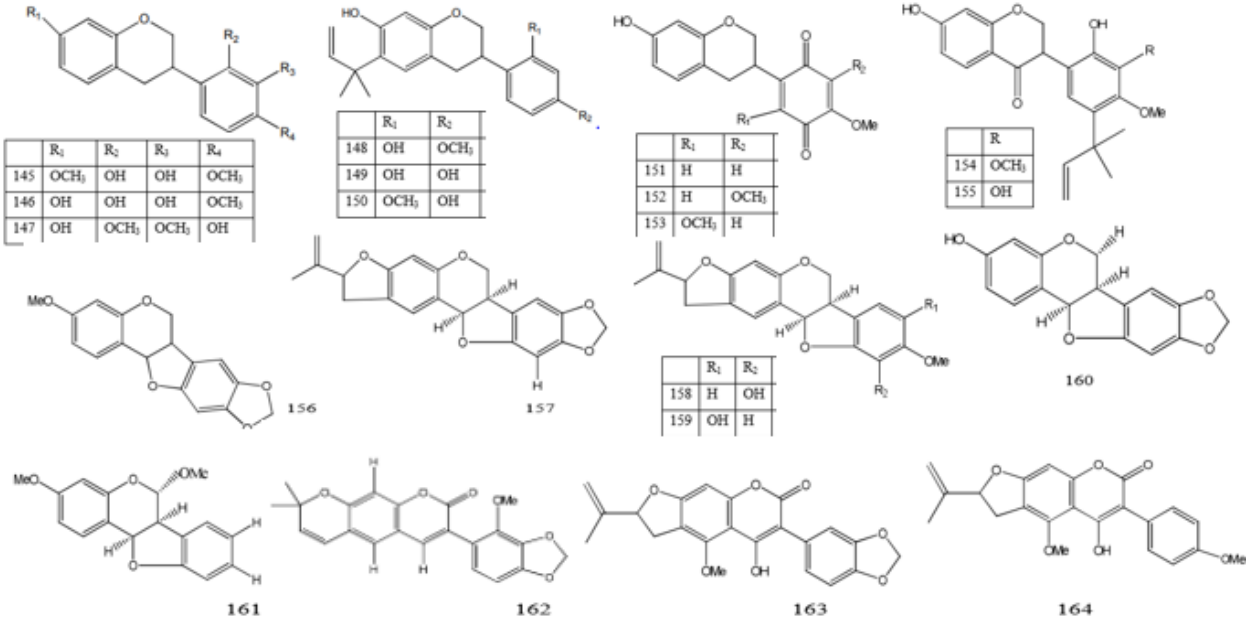
2.6.3.7 Minor Compounds

Phytochemical investigation of some of the *Millettia* species has led to the isolation of some isoflavanones, isoflavans, flavan, pterocarpanoids, 3-phenylcoumarins, alkaloids coumarins as well as triterpenoids. Table 2.7 and Fig. 2.15 list some of the minor compounds from the *Millettia* species

Table 2.6: Some examples of minor compounds from *Millettia* species

S..No	Compound	Species	Ref.	S.No	Compound	Speciess	Ref.
	Isoflavans				**Alkaloids**		
145	Isosativan	*M. dielsiana* (SB)	223	160	Maackiain	*M. pulchra* (AP)	213
146	Vesttitol	*M. dielsiana* (SB)	223	161	6-Methoxyhomopterocarpin	*M. pulchra* (AP)	213
147	Laxifloran	*M. racemosa* (HW)	224		**3-Phenylcoumarins**		
148	Isomillinol B	*M. racemosa* (HW)	224	162	Pervilleanine	*M.pervilleana* (RB)	45
149	Millinol	*M. racemosa* (HW)	224	163	Thonningine A	*M.thonningii* (RW)	227
150	Millinol B	*M. racemosa* (HW)	224	164	Thonningine B	*M. thonningii* (RW)	227
Isoflavan-quinone				165	Robustic acid	*M.thonningii* (RW)	263
151	Claussequinone	*M. pendula* (HW)	225		**Alkaloids**		
152	Pendulone	*M. pendula* (HW)	225	166	Millaurine	*M. laurentii* (LF)	207
153	Laurentiquinone	*M. laurentii* (HW)	225	167	O-acetylmillaurine	*M. laurentii* (LF)	207
Isoflavanones				168	β-Sitosterol	*M. brandisa* (LF)	208
154	Pervilleanone	*M.pervilleana* (RB)	226	169	Stigmasterol	*M. versicolor* (LF)	228
155	3'-O-Demethyl pervilleanone	*M. pervilleana* (RB)	226	170	24-methylenecycloartan-3β-ol	*M.versicolor* (LF)	228
Pterocarpanoids					**Triterpenes**		
156	Flemichapparin B	*M. ferruginea* (SB)	210	171	Lupeol	*M. versicolor* (LF)	228
157	Emoroidocarpan	*M.pervilleana* (RB)	45	172	Taraxasterol	*M. versicolor* (LF)	228
158	Pervilline	*M. pervilleana* (RB)	45	173	β-Amyrin	*M. versicolor* (LF)	228
159	Pervillinine (252)	*M. pervilleana* (RB)	45				

AP =LF Ariel Part; HW= Heart Wood Leaf; RB= Root Bark; RW= Root Wood; SB= Stem Bark; SW= Stem Wood



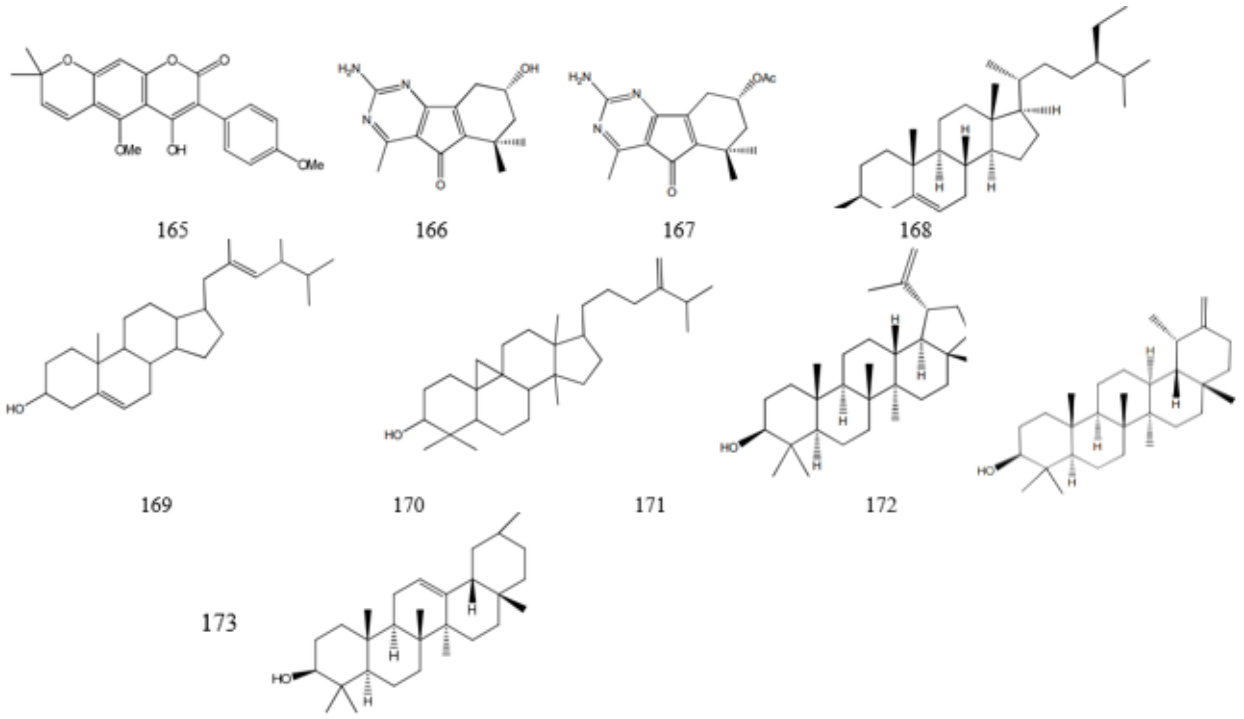


Figure 2.135: Some minor compounds of Millettia species

2.6.4 Biological Activities of Compounds from Millettia Species

2.6.4.1 Biologically Active Constituents of Millettia Species

The *Millettia* Species provides four basic types of active constituents [229]**.**

2.6.4.1.1 Flavonoids

Flavonoids are the dominant group of active constituents for *millettia* species. The species rich in flavonoids of six sub-groups namely flavanones, flavones, flavonols, flavan-3-ols, anthocyan ins and isoflavones. In plants, flavonoids contribute to: insect attraction and repulsion through colour of leaves, fruits and flowers; protection against viral, fungal and bacterial infections and UV light; nodulation in legume roots [230]. Flavonoids are effective antioxidants in plants as well as in animals. Flavonoids are identified as potential risk reducing components in the diet such as for cardiovascular disease, various cancers, and neurodegenerative diseases [230]**.** For example, quercetin-3-O-glucoside, a flavonoid compound ubiquitous in *millettia* species, has shown protective effect on human neuroblastoma cells (SH-SY5Y) against oxidative stress by a membrane injury recovery mechanism that is involved in up-regulation of genes involved in lipid and cholesterol synthesis [231]. Flavonoids are angiotensin converting enzyme (ACE) inhibitors in regulating blood pressure such as flavonols, flavones, isoflavones and flavonoids [232]. They

are shown to have hypolipidemic and anticancer activity. Flavonoids are also necessary for activity of cardiac glycosides [233].

2.6.4.1.2 Alkaloids

Alkaloids have pharmacological applications as anesthetics and CNS stimulants [230] such as oxymatrine and matrine found in root of most *millettia* species, which also have the effect of protecting bone marrow functions.

2.6.4.1.3 Saponins

Saponins including triterpenes, that are reputed to promote blood circulation, improve oxygen utilization, and protect against adverse influences (adaptogenic effect). Saponins are also important therapeutically as they are shown to have hypolipidemic and anticancer activity. Saponins are also necessary for activity of cardiac glycosides [233].

2.6.4.1.4 Polysaccharides

Polysaccharides that are used to counteract the bone marrow suppression induced by chemother apy and to enhance macrophage function [2297].

2.6.4.2 Anticancer Activities of Compounds from Millettia Species

In the search for cancer chemopreventive agents, in human brest tumor T47D cells, alpinum isoflavone and 4'-O-methylalpinumisoflavone from seeds and the root-bark of *M. thonningiia* inhibited hypoxia-induced HIF-1 activation with IC_{50} values of 5 and 0.6 μM, respectively [272]. This study revealed, at the concentrations that inhibited HIF-activation, 4'-O-methylal pinumisoflavone inhibited hypoxic induction of HIF-1 target genes (CDKN1A, GLUT¬1, and VEGF), tumor angiogenesis *in vitro*, cell migration, and chemotaxis 4'□O-methylalpinumiso flavone inhibits HIF-1 activation by blocking the induction of nuclear HIF-1α-protein, the oxygen-regulated subunit that controls HIF-1, activity. Mechanistic studies indicate unlike roteno ne and other mitochondrial inhibitors, 4'-O-methylalpinum isoflavone represents the first small molecule that inhibits HIF-1 activation by simultaneously suppressing mitochondrial respiration and disrupting protein translation invitro. This unique mechanism distinguishes 4'-O-methyl alpinumisoflavone from other small molecule HIF-1 inhibitors that are simple mitochondrial inhibitors or flavanoid-based [protein kinase inhibitors [234]. Isoflavonoids isolated from the leaves of *M. pachycarpa (M. taiwaniana*) were tested for the inhibitory potential to the human leukemia HL-60 cells, furowanin-A, warangalone, Isoerysenegalensein-E, and euchrenone b10 showed the most significant cytotoxicity. Furowanin-A, warangalone, and isoery senegalensein-

E, were found to induce apoptosis in HL-60 cells through activation of the caspase-9/caspase-3 pathway, which is triggered by mitochondrial dysfunction [232]. Isoerysenegalensein E and 6, 8-diprenylorobol were found to be the most potent antagonists of the receptor (ER) with their anti-estrogenic activity comparable to that of 4-hydroxy tamoxifen, a metabolite of tamoxifen and a stronger ER antagonist than tamoxifen. Warangalone and auriculasin were found to be weak ER inhibitors, and alpinumisoflavone and erysenegalensein E were non- ER inhibitors [235].

Compounds rotenone and 3α-hydroxyrotenone isolated from *M. pervilleana* [45] have shown inhibition of TPA-induced ornithine decarboxylase at the level of its mRNA expression and recommended as promising cancer chemo-preventive agents. The prenylated isoflavanone, pervi lleanone from the same plants also showed anticancer activity [45]. Isoflavonoid, griffonianone and maximaisoflavone charcterized from *M. griffoniana* showed significant cytotoxicity. Another flavonoid osajin isolated from *M. auriculata* showed anti-oxidant activity. The flavono id isolated from from the bark of *Millettia ovalifolia* displayed significant inhibition of cytosolic form of bovine carbonic anhydrase-II with IC_{50} value of 17.86 ± 0.07 μM [203]. The inhibitory effect of auriculasi isolated from *M. taiwaniana* and *M. auriculata* was investigated, and the compound exhibited significant inhibitory effect on mouse skin tumor promotion in an in vivo two-stage carcinogenesis test [216]**.** The cytotoxic and apoptotic effects of the constituents of the seeds of *M. pachycarpa* were also reported [44]. The studies of the cytotoxic effects against some cancer cell lines (HepG2, C26, LL2 and B16) and apoptotic effects against HeLa-C3 revealed that the isoflavonoid derivatives barbigerone, deguelin, tephrosin and millepachine demonstrated significant cytotoxic and apoptotic effects against cancer cells [203]. Pervilleanone and 3'-0-demethyl pervilleanone, isolated from *M pervilleana* showed mild growth inhibitory activities against human lung (NCI-H460), breast (MCF7) and CNS (SF-268) cancer cell-lines [45].

The study evaluating the estrogenicity and cytotoxicity toward tumoural cells of the isolates and the phenolic fraction (PF) of *M. macrophylla* [236] revealed that *Millettia macrophylla* has estrogenic properties, given its high amount of flavonoids as well as triterpernoids mainly, variabilin, aformorsin, pisatin, stigmastenone, flemichaparin B, formononetin, lupeol, which exhibited estrogenic effects in this work. The study confirmed that the estrogenic effects of phenolic fraction prepared from methanol extract of *M. macrophylla* appear to be more efficient than the methanol crude extract [238]. The MCF-7 cells proliferation is the reliable estrogenic

potency because this cell line is ER positive and it expresses aromatase and 5 α- reductase enzymes, which allows it to convert androgens to estrogens [237]. These authors reported that a relative proliferative effect (RPE) ≥ 80 corresponds to estrogenic activity and suggest that the compound may have agonistic activity to ERα. However, it is worthwhile to highlight that the MCF-7 cells can elicit an estrogen-induced response involving both genomic and non-genomic pathways**.** Hence, such as in vivo condition, the MCF-7 cells proliferation observed in E-screen assay is not limited to the activation of ERs [237].

2.6.4.3 Anti-inflamatory Activity of Compounds from Millettia Species

The constituents of *M. dielsiana* were evaluated for their potential to inhibit production of NO and TNF-a [218]. The study revealed that the tetra-methoxylated and prenylated isoflavone, millesianin C showed the most potent anti-inflammatory effect, decreasing nitric oxide (NO) production (1.37 ± 0.10 pM), similar to that of the positive control, dexamethasone and decreas ing TNF-a secretion (9.33 ± 1.08 ng/ml) better than that the control. The other tested compounds by Ye et al [218] for anti-inflammatory activity showed moderate to no activities with the results revealing the importance of 2,2-dimethylchromene moiety in increasing the inhibitory activity of NO production and TNF-a secretion. The C-27C-6 methoxyl groups were also observed to be an additional requirement for the enhancement of inhibitory activity of NO production and TNF-a secretion; while the formation methylenedioxy at C-4' and C-5' is only responsible for the decree sing of NO production inhibitory activity [218].

Antiinflammatory activity of various root extracts of *M. pinnata* evaluated by Singh and Pandey [276] showed significant antiinflammatory activity (compared to phenylbutazone) in carrageenin and PGE1 induced oedema models. Possible mechanism of action was attributed to prostaglandi n inhibition, especially by ethanolic extract and acetate extract. The butanol extract was found to be effective in carrageenin, but not in PGE1 model of inflammation. The anti-inflammatory prop erty appears to reside mainly in the intermediate polar constituents and not in lipophilic or extre mely polar constituents. Also, the petroleum ether extract of the seeds of *M. pinnata* showed pot ent acute anti-inflammatory effect whereas the aqueous suspension showed pro-inflammatory effects. In further studies, maximum anti-inflammatory effect was seen in the bradykinin induced oedema model. Possible mechanism of action could be inhibition of prostaglandin synthesis and decreased capillary permeability. Petroleum ether extract and aqueous extract inhibited histamine

and 5-hydroxytryptamine induced inflammation, probably by their lipophilic constituents prevent ing the early stages of inflammation. However, the fractions were not effective against Freund's adjuvant induced arthritic model. The later finding neglects the plant from its use in rheumatoid arthritis [238]. Mani Ganesh et al [239] were investigated for anti-inflammatory and analgesic activity of *M. glabra* leaf gall extract at the doses (p.o.) of 100, 200, and 400 mg/kg body weight. For evaluation of inflammation carrageenan-, histamine-and serotonin induced paw edema served as acute models and cotton pellet–induced granuloma served as a chronic model in rats. The acetic acid-induced writhing response and hot plate method using mice were used to assess analgesic activity. The higher doses of PG (200 and 400 mg/kg, p.o.) were inhibiting carrageenan, histamine and serotonin-induced paw edema as well as cotton pellet –induced granuloma successfully.

2.6.4.4 Antiviral Activities of Compounds from Millettia Species

Spedding [240] studied natural products that could inhibit key enzymes in the synthesis of DNA and reported that quercetin was one of the three most active naturally occurring flavonoids that inhibited three reverse transcriptases (RT) viz: avianmyeloblastosis RT, Rous-associated virus-2 RT, and Moloney murine leukemia virus (MMLV) RT when poly(rA)oligo(dT) 12–18 or rabbitglobin messenger RNA (mRNA) were used as templates. Myricetin, morin, quercetin, and fisetin are some of the flavonoids suspected to be potential nonpeptidic inhibitors of the HIV protease enzyme with IC_{50} values in the range of 10 to 50 μM as reported by Brinkworth [241] while quercetin has been found to also strongly inhibit integrase activity.

Rameshthangam and Ramasamy [242] evaluated the antiviral activity of bis (2-methylheptyl) phthalate isolated from *M. pinnata* leaves against White Spot Syndrome Virus (WSSV) of Penaeus monodon Fabricius. Oral administration of ethanolic extract and purified compound from the leaves of *M. pinnata* has increased the survival of WSSV infected Penaeus monodon.

2.6.4.5 Antiinfective Properties of Compounds from *Millettia* Species

The study [243] showed that the ethanol extract of *Millettia pachycarpa (M. taiwaniana*) was found to be the most toxic against the cabbage worm, while the acetone extract was most effective against housefly. The aqueous extract was found to be toxic to the housefly, cabbage worm and Pareva larvae. Rotenone and rotenoid group of compounds have been established as the active constituents in these extracts. Mukerja and Tripathi [244] reported that the ether extract of the seeds of *M. pachycarpa* was found to be one-third as toxic as dichlorodiphenyltri chloro methane

(DDT) when used as contact poison for houseflies. At a concentration of 0.5%, the extract was 80-90% lethal to silk worm larvae while at 1% it was 100% lethal to the eggs of silk worm. Bishnupada Roy [245] assayed crude ethanol, methanol, and acetone fractions of *M. pachycarpa* against Raillietinaechinobothrida in vitro and reported that exposure of the worm to the extracts at a concentration of 25 mg/mL in phosphate buffered saline (at 37°C ±1°C) caused distortions and disruption of mitochondria, nucleus, nucleolus, nuclear membrane and basal lamina.

Perrett et al [246], investigating the potential anthelmintic properties of *M. thonningii,* found that a chloroform extract of the seeds when topically applied to mouse skin prior to exposure to schistosoma mansoni cercariae, showed molluscicidal and cercaricidal activity. Subsequent re-infection was inhibited for as long as the extracts of *M. thonningii* were present on the surface of the animal skin perhaps justifying the traditional use of the plant as an anthelmintic and a purgative. The compounds implicated for this activity are thought to be the isoflavonoid alpinum isoflavone and its derivatives. In furtherance of the work done above, in vitro bioactivity study by Laddiard et al [247], showed that the extracts of the seeds of this plant are lethal to schistosoma mansoni miracedia, cercariae, and adult worms. Robustic acid and other coumarin compounds present in the seeds and alpinumisoflavones are said to be responsible for this observed bioactivity. In investigating the possible mode of this anti-schistosomal bioactivity using rat liver as the test organ, Laddiard [247], found that the seed extracts of *M. thonningii* inhibited the site I mitochondrial electron transport system's NADH dehydrogenase at 59 mg/l. Uddin et al. [248] investigated the antifilarial potential of the fruits and leaves extracts of *M. pinnata* on cattle filarial parasite. In their investigation, the aqueous and alcohol extracts of fruits and the alcohol extract of leaves caused an inhibition of spontaneous movements of the whole worm and the nerve-muscle preparation of S. cervi. The concentration required to inhibit the movements of the whole worm preparation was 250 µg/ml for aqueous, 120µg/ml for alcohol extract of fruits and 270µg/ml for alcohol extracts of the leaves. The concentrations of *M. pinnata* extracts required to produce an equivalent effect on the nerve-muscle preparation were 25, 5 and 20 µg/ml, respectively, suggesting a cuticular permeability barrier. Brijesh et al. [249] evaluated the crude decoction of dried leaves of *M. pinnata* for its antimicrobial (anti bacterial, antigiardial and antirotaviral) effect; and its effect on production and action of entero to xins (cholera toxin, CT; Escherichia coli labile toxin, LT; and E. coli stable toxin, ST); and adher ence of enteropathogenic E. coli and invasion of enteroinvasive

E. coli and shigella flexneri to epithelial cells. The decoction had no antibacterial, antigiardial and antirotaviral activity, but it was found to reduce the production of CT and bacterial invasion to epithelial cells. These results indicated that the crude decoction of *M. pinnata* has selective antidiarrheal action with efficacy against cholera and enteroinvasive bacterial strains causing bloody diarrheal episodes.

2.6.4.6 Insectcidal and Larvicidal Activities of Compounds from *Millettia* Species

The efficacy of *M. ferruginea* has been tested against many insect pests [288]. The study showed that water extracts of *M. ferruginea* seed powder at different concentration levels (10 – 40% w/v) caused 93 – 100% mortality against the different castes of adult Macrotermes termites at all conce ntration levels. The seed water extract also caused 45 – 60% mortality on the sorghum chaffer Pachnoda interrupta (Oliver) within 24 – 48 h, which was found to be significantly higher than the mortality caused by the standard insecticide, carbaryl, applied at the recommended rate (1.5 kg/ 400 l of water) [250]. The seed powder also caused 100% mortality in the bean weevil Zabrotes subfasciatus (Boheman). The seed extract of this plant has been observed to be effective in controlling storage insect pests, bean bruchid, Zebrotes subfaciatus and mosquito larvae [2851]. The aqueous suspension of the seeds showed high toxicity against the larvae of maize stalk borer Busseola fusca (fuller) (Lepidoptera: Noctuidae) on the first day after hatching of eggs [252].

The flavonoids; chalcones isolated from *M. ovalifolia* showed antimalarial activitiy [203]. *M. dura* contains 6α, 12α-didehydro-6-oxodeguelin had good insecticidal activity and played a key role as a phytotoxin inhibitor of ornithine decarboxylase. Ethyl acetate fraction of *M. ovalifolia* had good insecticidal activity and moderate antifungal activity [203]. Bosire et al. [236] revealed that usararotenoid A (139), a rotenoid from *M. usaramensis* ssp. *usaramensis* with trans-B/C ring junction and methylenedioxy group at C-2/C-3 showed high larvicidal activity (LC_{50} 4.3 ± 0.8 pg/ml, at 48 hrs) against the 4th instar A. aegyptii. The study [253] has showed that crude chloroform extract of seeds of *M. dura* (Leguminosae) showed high larvicidal activity (LD50 = 3.5 µg/ml at 24 hours) against second-instar larvae of the mosquito, Aedes aegypti (Diptera: Culicidae). Extractsobtained from the stem bark, root bark and leaves were inactive ev en at 20 µg/ml. Furthermore, the rotenoids deguelin and tephrosin isolated from the seeds of this plant also showed potent activities with LD_{50} values of 1.6 and 1.4 µg/ml at 24 hrs, respectively, and the related rotenoids millettone and millettosine were completely inactive even at 20 µg/ml. The

presence of methoxyl groups at C-2 and/or C-3 in deguelin and tephrosin appear to be impor tant for the observed larvicidal activity [253].

2.6.4.7 Antiplasmodial Activities of Compounds from *Millettia* Species

The rotenoids usaretonoid C, 12a-epimillettosin and 6a, 12a-dehydromillettone, isoflavone barbi gerone and chalcone 4'-O-geranylisoliquiritigenin from stem bark *M. usaramensis* were found to have antiplasmodial activity [254]. *M. pinnata* shows antiplasmodial activity against Plasmodiu m falciparum [255]. The study evaluated the antiplasmodial activity of some isoflavone derivatives isolated from stem bark of *M. oblata ssp. teitensis* and *M. dura*, in which the marginal antiplasmodial activity was observed for calopogonium isoflavone B, isoerythrin A-4'-(3-methylbut-2-enyl) ether, maximaisoflavoneB and 7,2'-dimethoxy-4',5'-methylenedioxyisoflav one against the chloroquine sensitive and resistant (Dd2) strains of Plasmodium falciparum [256].

2.7. Biosynthesis of Isoflavonoids

2.7.1 Biosynthesis of Isoflavonoids in Soybean

Isoflavonoids are synthesized by a legume specific branch of general phenylpropanoid pathway. Other branches of phenylpropanoid pathway are common to all plant species and produce lignin, proanthocyanidins, anthocyanin, phlobaphenes (Fig. 2.18). Chalcone [257] is the first step in the production of flavonoids and isoflavonoidswhich requires an enzymatic reaction catalyzed by the enzyme chalcone synthase (CHS), a plant specific polyketide synthase. Soybean contains nine CHS genes (CHS1- CHS9) among which CHS7 and CHS8 are critical for isoflavonoid biosynthesis [258]. Legume plants produce two kinds of chalcones, tetrahydroxy chalcones (naringenin chalcone) and trihydroxy chalcone (isoliquiritigenin chalcone) whereas nonlegume plants only produce tetrahydroxy chalcones (naringenin chalcone). The production of isoliquiritigenin in legumes is a result of additional enzymatic reaction catalyzed by a legume specific enzyme chalcone reductase (CHR). Both naringenin and isoliquiritigenin chalcones then get converted into their corresponding flavanones by chalcone isomerase (CHI). Soybean CHI gene family consists of seven members that are grouped into four subfamilies: CHI1, CHI2, CHI3, and CHI4 [259]. These genes are further categorized into different types based on their catalytic abilities where type I CHIs (such as CHI2) is common to all higher plants, convert naringenin chalcone to naringenin, and type II CHIs (such as CHI1) are almost entirely exclusive to legumes, possess additional capacity to catalyze the conversion of isoliquiritigenin to liquiritigenin [259].

The branch point enzyme that introduces isoflavonoid specific branch in the phenylpropanod pathway is 2-hydroxyisoflavanone synthase (isoflavone synthase, IFS). IFS is a microsomal cytochrome P450 monooxygenase enzyme that catalyzes a 2, 3 aryl ring migration of flavanones to their corresponding flavones and subsequent hydroxylation of the resulting C-2 radical. This reaction also requires reduced nicotinamide adenine dinucleotide phosphate (NADPH) and oxygen molecule and yields 2-hydroxyisoflavanone. The reaction product of IFS, 2-hydroxyisoflavanone, is extremely unstable and undergoes dehydration by forming a double bond between C-2 and C-3 by a dehydratase to form genestein or daidzein [260]**.** Two IFS genes, IFS1 and IFS2, have been identified in soybean [258] brought a major break through in the attempts to metabolically engineer isoflavonoids in nonlegumes. These two IFSs differ from one another by 14 amino acid residues; however, both IFS1 and IFS2 convert the naringenin and liquiritigenin flavanones to their corresponding isoflavones [258].

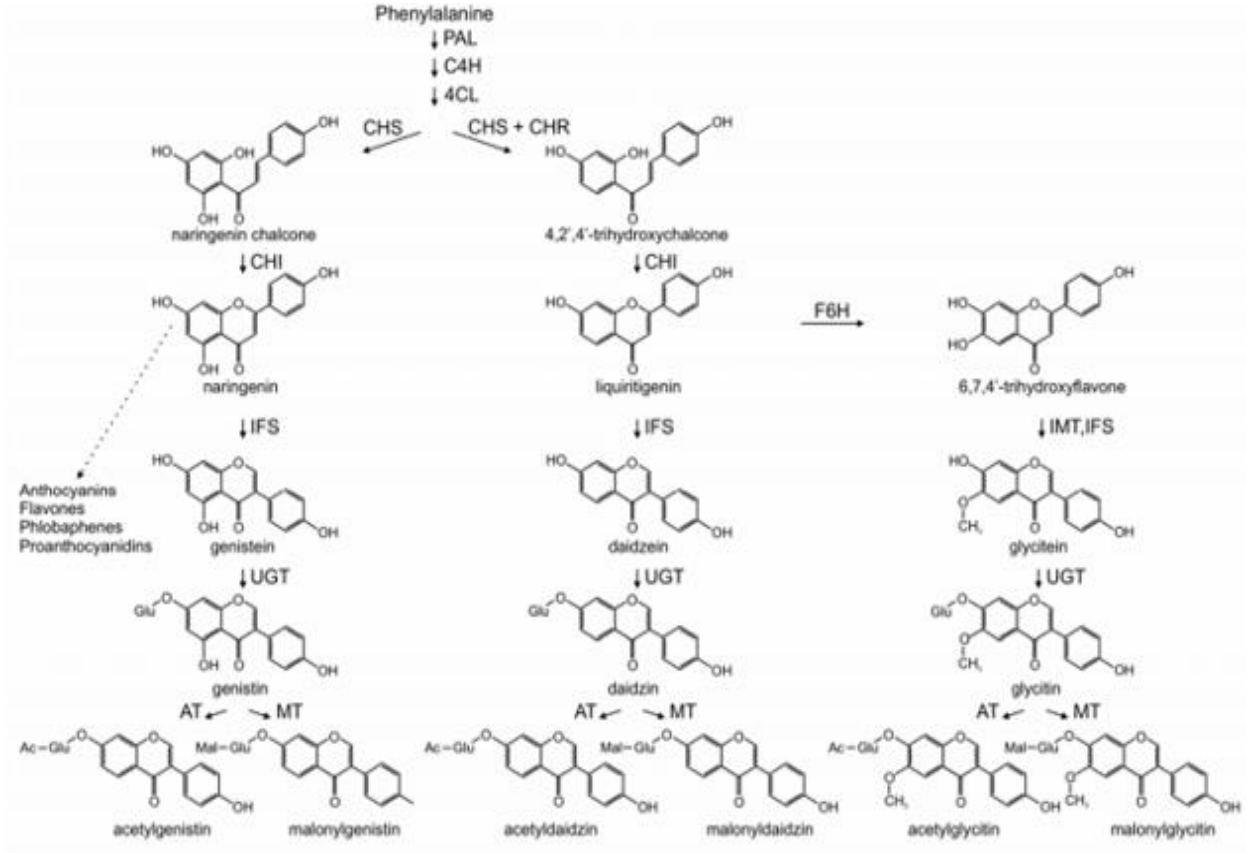


Figure 2.16: Soybean Seed Isoflavonoid Biosynthesis Pathway.

Dotted arrow indicates multiple steps involved in the synthesis of other phenylpropanoids. PAL, phenylalanine ammonia lyase; 4CL, 4-coumarate-CoA-ligase; C4H, cinnamate-4-hydroxylase; CHI, chalcone isomerase; CHR, chalcone reductase; CHS, chalcone synthase; F6H, flavonone-6-hydroxylase; UGT, glycosyl-transferase; IFS, 2-hydroxyisoflavanone synthase; IMT, isoflavone methyl-transferase; MT, malonyl-transferase; AT, acetyl-transferase [258]**.**

CHAPTER THREE

3. MATERIALS AND METHODS

3.1 Plant Material

3.1.1 Plant Selection Criteria

In this study, selection of plants was based on ethnobotanical literature and information obtained from local healers prescribing folk medicine. In selection, priority is given to species endemic to Ethiopia, braod spectrum tradional use, and species not studied yet for anticancer and antiviral activities. For this study, the three wild endemic plant species chosen were *Millettia ferruginea* seed, *Aloe sinana* (root and leave latex) and *Aloe pirottae* root.

3.1.2 Plant Collection

The selected plants were collected from different parts of Oromia state, Ethiopia in 2017. *M. ferruginea ssp ferruginea* seeds (Voucher No. A001/ 2017) was collected from Addis Ababa in March 2017. The root and leaves latex of *Aloe sinana* (Voucher No. A003/2017) were collected from Walanchiti (East Shawa zone) in November 2017. The root of *Aloe pirottae* (Voucher No. A004/2017) was collected from Addis Ababa in November 2017. The plants were identified and authenticated by prof. Teshome Soromesa of the Biology Department, Addis Ababa University. The voucher specimens were deposited in the Herbarium, Addis Ababa University, Ethiopia.

3.1.3 Plant Preparation

The Leaves and roots of plants were cleaned and the root were cut into small pieces. The seed and root were dried in open air under shade. The air-dried plant material was grind into fine powder using an electric grinder and kept for extractions. The leaves latex of *A. sinana* was collected by cutting the leaves transversally near the base and arranging them concentrically around a plate. The latex was then left in open air for three days to allow evaporation of water. Partially dried in open air was completely dried by lyophilizing in a vacuum freeze dryer.

3.2 Chemicals, Reagents and Equipment

3.2.1 Chemicals and Reagents

The commercially available standard anticancer agent used in this study was etoposide obtained from US Biological (Swampscott, MA). The commercially available standard antiviral agents used in this study were amantadine hydrochloride (AMT) and ribavirin (RBV) obtained from Sigma (St Louis, MO), oseltamivir carboxylate (OSV-C) from US Biological (Swampscott, MA), and arbidol

hydrochloride (ARB) from AK Scientific, Inc. (Mountain View, CA). Rupintrivir (T-705; 6-flouro-3-hydroxy-2-pyrazinecarboxamide) was synthesized in-house by I.Y. Lee. SRB was from the Korea Polar Research Institute, South Korea.

Methanol, *n*-hexane, chloroform, ethyl acetate and *n*- butanol used for extraction and purifications were HPLC-grade and obtained from Korea Research Institute of Chemical Technology (KRICT).

3.2.2 Equpements used for Structural Determination

Chromatographic separation was performed using silica gel silica gel 60 À (70-400 mesh). Analytical TLC was carried out on pre-coated silica gel 60 F254 plates (Merck 0.25 mm thickness). Chemical spots on TLC plates after development were detected using UV light at 254 nm and 366 nm. The ^{1} H, ^{13}C and DEPT NMR spectra were acquired in $CDCl_3$ solvent on a Bruker AMX-500 FT-NMR spectrometer (Bruker Analytische Messtechnik Gmbh, Rheinstetten, Germany), and the spectra were processed with the MestReNova 10.0 software, using tetra methyl silane (TMS) as chemical shift reference. LC-ESIMS were acquired on a PerkinElmer PE SCIEX API 150EX instrument equipped with a Turbolon spray ion source connected to a Gemini 5 mm RPCis 110 A column. Column chromatography was run on silica gel 60 A (70-400 mesh).

3.3 Plant Extraction

3.3.1 Extraction with Methanol

According to the literature on bioassay guided extraction, the most commonly used method for the initial extraction of plant extracts was involving exhaustive extraction with methanol and the partitioning of a methanol extract with different organic solvent of increasing polarity [262]. Shade-dried and grinded plant material was extracted with methanol three times (X 3) each for 3 days (72 hrs) and shaken ten times daily. Each time, methanol was used in the ratio of 3 ml for 1 g of dry powdered plant material. After 3 days each, methanol was squeezed out and filtered. The three filtrates were combined and concentrated to dryness in a rotary evaporator under reduced pressure below 34 °C. The small amounts of wet extracts were dried to the maximum by vacuum dryer. This solid or semisolid material is called the methanol extract. The small amounts of crude methanol extracts used for antiviral and cytotoxic activity test were completely dried by lyophilizing using a vacuum freeze dryer.

3.3.2. Fractionation of the Methanol Extract with Solvents of Increasing Polarity

A modified Kupchan Partition method [263] was used for the solvent-solvent partition of crude extracts. The MeOH extract was suspended in distilled water in the ratio of 1 g extract in 10 ml water. Aqueos - methanol suspended was succesively partitioned with *n*-hexane (Hx), chloroform (CH), ethyl acetate (EA) and *n*-butanol (Bu). Exhaustive extraction was carried out succesively with these solvents to extract as much as possible the most active components with highest biological activity. The water suspended in separating funnel was extracted three times each for 12 hrs (X 3) with the water immiscible organic solvent in increasing polarity (*n*-hexane < chloroform < ethyl acetate < n-butanol) by shaking in 30-minute interval. Each time, the volume of the organic solvent used was the same as that of the water layer. The organic layers were concentrated to dryness in a rotary evaporator under reduced pressure at temperature between 30-33 ºC. The aqueous layer was again extracted with the other organic solvent in the order of increasing polarity. Each time, the organic layers were combined and evap orated by rotary evaporator to dryness at temperature between 30-33 ºC, and the small amounts of wet extracts from each solvent were then lyophilized by using vacuum dryer.

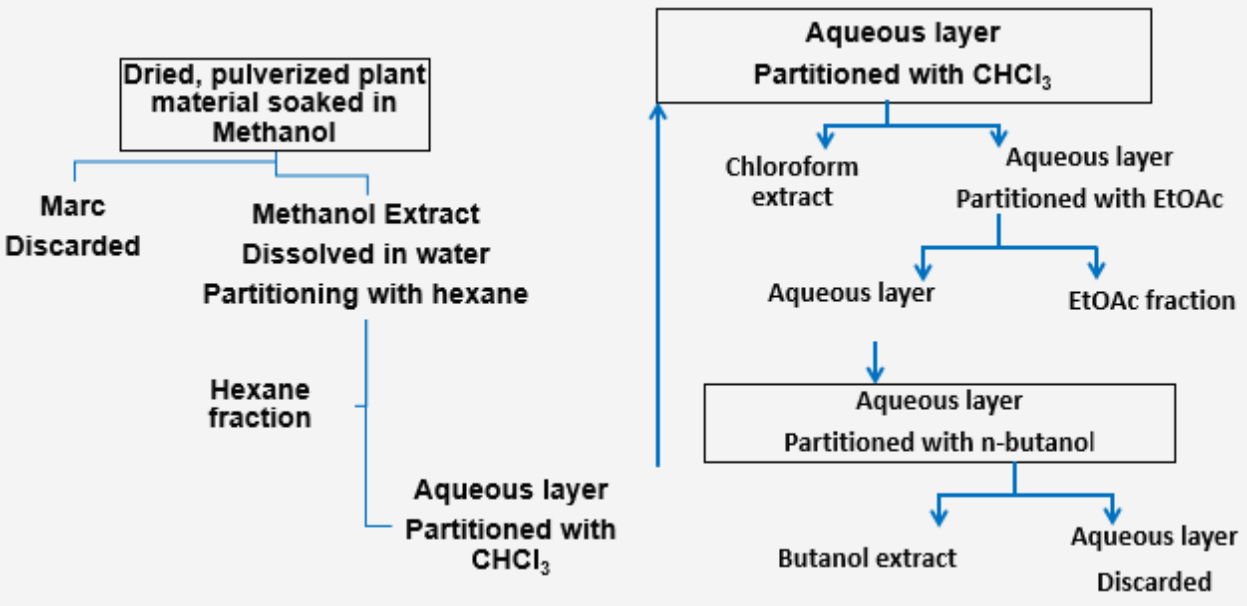


Figure 3.1: Extraction Scheme

3.4. Isolation of Compounds from the Chloroform Fraction of the Seed of *M. ferruginea*

Compounds were isolated from chloroform fraction by Vacuum Liquid Chromatography (VLC), TLC and UV light.

The chloroform fraction (1.0 g) was subjected to Vacuum Liquid Chromatography (VLC) for separation. The column was packed with *n*-hexane (Hx): ethyl acetate (EtOAc) in 95:5 ratios with fine TLC grade silica (Kiesel gel 60À, mesh 70 - 400) under vacuum as discribed by Pelletier *et. al.* [264] and Coll and Bowden [265]. The column was washed with 100 % *n*-Hx to facilitate compact packing. A sample was dissolved in very small amount of chloroform and adsorbed onto silica gel Kiesel gel 60À (mesh 70 - 400) allowed to dry. The column was eluted with a mixture of n-HxF: EtOAc (95:5 to 50:50) in gradient systems of increasing polarity and the fractions were collected in 20 ml test tube. The presence of the compounds in each fractions were monitored by TLC profile, which were detected by visually for colored compounds and under UV light of both long and short wavelength (254nm and 366nm). The eluents were combied into ten major fractions (MF1 – MF10) after TLC analysis. Further TLC analysist showed five major fractions (MF: 1,3,5,7, add 9) with single single spot each and five major fractions (MF: 2,4,6,8, and 10) with mixed spots each under UV. One major fractionMF1 crystalized overnight and filtered to afford 40 mg of a pure white crystal labeled as 10CH-01. The four major fractions MF3, 5, 7, and 9 each were concentrated under reduced pressure by a vacuum rotary evaporator to afford 86 mg a white solid powder labeled as 10CH-02, 91 mg of a white solid powder labeled as 10CH-03, 28 mg a yellow solid powder labeled as 10CH-04 and 99 mg a white solid powder labeled as 10CH-05. Five major fractions with closer TLC profile combined and concentrated under reduced pressure by a vacuum rotary evaporator to afforded five mixtures and kept for further purifications.

3.5 Biological Studies

3.5.1 Anticancer Assay

3.5.1.1 Tumor Cell Lines.

In this study, human non-small cell lung cancer cell line A549, ovarian cancer cell line A2780, pancreatic cancer cell line MIA-Paca-2 and stomach cancer cell line SNU-638 were used. All the cell lines were maintained in RPMI-1640 complete growth medium (GIBCO, Carlsbad, CA) and supplemented with 5% fetal bovine serum (FBS, GIBCO). Carbon dioxide incubator (Heraeus, GmbH, Germany) at 37°C with 90% humidity and 5% CO_2 was used to grow cell cultures.

3.5.1.2 Cytotoxicity Assessment: Sulforhodamine B (SRB) Assay Method

The cytotoxicity of the methanol extract, its solvent fractions and compounds against cultured human tumor cell lines, A549, A2780, MIA-Paca-2 and SNU-638**,** were evaluated following the sulforhodamine B (SRB) method [266,267] at different concentrations of 0.1, 0.3, 1.0, 3.0, 10.0,

and 30 µg/ml to determine percentage of growth inhibition and the IC_{50} (50% growth inhibition). The human cancer cell lines were grown in tissue culture flasks at 37 °C in 90% humidified atmosphere of 5% CO_2 in complete growth medium. Each human cancer cell line was incubated over standard 96-well flat-bottom microplates for 24 h at 37 °C in a humidified atmosphere of 5% CO_2. Serially diluted samples (0.1, 0.3, 1.0, 3.0, 10.0 and 30.0 µg/ml) were added to the 96-well cell culture plates. The culture medium was removed from each well after continuous exposure to the compounds for 72 hrs, and 10% cold trichloroacetic acid was used at 4 °C for 1 hr to fix the cells attached to the bottom of the wells. The plates were then washed 5-6 times with distilled water and then air-dried. The cells were stained with 0.4% SRB dye and incubated at room temperature for 30 min, and washed again with 1% acetic acid. The plates were again air-dried and each well was solubilized with 10 mM buffered Tris base solution (pH10.5). The optical density (OD) of the plate wells was measured spectrophotometrically at 520 nm with a micro titer plate reader and data were maintained. Triplicate wells were conducted for each drug concentration tested, The IC_{50} values of compounds were calculated by the nonlinear regression analysis.

The IC_{50} was expressed as the concentration of drug reducing the plaque number by 50% as compared to mock-treated controls. It was calculated from a dose–response line obtained by plotting the percentage plaque reduction, with respect to the control plaque count, versus the logarithm of sample dose. Triplicate wells were utilized for each drug concentration tested. The percentage growth inhibition was calculated using following formula: % cell growth inhibition = 100-{(At-Ab)/ (Ac-Ab)} x100 where, At = Absorbance value of test compound; Ab = Absorbance value of blank; Ac = Absorbance value of control.

3.5.2 Antiviral Assay

3.5.2.1 Cells and Viruses

Madin-Darby canine kidney (MDCK) cells and C6/36 mosquito cells (ATCC, Manassas, VA) were grown in minimum essential medium (MEM; Gibco/ Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Invitrogen) at 37 ºC and 28 ºC, respectively. Influenza virus strains A/Puerto Rico/8/34 (H1N1) (PR8), Lee and A/Hong Kong/8/68 (H3N2) (HK) were obtained from ATCC. Influenza A viral PR8 and HK strains were propagated in 10-day-old chicken embryos at 37 ºC for 3 days and influenza B virus (Lee) by infection of MDCK cells under serum-free conditions.

Picornaviruses were provided by the Bio and Drug Discovery Division, Korea Research Institute of Chemical Technology. African green monkey kidney cells (Vero, CCL-81) were purchased from the American Type Culture Collection (Manassas, VA, USA). African green monkey kidney Vero cell (ATCC) was maintained in RPMI 1640 supplemented with fetal bovine serum (10% v/v). Cells were incubated at 37^{o}C in a 5% CO2 humidified atmosphere. Picornaviruses were propagated in Vero cells.

DENV-2 (New Guinea C strain) was purchased from the National Collection of Pathogenic Viruses, Culture Collections of Public Health England (Salisbury, Great Britain) and propagated in C6/36 cells. DENV viral titers were quantified by focus-forming assay on Vero76 cells as described previously [268].

3.5.2.2. Antiviral Assay LOOK THIS PART AGAIN =THE BLUE AND OTHER PARTS

Cytopathic effect (CPE) reduction assay: CPE reduction assay was used for influenza and picornavirus antiviral assay**.**

In the CPE reduction assay of influenza virus, MDCK cells were seeded in 96-well plates and either mock-infected or infected with influenza virus at a multiplicity of infection (MOI) of 0.001 [50 plaque-forming units (PFU) of influenza virus per well]. After incubation for 1 h at 33 C (mock, Lee) or 35 ^{0}C (PR8 and HK), the medium was removed, and test and standard chemicals were added, which were serially diluted in MEM containing 2 µg/ml TPCK-trypsin (Sigma). On day 2 or 3 post-infection (p.i.), the cell viability was measured after treatment with fluorescein diacetate (FDA; Sigma), as described by Kim et al. [269] and Schols et al. [270]. The 50% cytotoxic concentration (CC_{50}) and the 50% effective concentration (EC_{50}) values were calculated using SoftMax Pro Software (Molecular Devices, Sunnyvale, CA). The percent of protection was calculated as follows:

Percent protection = [(ODT) V - (ODC) V] / [(ODC) M - (ODC) V] ×100

Where (ODT) V, (ODC) V and (ODC) M indicate absorbance of the sample, the virus-infected control (no compound) and mock-infected control (no virus and no compound), respectively.

Vero cells were seeded in 96-well plates and either mock-infected or infected with picornaviruses at a multiplicity of infection (MOI) of 0.001 50 plaque-forming units (PFU) of picornaviruses per well. After incubation for 1 h at 37 ^{0}C (mock, picornaviruses), the medium was removed and the

cells were washed. Then sample and standard chemicals were added, which were serially diluted in MEM containing 2 µg/ml TPCK-trypsin (Sigma). On day 2 and 3 post-infection (p.i.), the cell viability was measured after treatment with fluorescein diacetate (FDA; Sigma), as described by Kim et.al. [269] and Schols et. al. [270]. The percent of viability was calculated as follows:

Percent viability = [(ODT) V - (ODC) V] / [(ODC) M - (ODC) V] ×100

Where (ODT) V, (ODC) V and (ODC) M indicate absorbance of the sample, the virus-infected control (no compound) and mock-infected control (no virus and no compound), respectively.

50% cytotoxic concentration (CC_{50}) is defined as the tested sample concentration that produces cellular toxicity of 50%. 50% effective concentration (EC_{50}) is defined as tested sample concentration that reduces the replication of viruses by 50% in the CPE reduction assay. The selectivity index (S.I.) is the ratio of CC_{50} to EC_{50} (CC_{50} / EC_{50}).

Immunofluorescence assay (IFA). IFA assay was used for DENV-2 antiviral assay. Vero cells were seeded on 96-well plates for DENV antiviral assay. After overnight incubation, cells were inoculated with DENV-2 at a multiplicity of infection (MOI) 0.2 for 2 h at 37°C. Crude extracts were added at two different concentrations (20 µg/ml and 100 µg/ml). An IFA used to detect dengue infection was optimized for the dengue high-throughput content imaging assay. Briefly, DENV-infected cells were detected by probing anti-DENV E (4G2) monoclonal antibody and Alexa Fluor 488 (A488)-conjugated goat anti-mouse IgG (H+L) (Invitrogen Molecular Probes, USA) as secondary antibody. Cell nuclei counterstained with 5 µg/ml 4',5-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, USA). After washing, digital images were acquired using Operetta® high content imaging system (Perkin Elmer, USA). The digital images were taken from 4 different fields of each well at 20X magnification.

The percentage of inhibition was derived by using the formula; [1-(A488-positive cells/total cells)] x 100%.

CHAPTER IV

4. RESULTS AND DISCUSSION

4.1 Structural elucidation of the compounds from the Seed of *Millettia ferguinea*

The chloroform fraction (1.0 g) was subjected to Vacuum Liquid Chromatography (VLC) for separation. The column was eluted with a mixture of *n*-Hxf: EtOAc (95:5 to 50:50) in gradient systems of increasing polarity and led to the identification of one new isoflavone and seven known isoflavones. Isolates were characterized on the basis of their spectroscopic data and by comparison with literature report. The structural characterization of the compounds is discussed in this section.

4.1.1 Durmillone (90)

Compound 10-CH-01 was isolated as white solid. The ESIMS showed a $[M+H]^+$ ion peak at m /z 379.1195 compatible with the molecular formula $C_{22}H_{18}O_6$. The ^1H and ^{13}C NMR spectra (Table 4.1), ^1H (δH 7.94, s. H-2) and ^{l3}C (δC 151.7 for C-2, 124.3 for C-3 and 175.4 for C-4) indicated that this compound is an isoflavone derivative [210]. Furthermore, the ^1H and ^{13}C NMR spectra showed the presence of a methylenedioxy (δH 5.99, s; δC 101.2) and a methoxyl (δH 3.97, s; δC 56.4) groups. The ^1H NMR spectrum also showed olefinic proton consisting of two set of doublets at δH 6.81 (d, J= 10.2 Hz) and 5.75(d, J= 10.2) which together with singlet at δ_H 1.56 integrating for six protons, suggesting the presence of a 2, 2-dimethylpyrano substituent. It also showed a deshielded aromatic proton at 7.56 and an AMX spin system aromatic protons at δH7.11 (d, J = 1.8), 6.97 (d, J = 7.8), 6.85 (dd, J = 1.8, 7.8 Hz).

Thus the isoflavone must have one methoxyl and the 2,2-dimethylpyran substituents in ring A suggesting that the 2,2-dimethychromene group is located at C-7/C-8 on ring A. The fact that there is only one aromatic singlet at δ_H 7.56 (assigned to H-5) in this ring, suggested that C-6 is substituted, which in this case should be a methoxyl group (δ_C 3.97; δC 56.4). This placement was confirmed from the ^1H NMR spectrum which revealed the existence of meta coupling. This meta coupling only exists between the two B-ring protons which must be placed at H-2' and H- 6'. This leaves only two options, C-6 of ring A and C-5’ of ring B, for placement of methoxyl group. Furthermore, if methoxyl group is placed at C-5’, the fact that characteristic peak and the only one aromatic singlet at δ_H 7.56 will be left unassigned which will lead to wrong characterization. The problem is resolved when the methoxyl is placed only at C-6 of ring A. Based on these and

previously reported data [210], compound 10-CH-01 was identified as a known compound with trivial name durmillone. Durmillone has been previously isolated from bark of *M. ferruginea* subsp. *darassana* [210], M. *dura* [271] and root bark of *M. graffoniana* [272]. This is the first report of durmillone from seed of *M. ferruginea* subsp *ferruginea*.

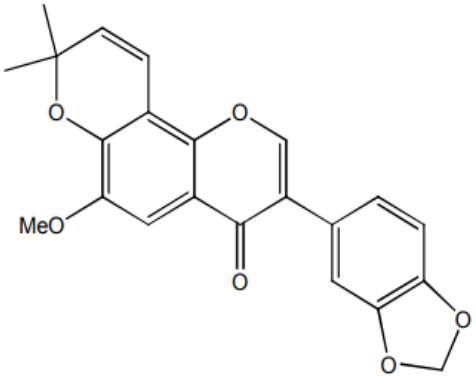


Figure 4.1: Structure of durmillone (90)

Table 4.1: ^{1}H ($CDCl_3$,500.13 MHz) and ^{13}C ($CDCl_3$,125.13 MHz) NMR data of compound 10CH-01 and durmillone [210] (chemical shift, δ in ppm and multiplicity, J in Hz)

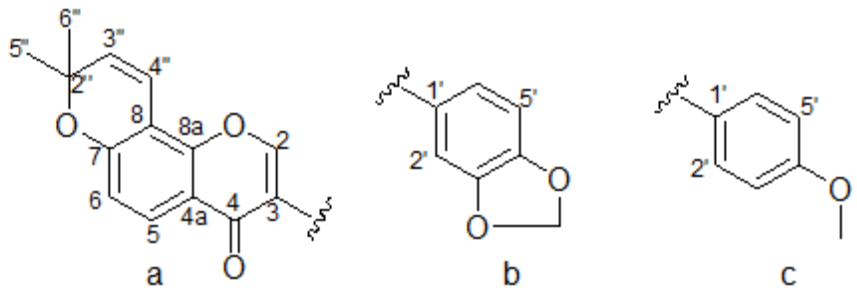
position	compound10CH-01		Durmillone [210]		Position	Compound 10CH-01		Durmillone [210]	
	δH (m,J)	δC	δH (m, J)	δC		δH (m, J)	δC	δH (m, J)	δC
2	7.94 (s)	151.7	7.94	150.2	2'	7.11 (d, J 1.8)	109.8	7.10 (d, J 1.8)	110.0
3		124.4		125.3	3'		147.6		147.5
4		175.4		175.0	4'		147.7		147.5
4a		117.6		118.4	5'	6.85 (d, J 7.8)	108.4	6.87 (d, J 7.8)	108.2
5	7.56 (s)	108.4	7.55	105.4	6'	6.97 (dd, J 7.8)	122.4	6.97 (dd, J 7.8)	122.5
6		147.2		147.0	2''		78.2		78.1
7		147.3		147.4	2''-CH_3	1.56 (s)	28.0	1.55	28.0
8		110.2		110.3	3''	5.75 (d, J 9.6)	115.2	5.74 (d, J 9.6)	114.9
8a		147.4		153.0	4''	6.81 (d, J 8.0)	130.4	6.80 (d, J 8.0)	129.0
1'		125.94		125.6	6-OCH_3	3.97 (s)	56.4	3.67	56.3
					-CH_2O-	5.99 (s)	101.2	5.99	101.1

4.1.2 Calopogonium isoflavone B (76) and Calopogonium isoflavone A (82)

10-CH-02 was isolated as white solid from chloroform fraction of the seed of *M. ferguinea*. The positive ESI-MS displayed a 100% peak at m/z = 350.05 $[M + 2]^+$ (calcd. for $C_{21}H_{16}O_5$ + 2H), for which $[M]^+$ = 348.357 is compatible with the molecular formula $C_{21}H_{16}O_5$ (cal. for 348.357), and

a second 60% peak at m/z = 335.26 $[M + 1]^+$ (calcd. for $C_{21}H_{18}O_4$ + H), for which $[M]^+$ = 334.37 is compatible with the molecular formula $C_{21}H_{18}O_4$. This ESI-MS data indicated that 10-CH-02 actually contained mixtures of two compounds and not just one compound.

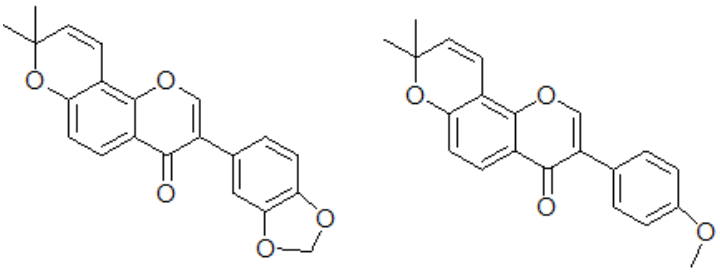
^{1}H NMR data (Table 4.2), analysis revealed the existence of paired equivalent ^{1}H NMR signals at ^{1}H (δ 7.93 (s) and 7.92 (s), two hydrogens oxygenated methine and similarly the ^{13}C NMR revealed two oxygenated methine signal at *δ*c 151.90 and 157.74; two quaternaries at δc 125.7 and 124.8; and two carbonyls at *δ*c 175.9 and 175.7, which is consistent with two isoflavone skeletons representing each of the two compounds contained in 10-CH -02, in agreement with what was observed in the ESI-MS data. This implied that 10-CH-02 was composed of two isoflavone derivatives. The 1D NMR spectral data further revealed the presence of paired equivalent ^{1}H and ^{13}C NMR data for each of the following: doublet at δ_H 8.08 with δ_C 130.3 for C-5 methines (see Figure 4.2), doublet at δ_H 6.97 with δ_C 114.92 due to C-6 methines, δ_C 108.4 and 109.8 quaternaries at C-8; two olefenic protons at C-3'' and C-4'' δ_H (6.81 d and 5.72 d) with δ_C 126.7 and 114.97. In the ring A of both compounds in 10-CH-02, the ^{1}H NMR spectrum showed, in addition to the olefin protons mentioned above, a singlet at δ_H 1.56 integrating for 12 protons, suggesting the presence of a 2'', 2''-dimethylpyrano substituent at C-7/C-8 junction of both compounds. These are what the two compounds had in common.



a: part of the structure which the two compounds had in common, b: part which is present in 10CH-02-I (compound 76) and c: part found in compound 10CH-02-II (compound 82)

In the ring B of compound10CH-02-I (76), the 1D NMR spectral data further revealed the presence of a methylenedioxy group (δ_H .99, s; δ_C 101.2) at C-3'/C -4'; one singlet aromatic proton at C -2' (δ_H 6.88, s), two doublets aromatic proton at C-5' and C-6' δ_H (7.93 d and 7.09 d) with δ_C 114.9 and 126.7. Compound 10CH-02-I was therefore, identified as calopogonium isoflavone B, a

compound previously reported from the root bark of bark of *M. ferruginea* subsp. *darassana* [210]. In the ring B of compound 10CH-02-II (82), the 1D NMR spectral data revealed the presence of methoxy at C-3' ($^{\delta}$H 3.84, s), four doublets aromatic proton at C- 2', C-3', C-5' and C-6' $^{\delta}$H (7.93 d, 6.97 d, 7.93 d and 7.09 d) with δ_C 114.9, 126.7, 114.9 and 126.7. Compound 10CH-02-II was therefore, identified as calopogonium isoflavone A, a compound previously reported from stem bark of M. *dura* [271]. There fore, 10-CH-02 has two compounds as given in Fig. 4.2.



10CH-02-I (compound 76) 10CH-02-II (compound 82)

Figure 4.2: Structure of calopogonium isoflavone B (76) and calopogonium isoflavone A (82)

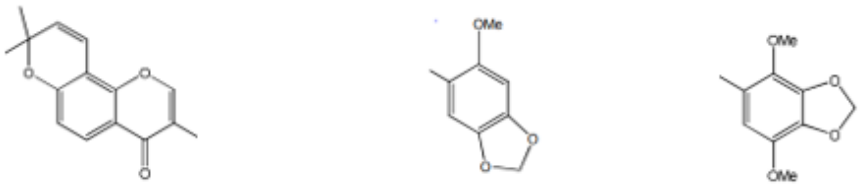
Table 4.1: ^{1}H (500.13 MHz) and ^{13}C (125.13 MHz) NMR data for compound 76 and 82 (chemical shift, ***δ*** in ppm and multiplicity, J in Hz)

Position	Compound 76		Compound 82		Position	Compound 76		Compound 82	
	δ_H (m, J)	δ_C	δ_H (m, J)	δ_C		δ_H (m, J)	δ_C	δ_H (m, J)	δ_C
2	7.51(s)	151.7	7.48 (s)	151.9	2'	6.88 (s)	108.3	7.93(d, J=2.4)	122.2
3		125.7		124.9	3'		130.3	7.09 (d, J =1,8)	130.3
4		175.8		175.9	4'		130.2		147.63
4a		118.3		118.4	5'	7.93(d, J 6.4)	124.8	6.97 (d, J= 9.6)	159.5
5	8.05 (d, J 8.4)	114.9	8.08 (d,J 8.4)	114.9	6'	7.09 (d, J 6.4)	113.9	7.93(d, J= 6.4)	113.9
6	6.85 (d,J 6.8)	109.3	6.85 (d,J 6.8)	109.3	3''	5.72(d,J 9.6)	130.1	5.72(d,J=9.6)	127.9
7		157.3		157.3	4''	6.81(d, J 9.6)	125.7	6.81(d, J= 9.6)	130.1
8		109.8		109.8	2''		78.2		78.2
8a		152.4		152.4	CH_3	1.5 (s)	28.2	1.5(s)	28.2
1'		125.7		124.7	$-CH_2O-$	5.99 (s)	101.2	3.84(s)	55.6

4.1.3 Jamacin (74) and Ferrugone (73)

10CH-03, was isolated as white solid from chloroform fraction of the seed of *M. ferguinea*. The positive ESI-MS displayed a 100% peak at m/z = 410.08 $[M + 2]^+$ (calcd. for $C_{23}H_{20}O_7$ + 2H), for which $[M]^+$ = 408.36 is compatible with the molecular formula $C_{23}H_{20}O_7$ (cal. for 408.36), and a second 100% peak at m/z = 379.21 $[M + 1]^+$ (calcd. for $C_{22}H_{18}O_6$ + H), for which $[M]^+$ = 378.384 is compatible with the molecular formula $C_{22}H_{18}O_6$. This ESI-MS data indicated that 10-CH-03 actually contained mixture of two compounds and not just one compound.

1D NMR data (Table 4.3), analysis revealed the existence of paired equivalent ^{1}H NMR spectral data of ^{1}H (δ 7.91(s) and 7.91 (s), two hydrogens oxygenated methine and similarly the ^{13}C NMR revealed two oxygenated methine signal at δc 153.80 and 153.80; two quaternaries at δc 122.1 and 121.9; and two carbonyl at δc 175.8 and 175.8, which is consistent with two isoflavone skeletons representing each of the two compounds contained in 10-CH-03, in agreement with what was observed in the ESI-MS data. This implied that 10-CH-03 was composed of two isoflavone derivatives. The 1D NMR spectral data further revealed the presence of paired equivalent ^{1}H and ^{13}C NMR data for each of the following: doublet at δ_H 8.05 with δ_C 130.3 for C-5 methines (see Figure 4.3), doublet at δ_H 6.86 with δ_C 111.2 due to C-6 methines, δ_C 112.8 and 112.8 quaternaries at C-8; *cis* two olefenic protons at C-3'' and C-4'' δ_H (5.72 d and 6.81 d) with δ_C 126.7 and 115.1. In the ring A of both compounds in 10-CH-03, the ^{1}H NMR spectrum showed, in addition to the olefin protons mentioned above, a singlet at δ_H 1.50 integrating for 12 protons, suggesting the presence of a 2'', 2''-dimethylpyrano substituent at C-7/C-8 junction of both compounds. These are what the two compounds had in common.

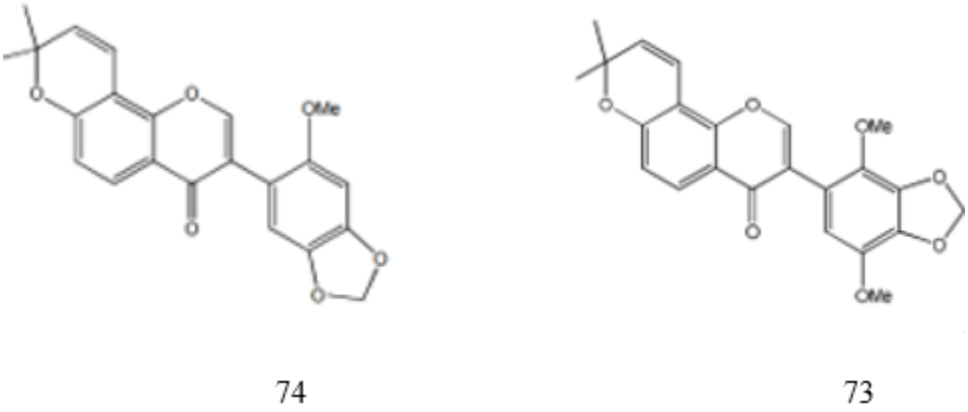


a b c

a: part of the structure which the two compounds had in common b: part which is present in compound 10CH-03-I (compound 74) and c: part found in compound 10CH-03-II (compound 73)

In ring B of compound 74, two para-oriented aromatic singlets were displayed at δ_H 6.84 (H-2'), 6.02 (H-5') with δ_C 111.2 for C-2' and 110.1 for C-5'; methoxyl group (δ_H 3.84; δ_C 56.9) is attached

to C-6'. The methylenedioxy group (δ_H 5.95; δ_C 101.4) was then placed at C-3'/C-4' accounting for the para orientation of the two protons, H-2'' and H-5''. Therefore, compound 74 was characterized as jamacin, previously isolated from the stem bark of *Millettia ferruginea subsp. Darassana* [210]. In ring B of compound 73, two para-oriented aromatic methoxy were displayed at C-2' and C-5'with δ_C 141.2 and 139.1, respectively and one aromatic singlet was displayed at δ_H 6.52 with δ_C 110.0 for C-6'. The methylenedioxy group (δ_H 6.02; δ_C 101.8) was then placed at C-3'/C-4' accounting for the para orientation of the two methoxy groups. Therefore, compound 73 was characterized as ferrugone, previously isolated from the stem bark of *M. ferruginea subsp. Darassana* [210] (Fig. 4.3).



74 73

Figure 4.3: Structure of jamacin (74) and ferrugone (73)

Table 4.2: ^{1}H ($CDC1_3$, 500.132 MHz) and ^{13}C ($CDCl_3$, 125.77 MHz) NMR data of compound 74 and 73 (chemical shift, δ in ppm and multiplicity, J in Hz)

Position	Compound 74		Compound 73		Position	Compound 74		Compound 73	
	δ_H (m, J)	δ_C	δ_H (m, J)	δ_C		δ_H (m, J)	δ_C	δ_H (m, J)	δ_C
2	7.91(s)	153.8	7.91(s)	153.4	2'		141.2		139.1
3		122.0		121.9	3'	6.84 (s)	110.1		137.1
4		175.8		175.8	4'		138.9		137.1
4a		118.4		118.3	5'		153.0		141.2
5	8.05(d, J 9)	126.8	8.05(d, J 9)	126.7	6'	6.83 (s)	95.5	6.52 (s)	110.2
6	6.86 (d, J 9)	115.1	6.86 (d, J 9)	115.1	2''		77.7		77.7
7		153.0		152.4	3''	5.72 (d,J 10.2)	126.7	5.72 (d,J 10.2)	126.7
8		109.3		109.3	4''	6.81(d J 10.2)	115.1	6.81(d, J 9)	115.1
8a		157.2		157.2	2 X CH_3	1.5 (s)	28.1	1.5(s)	28.1
1'		118.3		118.3	-CH_2O-	5.97 (s)	101.9	6.02 (s)	101.9
2'- 0CH_3	3.73 (s)	56.9	3.87 (s)	60.2	5'- 0CH_3	--	---	3.85 (s)	56.9

4.1.4 Barbigerone (83)

Compound 10CH-04 was isolated colorless solid from chloroform fraction. ESI-MS analysis gave a molecular ion peak $[M+2H]^+$ at m/z 396.2 coresponding to a molecular formula $C_{23}H_{22}O_6$. The ^{13}C and DEPT spectra showed 23 carbons coresponding to three methoxyl, two methyl, seven methines and eleven quaternary carbons. A singlet olefinic peak at δ_H 7.97 in the ^1H NMR spectrum, and the corresponding carbon peak at δ_C 153.9 (oxygenated sp^2 carbon) indicated that compound 83 is an isoflavone derivative. Both ^1H and ^{13}C NMR spectra (Table 4.4) revealed the presence of three methoxyl (a singlet peaks at δ_H 3.78, 3.86 and 3.93, and δ_C 58.9, 56.2, and 56.6) and a 2,2-dimethylchromene [δ_H 7.2 (d, J 9.6 Hz, H-3''), 6.82 (d, 9.6 Hz, H-4''), 1.50 (s, H-5''and H-6'')] substituents on the isoflavone skeleton. The ^1H NMR spectrum further showed two ortho coupled (J 8.8 Hz) aromatic protons at δ_H 8.05 and 6.85 corresponding to H-5 and H-6, respectively in ring A, suggesting that C-7 and C-8 are substituted. Moreover, the existance of the olefinic doublets at δ_H 5.71 and 6.82 and of the 2,2- dimethylchromene ring with δ_C 157.2 (C-7), 109.4 (C-8) and 152.4 (C-8a) allowed the placement of the 2,2-dimethylchro mene group at C-7/C-8 of the ring A. This means all the three methoxyl groups are located in ring B. The appearance of two singlets aromatic protons at δ_H 6.62 and 6.95 in this ring indicated that the two aromatic protons were para- oriented. Two singlets of aromatic protons at δ_H 6.62 and 6.95 were then assigned to

H-3’ and H-6’, respectively. The exact placement of three methoxyl groups at $ð_H$ = 3.78, 3.86 and 3.93, and $δ_C$= 58.9, 56.2, and 56.6 were there fore at C-2’, C-4’ and C-5’, respectively.

Thus, based on this data and comparsion with reported data [211], compound 83 was characterized as 2',4',5'-trimethoxy- 2",2"-dimethylpyrano [5",6":7, 8] isoflavone, trivial name barbigerone, a compound previously reported from the stem bark of *M*. ferruginea ssp. darasana [211] and seeds of *M. pachycapa* (273).

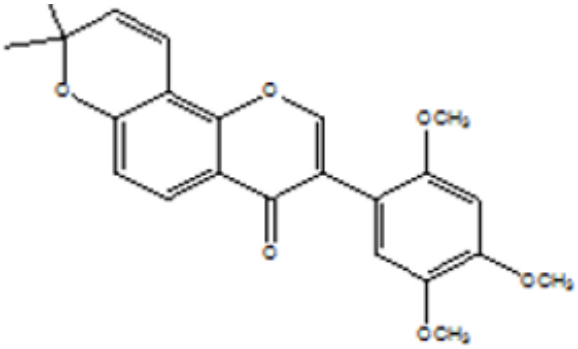


Figure 4.4: Structure of barbigerone (83)

Table 4.3: ^{1}H ($CDC1_3$, 500.132 MHz) and ^{13}C ($CDCl_3$, 125.77 MHz) NMR data of compound 83 and Barbigerone [211] (chemical shift, ***δ*** in ppm and multiplicity, J in Hz)

Posi tion	Compound 83		Barbigerone [211]		Position	Compound 83		Barbigerone [211]	
	$δ_H$ (m, J)	$δ_C$	$δ_H$ (m, J)	$δ_C$		$δ_H$ (m, J)	$δ_C$	$δ_H$ (m, J)	$δ_C$
2	7.97 (s)	153.9	7.97(s)	153.9	4’		149.7		149.8
3		121.5		121.5	5’		143.0		143.1
4		175.87		175.6	6’	6.95 (s.)	115.3	6.95 (s)	115.3
4a		118.4		118.5	2’’		77.6		77..6
5	8.05 (d,J = 8.8)	126.1	8.05 (d, J= 8.8)	126.7	3’’	5.72(d, J 9.6)	130.2	5.72 (d, J 9.6)	130.2
6	6.85 (d,J =8.8)	115.1	6.85 (d, J= 8.8)	115.1	4’’	6.82 (d, J 9.6)	115.1	6.82 (d, J 9.6)	115.1
7		157.1		157.2	5’’	1.50(s)	28.1	1.49 (s)	28.1
8		109.3		109.3	6’’	1.50 (s)	28.1	1.49 (s)	27,1
8a		152.3		152.4	2’-OCH_3	3.78 (s)	58.9	3.77 (s)	56.9
1’		112.2		112.4	4’- OCH_3	3.93 (s)	56.1	3.93 (s)	56.2
2’		151.9		151.9	5’- OCH_3	3.86 (s)	56.5	3.85 (s)	56.6
3’	6.62 (s,)	98.3	6.62 (s,)	98.3	--	---	----	--	--

All spectra run in CDCI3; ^{1}H NMR at 500.132MHz and ^{13}C NMR at125.77MHz.

4.1.5 Ichthynone (75) and 6'-hydroxyl calopogonium isoflavone B (10CH-05-II)

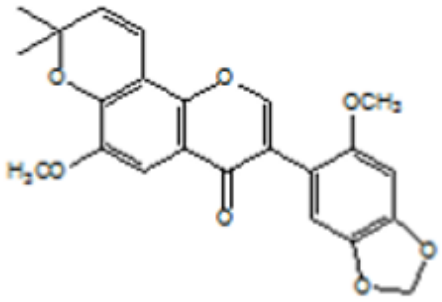
10CH-05, was isolated as an amorphous white powder from chloroform fraction. The positive ESI-MS gave a 100% peak at m/z = 410.13 $[M + 2]^+$ (calcd. for $C_{23}H_{20}O_7$ + 2H), for which $[M]^+$ 408.425 is compatible with the molecular formula $C_{23}H_{20}O_7$ (cal. for 408.425), and a second 100% peak at m/z = 365.23$[M + 1]^+$ (calcd. for $C_{21}H_{16}O_6$ + H), for which $[M]^+$ 364.338 is compatible with the molecular formula $C_{21}H_{16}O_6$. This ESI-MS data indicated that it is actually contained mixtures of two compounds and not just one compound.

1D NMR data (Table 4.5), analysis confirmed the existence of paired equivalent 1D NMR spectral data of ^1H (δ_H 7.91(s) and 7.91 (s), H-2), two hydrogens oxygenated methine and similarly the ^{13}C NMR revealed two oxygenated methine signal at δc 153.8 and 153.4; two quaternaries at δc 122.02 and 122.0; and two carbonyls at δc 175.8 and 175.7, which is consistent with two isoflavone skeletons. In agreement with what was observed in the ESI-MS data. The ^1H and ^{13}C NMR spectral data further revealed the presence of paired 2,2- dimethylpyrano signal at δ_H (5.72 d, J 10.2 and 6.87 d, J 10.2) and δ_C (130.3 and 115.1) with a singlet at δ_H 1.50 integrating for 12 protons and methylenedioxy signal at δc (101.3 amd 101.8), and δ_H (5.95 and 6.02) substituents constituting common structural feature.

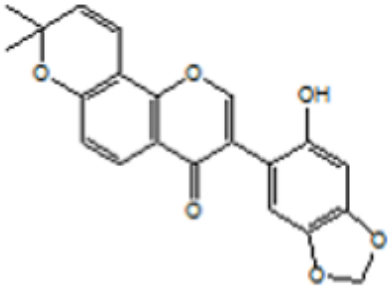
The ^1H and ^{13}C NMR spectral data analysis for compound 75, both ^1H and ^{13}C NMR spectra (Table 4.5) revealed the presence of two methoxyl (a singlet peaks at δ_H 3.86 and 3.86, and δ_C 56.0 and 60.2). The existence of two methoxyl substituents is compatible with the molecular formula $C_{23}H_{20}O_7$. The 1D NMR spectral data further revealed the presence of three singlets aromatic proton for compound 75, which allows one methoxy in ring A and the second methoxy in ring B. Further, it confirms the placement of the methylenedioxy at C-3'/C -4', one methoxyl at C-6' in ring B and one methoxyl at C-6 in ring A. Thus, based on this data and comparison with reported data [210], compound 75 was characterized as trivial name ichthynone (75), a compound previously reported from an ethanolic extract of the bark of *M. ferruginea subsp, darassana* [210].

The ^1H and ^{13}C NMR spectral data analysis for 10CH-05-II revealed the presence of a methylenedioxy group (δ_H 5.95, s; δ_C 101.37) at C-3'/C -4', two doublets aromatic proton at C-5 and C-6 (δ_H 8.06 and 6.87), two singlets aromatic proton at C- 2' and C-5' δ_H (6.85, s and 6.52 s) and δ_C (126.7 and 153.8) and hydroxyl group at C-6' (δ_H 6.62, s; δ_C 130.3). Thus, based on this

data, compound 10CH-05-II was characterized as 6'-hydroxyl calopogonium isoflavone B, a new compound isolated from *Millettia* species.



75



10CH-05-II

Figure 4.5: Structure of Ichthynone (75) and 6'-hydroxyl calopogonium isoflavone B (10CH-05-II)

Table 4.4: ^{1}H ($CDC1_3$, 500.132 MHz) and ^{13}C ($CDCl_3$, 125.77 MHz) NMR data of compound 75 and 10CH-05-II (chemical shift, δ in ppm and multiplicity, J in Hz)

Position	Compound 75		10CH-05-II		Position	Compound 75		10CH-05-II	
	δ_H (m, J)	δ_C	δ_H (m, J)	δ_C		δ_H (m, J)	δ_C	δ_H (m, J)	δ_C
2	7.91(s)	153.8	7.91(s)	153.4	4'		137.1		137.1
3		122.0		122.0	5'		139.1		139.1
4		175.8		175.7	6'	6.85(s)	95.8	6.85(s)	95.8
4a		118.4		118.3	2''		77.7		77.6
5	8.04 (s)	111.2	8.06(d, J 8.8)	126.7	3''	5.73 (d, J 10.2)	115.0	5.70 (d, J 10.2)	115,1
6		152.4	6.87(d, J 8.8)	115.0	4''	6.87 (d, J 0.2)	130.3	6.8 (d, J=10.2)	130.3
7		152.9		148.4	5''-CH_3	1.5(s)	28.1	1.5(s)	28.1
8		109.2		109.2	6 -OCH_3	3.86(s)	60.2	-	-
8a		157.2		157.7	2'-OCH_3	3.86(s)	56.0	-	-
1'		118.3		118.4	-OCH_2O-	5.95(s)	101.3	6.02(s)	101.8
2'		153.4		153.4	2'-OH	-	-	6.562(s)	130.3
3'	6,02 (s)	111.2	6,52 (s)	111.2	-	-	-	-	-

All spectra run in CDCI3; ^{1}H NMR at 500.132 MHz and ^{13}C NMR at 125.77 MHz.

4.2 Anticancer Activities

4.2.1 Anticancer Activities of the Plants Methanol Extract and Solvent Fractions

The results of the *in vitro* cytotoxicity using SRB assay are presented herein based on the recommendation of the National Cancer Institute (NCI, USA) plant screening program that a crude extract is generally considered to have *in vitro* cytotoxic activity and considered promising for purification of a crude extract, if the IC_{50} value following incubation between 48 and 72 h, is less than 20 µg/ml and less than 4 µg/ml for pure compounds [274]. This cut-off point for good cytotoxic compound has also been defined less than 10 µM [275]. Additionally, the activity rating and recommending for further study is based on comparision with the anticancer activity of anticancer drug etoposide that evaluated under the same condition with that of plant extracts.

Prior to this study, no report of anticancer activity of *M. ferrguinea, A. sinana* and *A. pirottea*, wild endemic medicinal plants to Ethiopian, was seen from extensive literature search. Therefore, the present study is the first report on anticancer activity of the extracts/ solvent fractions of these plants and isolated active components from *M. ferrguinea* seed against A 549, A2780, SNU 638, PANC-1 and MIP-PaCa-2 cell lines.

4.2.1.1 Anticancer Activities of the Methanol Extracts and Solvent Fractions of *M. ferruginea* Seed.

The cytotoxic activity of methanol extract (MeOH), *n*-hexane fraction (Hxf), chloroform fraction (CHf) and *n*-butanol fraction (Buf) of *M. ferruginea* seed on A 549, A2780, MIA-Paca-2 and SNU-638 cancer cell lines were investigated *in vitro* using SRB assay method at different concentrations including 0.1, 0.3, 1.0, 3.0, 10.0, and 30.0 µg/ml. Standard anticancer compound etoposide was served as reference compound. The results are given in Table 4.6 and Figure 4.6.

As can be seen in Fig. 4.6 and Table 4.6, methanol extract and its solvent fractions of *M. ferrguinea* seed exhibited anti-cancer activities *in vitro* with different degrees of potency in a dose-dependent manner. The percentage growth inhibition was found to be increasing with increasing concentratio n of test compounds. The methanol extract, n-Hxf and CHf exhibited potent and broad cytotoxicity against A549, A2780 and SNU638 cell lines with IC_{50} value ranging from 0.07- 0.50 µg/ml. However, Buf was found to be weakly active as cytotoxic agents against all four cancer cell lines with IC_{50} value > 30 µg/ml. CHf exhibited exceptionally the highest cytotoxicity against A549, A2780 and SNU638 cell lines with IC_{50} values 0.15, 0. 11 and 0.07 µg/ml, respectively. The MeOH extract, *n*-Hxf and CHf were found to be significant against MIA-PaCa-2 cell lines with

IC_{50} value 0.5, 13.66 and 2.53 µg/ml, respectively. MeOH extract, Hxf and CHf were more potent as nearly from 1.5-fold to 8-fold cytotoxic IC_{50} values than reference standard anticancer drug etoposide in killing A549. The sensitivity of extract/fractions and etoposide in killing A549 cancer cells decreases in the order: CHf > MeOH > Hxf > etoposide > Buf. MeOH extract and CHf were more potent and sensitive by 2-fold and 5-fold cytotoxic IC_{50} values, respectively than etoposide in killing A2780. The sensitivity of extract/fractions and etoposide in killing A2780 cancer cells decreases in the order: CHf > MeOH > etoposide > Hxf > Buf. CHf was more potent by 3.5-fold cytotoxic IC_{50} values than etoposide in killing SNU-638. The sensitivity of extract/fractions and etoposide in killing SNU 638 cancer cells decreases in the order: CHf > etoposide > MeOH > Hxf > Buf. MeOH extract was more potent as nearly 2-fold cytotoxic IC_{50} values than etoposide in killing MIA-PaCa-2. The sensitivity of extract/fractions and etoposide in killing MIA-PaCa-2 cancer cells decreases in the order: MeOH > etoposide > CHf > Hxf > Buf. The sensitivity of extracts against all four cancer cell lines decreased in the order: CHf > MeOH > Hxf > Buf. The sensitivity of cancer cells to the extracts decreases in the order: A549 > SNU-638 > A2780 >MIP-PaCa-2.

Table 4.5: Net growth as percent of control and IC_{50} value for *M. ferruginea* seed extract /solvent fractions against the cancer cell lines.

Cells		Net growth as % of control						IC_{50}
	Concentration (µg/ml)	0.1	0.3	1.0	3.0	10.0	30.0	
A549	MeOH	70.44	48.65	15.59	9.29	5.89	4.76	0.25
	Hxf	93.34	80.95	39.75	22.58	9.65	-31.04	0.83
	CHf	64.79	29.36	4.96	-57.36	-42.80	-58.68	0.15
	Buf	102.10	97.10	94.75	98.26	97.85	58.83	> 30.0
A2780	MeOH	77.36	51.76	23.67	15.02	8.51	3.80	0.34
	Hxf	97.13	90.07	58.18	47.52	8.27	-67.81	1.71
	CHf	51.34	28.97	5.89	1.56	-13.30	-13.27	0.11
	Buf	103.31	100.93	106.26	105.51	95.30	89.25	> 30.0
MIA-PaCa-2	MeOH	66.45	52.24	45.13	33.65	27.45	3.81	0.50
	Hxf	97.35	98.66	99.04	91.04	72.80	7.34	13.66
	CHf	98.72	98.31	69.28	52.88	16.98	-69.56	2.53
	Buf	98.29	99.75	99.89	93.13	98.72	64.05	> 30.0
SNU-638	MeOH	72.42	47.66	18.08	9.97	6.95	6.08	0.26
	Hxf	100.64	92.64	60.17	33.08	0.93	-40.96	1.48
	CHf	37.92	14.31	0.73	-23.85	-18.37	-25.53	0.07
	Buf	97.81	99.08	92.95	96.01	95.13	59.63	> 30.0

Date of measurement: MeOH on 2017/01/30, CHf and *n*-Buf on 2017/01/31, *n*-Hxf on 2017/ 02/ 01.

Solubility: Good. The results are expressed as the mean of 3 different experiments.

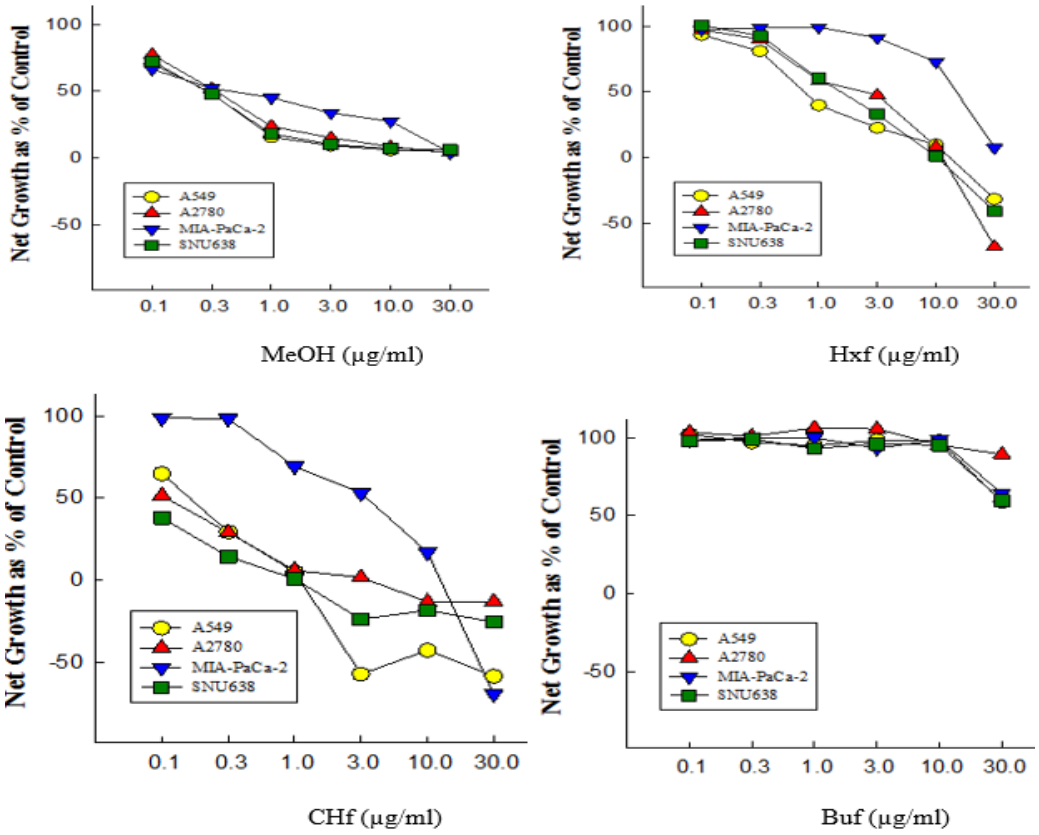


Figure 4.3: Dose response effects of MeOH extract and solvent fractions of *M. ferruginea* seed on the viability of the cell lines.The results are expressed as the mean of 3 different expreiments**.**

Compared to control by using SBN assay, methanol extract showed significantly strong cytotoxicity against all the tested cancer cell lines in broad concentration range between 0.3 µg/ml and 30.0 µg/ml with 50.76 to 96.20 percent of cell growth inhibition. This extract had the highest inhibitory effects against all tested cancer cell lines at 30 µg/ml. The sensitivity of cells to the MeOH extract was in the order of A549 > SNU638 > A2780 > MIP-PaCa-2.

The *n*-hexane fraction exerted cytotoxic effect against A549 and A2780 cancer cell lines in concentration range between 1.0 µg/ml and 30.0 µg/ml, against SNU-638 between 3.0 µg/ml and 30.0 µg/ml and against MIA-PaCa-2 between 10.0 µg/ml and 30 µg/ml. This fraction had the highest inhibitory effects on all four tested cancer cell lines at 30 µg/ml. The sensitivity of cells to the *n*-HxF was in the order: A549 > SNU-638 > A2780 > MIP-PaCa-2.

The chloroform fraction showed extremely potent cytotoxicity on the A549 and A2780 cell lines in concentration range between 0.1µg/ml - 30.0 µg/ml, on SNU-638, 0.10 µg/ml - 30.0 µg/ml and

on MIP-PaCa-2 cell line 0.30 µg/ml - 30.0 µg/ml. All fractions had the highest inhibitory effects on all cell lines at 30.0 µg/ml. The sensitivity of cells to the CHf was in the order: SNU638 > A2780 > A549 > etoposide > MIP-PaCa-2.

The Buf had no or weak inhibitory activity against all cell lines tested. The percentage cell growth inhibition was less than 41.17% with IC_{50} vaues of > 30 µg/ml on all the cell lines.

Based on the literature, *M. ferrguinea* seed exhibited high anti-proliferative activity that has not been observed even for *M. pinnata*, a plant well known for its several medicinal properties with the most potent cytotoxic activity against many cancer types than species in the *Millettia* genus [276]. For instance, a previous study has reported that chloroform leaf extract of petroleum ether and ethyl acetate fractions of *M. pinnata* exhibited cervical cell growth inhibitory activity with IC_{50} value of 552 and 91 µg/ml, respectively [277]. Some extracts of Chinese herbs showed strong toxicity against many cancer types, such as methanol extract of the *Adlay* seed, known medicinal plant inhibited the proliferation of A549 cancer cells with an IC_{55}-IC_{65} values of 100 µg/ml [278], acetone extract of the root of *Angelica sinensis* exhibited anti-proliferative effects in A549, HT29 and J5 human cancer cells with IC_{50} values ranging from 35-50 µg/ml, acetone extract of stem bark of *Physocarpus intermedius* Schn exhibited anti-proliferative effects in A549 with an IC_{50} values of 3.7 mg/ml [279]. Methanol extract of *C. roseus* leaves, a plant well known for its several medicinal properties**,** has shown anticancer activity against MCF-7 cell line with IC_{50} values of 15 µg/ml [280]. Other medicinal plants have been reported like extracts of *Ononis hirta, Inula viscose* and *Zingiber officinale* inhibited growth of A549 cancerous cells with IC_{50} values of 27.96 µg/ml, 15.78 µg/ml and 34.8 µg/ml respectively [281]. Solamargine [282] is an alkaloid isolated from an herb, *Solanum incanum*. It was found to be a powerful cytotoxic agent in four human lung cancer cell lines with IC_{50} value ranging from 3.0 - 7.2 µM. Kim et al. [283] found that 3-O-caffeoyloleanolic acid, betulinic acid, and the methyl ester of euscaphic acid from the stem bark of *Physocarpus intermedius* Schn. (Rosaceae), in order of decreasing potency, were the most active in vitro against A549 cells with ED_{50} (median effective dose) values of 1.6, 2.0, and 3.7 mg/ml, respectively. It was reported that acacetin (5, 7-dihydroxy-4'-methoxyflavone) can inhibit A549 cell proliferation in a dose-dependent manner with IC_{50} value 9.46 µM [285]. A study reported that 8-epi-xanthatin and its epoxide, two xanthanolide sesquiterpene lactones from the methanolic extract of the leaves of *Xanthium strumarium* L. (*Asteraceae*), potently inhibited

proliferation of cultured human tumor cells, including A549 cells with IC_{50} values of 4.5 and 3.0 µM, respectively [286].

MeOH extract, Hxf and CHf showed a significant cell growth inhibitory effects against A549, A2870, MIP-PaCa-2 and SNU 638 cell lines comparable to that observed for purified compounds in clinical trials silvestrol, clinical use paclitaxel and camptothecin [287], and in advanced preclincal trals such as neopeltolide [288], spiruchostatin B [289], smplocamide A [290], myricetin (flavonol aglycones) and tangeretin [291]. This study revealed that extracts of *M. ferruginea* seed had high anti-proliferative component(s) than extracts of many medicinal plants and purified compounds in advanced preclincal trals, and had comparable component(s) to many of compounds in clinical use. The results support that bioactive pure compound (s) isolated from Hxf, CHf and MeOH extract of *M. ferruginea* seed could definitely exhibit higher anti-cancer activity and inhibiting cancerous cell growth more effectively than standard control compound etoposide and many of compounds in clinical use, clinical trials and advanced preclinical trials. Thus purified compounds from these fractions could serve as the basis for the development of a new group of cancer chemotherapeutics and certainly holds great promise towards good active leads.

Overall, this study evaluates that chloroform fraction, n-hexane fraction and MeOH extract had potential cytotoxic activity against all the tested cancer cell lines, indicating the presence of cytotoxic compounds in these extracts. In the NCI of US plant screening program, these plant extracts were identified as promising anticancer products and should be promising for continued research in these cells and screened for more cancer cell lines [274]. Therefore, their constituents may be a highly effective candidate as a future anti-cancer drug.

Table 4.7: Net growth as percent of control and IC_{50} (in µM) value for etoposide against the cancer cell lines. The results are expressed as the mean of 3 different expreiments.

Cells	Date of measurement	Concentration (µg/ml) / Net Growth as % of Control						IC_{50}
		0.1	0.3	1.0	3.0	10.0	30.0	
AS49	2017/01/31	90.74	57.74	8.84	0.44	-55.88	-88.82	0.34
	2017/02/01	98.60	100.18	70.77	0.82	-66.36	-92.70	1.18
A2780	2017/01/31	95.62	77.29	25.50	11.19	4.22	-92.56	0.58
	2017/02/01	96.50	48.22	25.27	10.40	4.72	-89.19	0.37
MIA-PaCa-2	2017/01/31	92.70	86.91	32.28	11.41	6.21	-24.32	0.72
	2017/02/01	99.70	92.04	64.36	23.64	11.86	1.56	1.48
SNU-638	2017/01/31	88.02	37.34	5.81	0.08	-37.06	-77.91	0.24
	2017/02/01	87.71	22.86	10.77	2.93	-9.63	-67.90	0.20

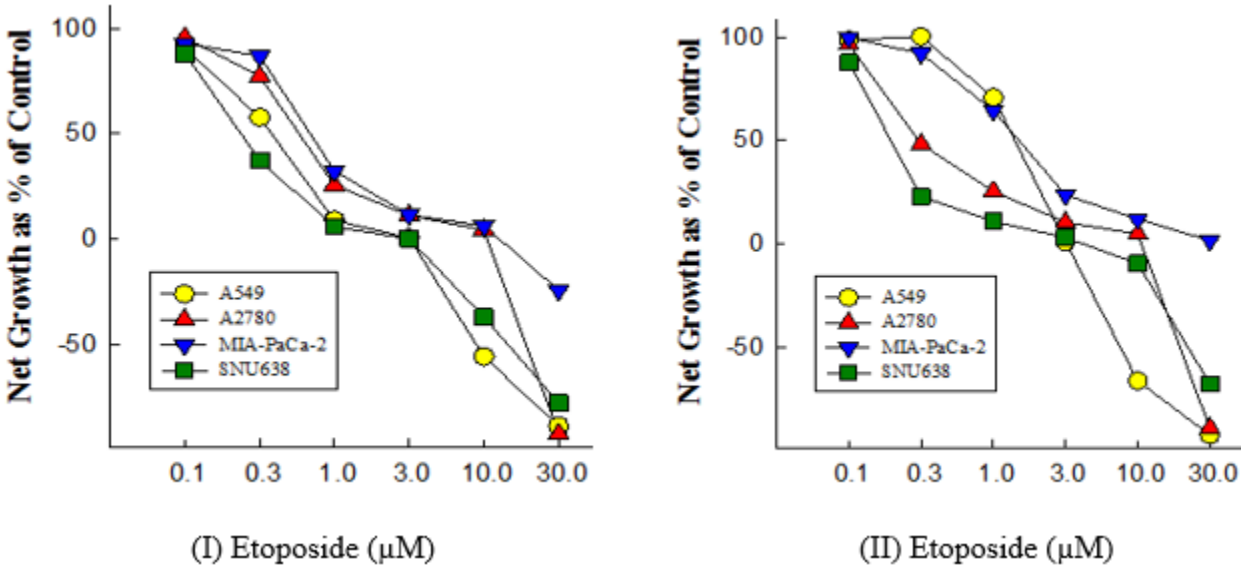


Figure 4.4: Dose response effects of reference etoposide, (I) 2017/01/31 and (II) 2017/02/01, on the viability of cancer cell lines. The results are expressed as the mean of 3 different experiments.

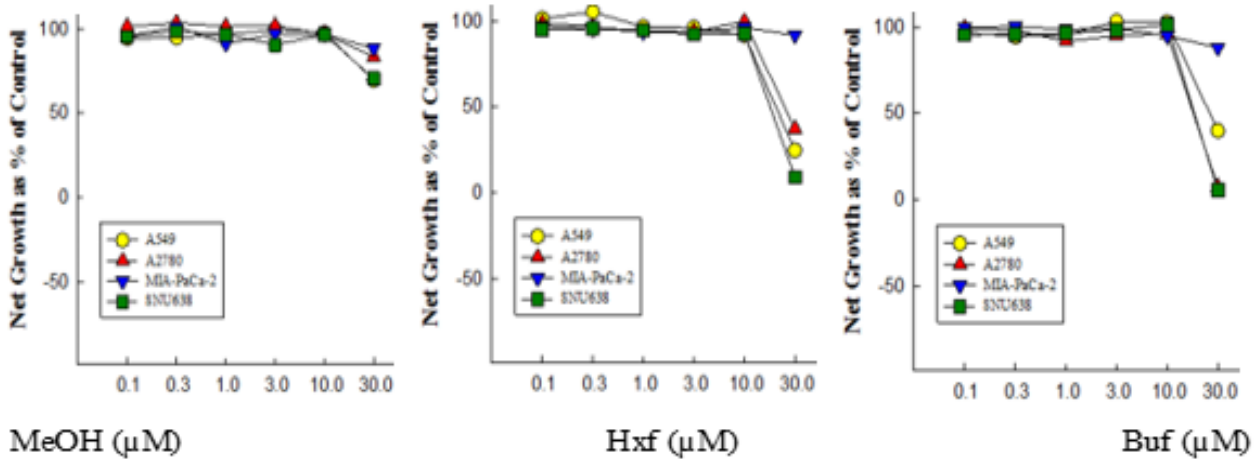
4.2.1.2 Anticancer Activities of the Methanol Extract and Solvent Fractions of *Aloe sinana* root.

The cytotoxic activity of methanol extract and its Hxf, CHf, EAf and Buf of *A. sinana root* were investigated in vitro against A549, A2780, MIA-Paca-2 and SNU 638 cancer cell lines at different concentrations including 0.1, 0.3, 1.0, 3.0, 10.0, and 30.0 µg/ml using SRB assay to determine cell

growth inhibition as percent of control and IC_{50} (50% growth inhibition). Standard anticancer drug etoposide (Table 4.7 and Figure 4.7) was used as reference compound. The results are given in Table 4.8 and Figure 4. 8.

Table 4. 6: Net growth as percent of control and IC_{50} for *A. sinana* root extract/solvent fractions against the cancer cell lines.

Cells	Solvent	Concentration (µg/ml)						IC_{50}
		0.1	0.3	1.0	3.0	10.0	30.0	
A549	Me	94.39	94.81	96.51	99.71	97.85	69.53	>30
	Hx	101.39	105.39	96.53	96.08	91.83	24.54	21.13
	CH	94.89	93.69	93.96	92.99	59.40	-61.81	10.26
	EA	95.94	100.54	96.64	101.76	101.76	23.96	27.54
	BU	96.85	94.36	95.51	103.11	102.67	39.90	29.14
A2780	Me	101.57	103.75	101.65	101.97	96.91	83.49	>30
	Hx	98.89	96.67	93.90	98.80	99.70	37.28	27.39
	CH	100.1	98.43	105.30	105.31	62.62	-33.98	10.37
	EA	98.43	104.22	100.07	103.72	100.49	50.96	>30
	BU	99.40	98.03	91.71	94.93	95.82	7.21	18.31
MIA-PaCa-2	Me	95.63	101.34	91.08	97.04	96.74	88.36	>30
	Hx	97.24	95.63	93.75	93.53	96.16	91.92	>30
	CH	98.60	96.57	101.86	97.68	96.73	73.97	>30
	EA	101.08	100.78	99.21	102.48	100.27	62.04	>30
	BU	99.44	100.39	98.38	99.01	94.91	88.02	>30
SNU-638	Me	95.44	98.58	96.70	90.52	96.61	70.49	>30
	Hx	94.91	95.51	94.58	92.32	92.69	8.94	17.74
	CH	93.02	98.61	93.71	91.47	43.69	0.47	8.82
	EA	96.48	88.20	92.63	92.12	97.06	-11.32	12.38
	BU	95.35	95.50	96.33	98.33	101.34	5.51	24.88



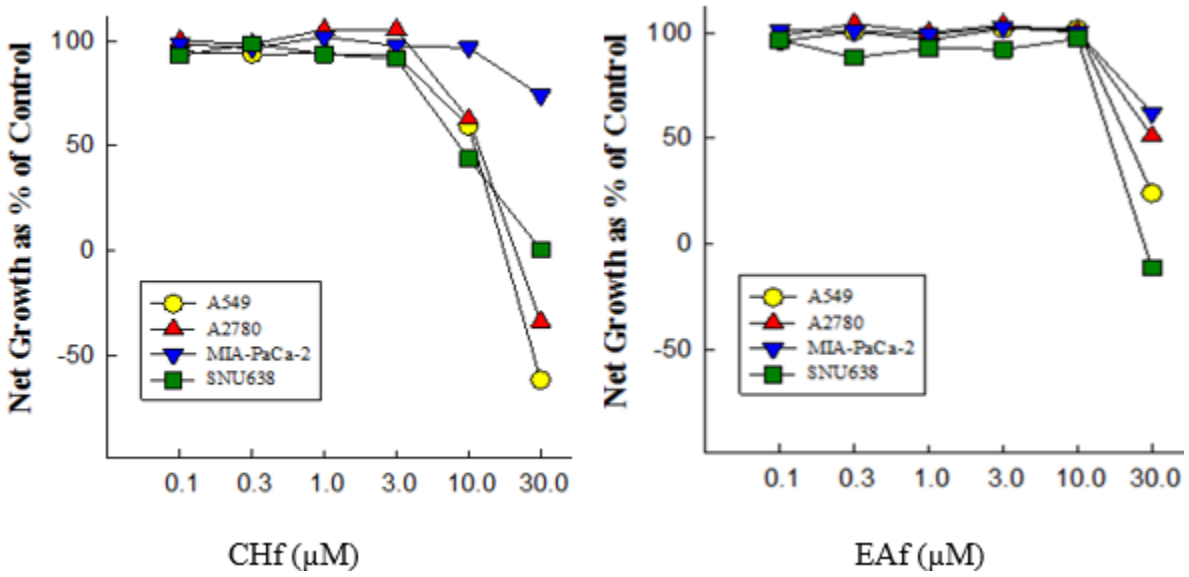


Figure 4.5: Dose response effects of MeOH extract and solvent fractions of *A. sinana* root on the viability of the cancer cell lines.

The result presented in Table 4. 8 and Figure 4.8 revealed that the extract/solvent fractions, showed anti-cancer activities *in vitro* wth different degrees of potency in a dose-dependent manner. The percentage growth inhibition was found to be increasing with increasing concentration of test compounds.

The CHf of *A. sinana* root effectively inhibited the growth of A549 and A2780 cell lines at concentrations between 10.0 µg/ml and 30 µg/ml and SNU-638 cell line at concentrations between 3.0 µg/ml and 30 µg/ml. Other solvent fractions, except MeOH, inhibited the growth of A549, SNU-638 and A2780 cell lines at slightly higher concentrations of 10.0 µg/ml. For MIA-PaCa-2, the SNB assay failed to predict an IC_{50} values because cell density was > 50% at all concentrations tested (Table 4.8). The sensitivity of cancer cell lines to the extract/ fractions decreases in the order: SNU-638 > A2780 > A549 > MIP-PaCa-2 (Table 4.8 and Figure 4.8). All solvent fractions except MeOH extract of *A. sinana* root exhibited strong to moderate cytotoxic activity against A549 and SNU-638 cell lines with IC_{50} values ranging from 8.82 - 29.14 µg/ml. The CHf, Buf and Hxf exhibited strong to moderate cytotoxic activity against A2780 cell line with IC_{50} values ranging from 10.37 - 27.39 µg/ml. It showed weak and/or no cytotoxic activity against MIP-PaCa-2 cell line with a maximum of 37.96% growth inhibition at highest concentration tested and IC_{50} value > 30 µg/ml. The CHf was the strongest and most toxic on A549, A2780 and SNU-638 cell lines with IC_{50} values of 10.26, 10.37 and 18.82 µg/ml, respectively.

The methanol extract and all solvent fractions were less potent and sensitive than etoposide in killing all the cancer cells tested. The sensitivity of extract/fractions and etoposide in killing cancer cells decreases in the order: etoposide > CHf > Hxf > EAf > Buf > MeOH.

Overall, this study evaluates that CHf, EAf, Buf and *n*-Hxf on A549 and SNU-638, and CHf, Buf and *n*-Hxf on A2780 have potential cytotoxic activity, indicating the presence of cytotoxic compounds in these fractions. In the NCI plant screening program, these plant extracts were identified as promising anticancer products and should be promising for continued research in these cells and screened for more cancer cell lines. Therefore, their constituents could serve as the basis for the development of a new group of cancer chemotherapeutics and certainly holds great promise towards good active leads.

4.2.1.3 Anticancer activities of Methanol extract and Solvent Fractions of *Aloe sinana* Leaves Latex.

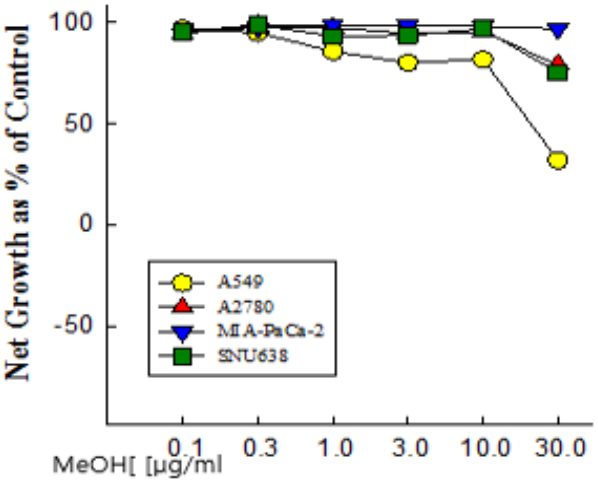
The cytotoxic activity of methanol extract, CHf, EAf and Buf of *A. sinana* leaves latex were investigated in vitro against A549, A2780, MIA-Paca-2 and SNU 638 cell lines at different concentrations including 0.1, 0.3, 1.0, 3.0, 10.0, and 30 µg/ml using SRB assay to determine % of cell growth inhibition and IC_{50}. Etoposide (Table 4.7 and Figure 4.7) was used as reference compound. The results are given in Table 4.9 and Figure 4.9.

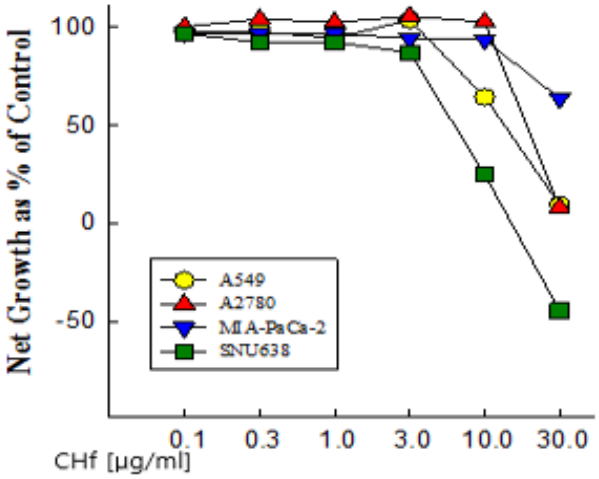
Table 4.7: Net Growth as percent of control and IC50 value for *A. sinana* leaves latex extract/ solvent fractions against the cancer cell lines.

Cells	Solvent	Concentration (μg/ml)						IC_{50}
		0.1	0.3	1.0	3.0	10.0	30.0	
A549	Me	96.88	95.07	85.72	80.17	81.86	31.89	20.19
	CH	97.66	97.84	94.48	103.82	64.42	9.40	12.54
	EA	101.80	96.37	98.72	102.54	98.64	74.80	>30
	BU	98.84	103.14	94.23	99.91	101.17	69.30	>30
A2780	Me	94.61	98.58	97.56	94.76	95.48	79.37	>30
	CH	100.31	104.24	102.59	105.59	102.80	8.06	25.58
	EA	103.77	101.05	103.02	99.64	104.63	101.34	>30
	BU	98.17	95.99	93.96	97.62	97.08	95.08	>30
MIA-PaCa-2	Me	96.52	97.38	99.03	98.68	98.29	97.01	>30
	CH	97.16	96.90	96.87	94.35	93.65	63.73	>30
	EA	97.66	96.72	98.55	98.81	97.45	92.57	>30
	BU	100.10	99.70	98.61	100.13	98.71	87.68	>30
SNU-638	Me	95.58	98.91	93.41	93.73	96.98	75.30	>30
	CH	96.71	92.45	92.47	87.06	24.82	-44.78	6.37
	EA	96.54	96.01	96.18	98.13	93.46	54.66	>30
	BU	95.55	93.99	93.87	96.01	84.98	39.77	24.22

Date of measurement: Me on 2017/01/30, Hx, CH, and BU on 2017/02/01, EA on 2017/01/31

Solubility: Good. The results are expressed as the mean of 3 different experiments





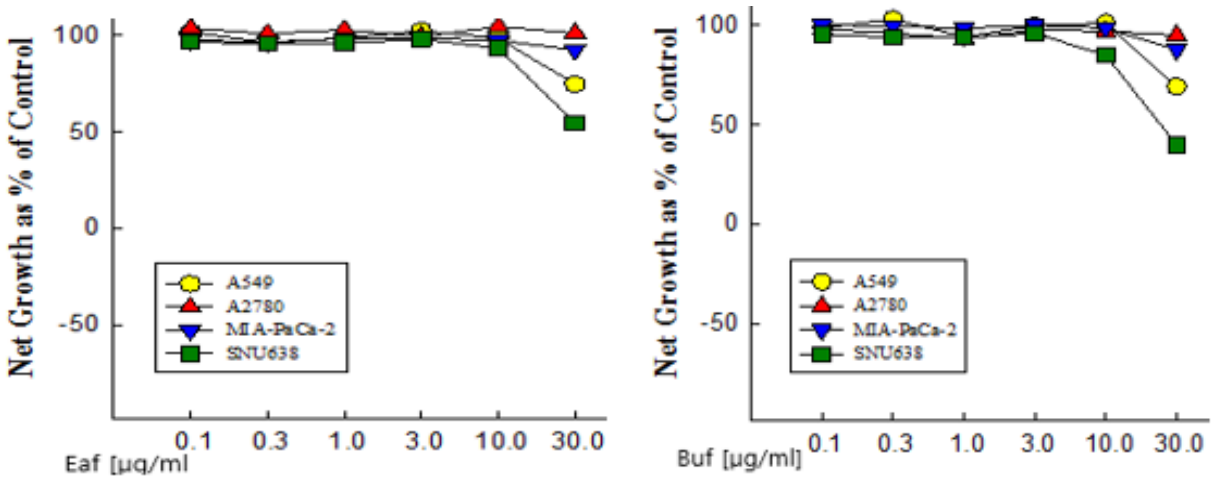


Figure 4.6: Dose response effects of MeOH extract and solvent fractions of *A.sinana* leaves latex on the viability of the cancer cell lines. The results are expressed as the mean of 3 different experiments.

The results in Table 4.9 and Figure 4.9 indicated that the extract/solvent fractions, showed anti-cancer activities *in vitro* with different degrees of potency in a dose-dependent manner. The percentage growth inhibition was found to be increasing with increasing concentration of test compounds. The MeOH extract and CHf exhibited strong cytotoxic activity against A549 cell line with IC_{50} values of 12.54 and 20.19 µg/ml, respectively. The CHf and Buf exhibited strong and moderate cytotoxic activity against SNU-638 cell line with IC_{50} values of 6.37 and 24.22 µg/ml, respectively. Only CHf exhibited moderate cytotoxic activity against A2780 cell line with IC_{50} values of 25.58 µg/ml. The extract/solvent fractions showed weak cytotoxic activity against MIP-PaCa-2 cell line with a maximum of 36.27% growth inhibition at highest concentration tested and IC_{50} value > 30 µg/ml. The CHf was the strongest and the most toxic on A549, A2780 and SNU 638 cell lines with IC_{50} values of 12.54, 25.58 and 6.37 µg/ml, respectively. It was found that the sensitivity of cancer cells to the extract/fractons decreases in the order: SNU 638 > A549 > A2780 > MIP-PaCa-2.

Overall, this study evaluates that MeOH extract and CHf against A549, CHf against A2780, and CHf and Buf against SNU 638 had potential cytotoxic activity, indicating the presence of cyto-toxic compounds in these fractions. Based on National Cancer Institute of U S reccommandation, these extract/ fractions of the leaves latex of *A. sinana* were significantly active ($IC_{50} \leq 20$µg/ml) [236] against A549, A2780 and SNU-638 cancer cell lines and considered promising for

purification and identification of biologically active substances..Thus, *A. sinana* leaves latex possesses significant anticancer activity supporting further extensive study to isolate and identify the specific bioactive molecules responsible for the observed activity and and could serve as the basis for the development of a new group of cancer chemotherapeutics and certainly holds great promise towards good active leads.

4.2.1.4 Anticancer Activities of the Methanol Extract and Solvent Fractions of *Aloe pirottae* Root.

The cytotoxic activity of MeOH extract, Hxf, CHf, EAf and Buf of *A. pirottae* root were investigated *in vitro* against A549, A2780, MIA-Paca-2 and SNU-638 cell lines at different concentrations including 0.1, 0.3, 1.0, 3.0, 10.0, and 30 µg/ml using SRB assay to determine % of cell growth inhibition and IC_{50}. Etoposide (Table 4.7 and Figure 4.7) was used as reference compound. The results are given in Table 4.10 and Figure 4.10.

Table 4.8: Net growth as percent of control and IC_{50} value of *A. pirottae* root extract/solvent fractions against the cancer cell lines.

A549	CH	103.17	103.42	99.51	104.03	98.59	12.83	21.33
	EA	97.82	99.69	96.95	101.63	94.72	49.76	29.89
	BU	98.76	99.15	94.33	100.81	97.20	80.65	>30.0
A2780	Me	97.30	94.94	93.92	93.51	94.90	79.02	>30
	Hx	96.95	92.22	99.00	92.46	87.37	31.34	21.70
	CH	103.29	103.36	103.14	104.20	104.05	8.10	25.67
	EA	97.70	98.95	98.71	100.54	100.57	52.07	>30
	BU	98.19	98.43	92.72	98.33	96.46	89.26	>30.0
MIA-PaCa-2	Me	100,33	98.93	98.40	96.83	93.09	86.92	>30
	Hx	99.27	98.59	98.30	100.10	99.38	74.44	>30
	CH	98.56	107.38	94.07	94.28	95.73	9.74	18.96
	EA	98.69	98.43	97.70	98.33	96.72	50.53	>30
	BU	00.35	98.77	99.13	83.25	100.60	56.75	>30.0
SNU-638	Me	95.61	95.44	86.22	87.45	89.38	72.68	>30
	Hx	96.01	96.09	76.34	92.44	72.45	14.92	14.69
	CH	95.31	97.76	95.73	95.84	85.50	0.20	12.88
	EA	110.93	94.31	96.37	93.85	63.05	11.18	12.47
	BU	95.13	93.65	96.33	88.07	86.20	69.14	>30.0

Solubility: Good: The results are expressed as the mean of 3 different experiments

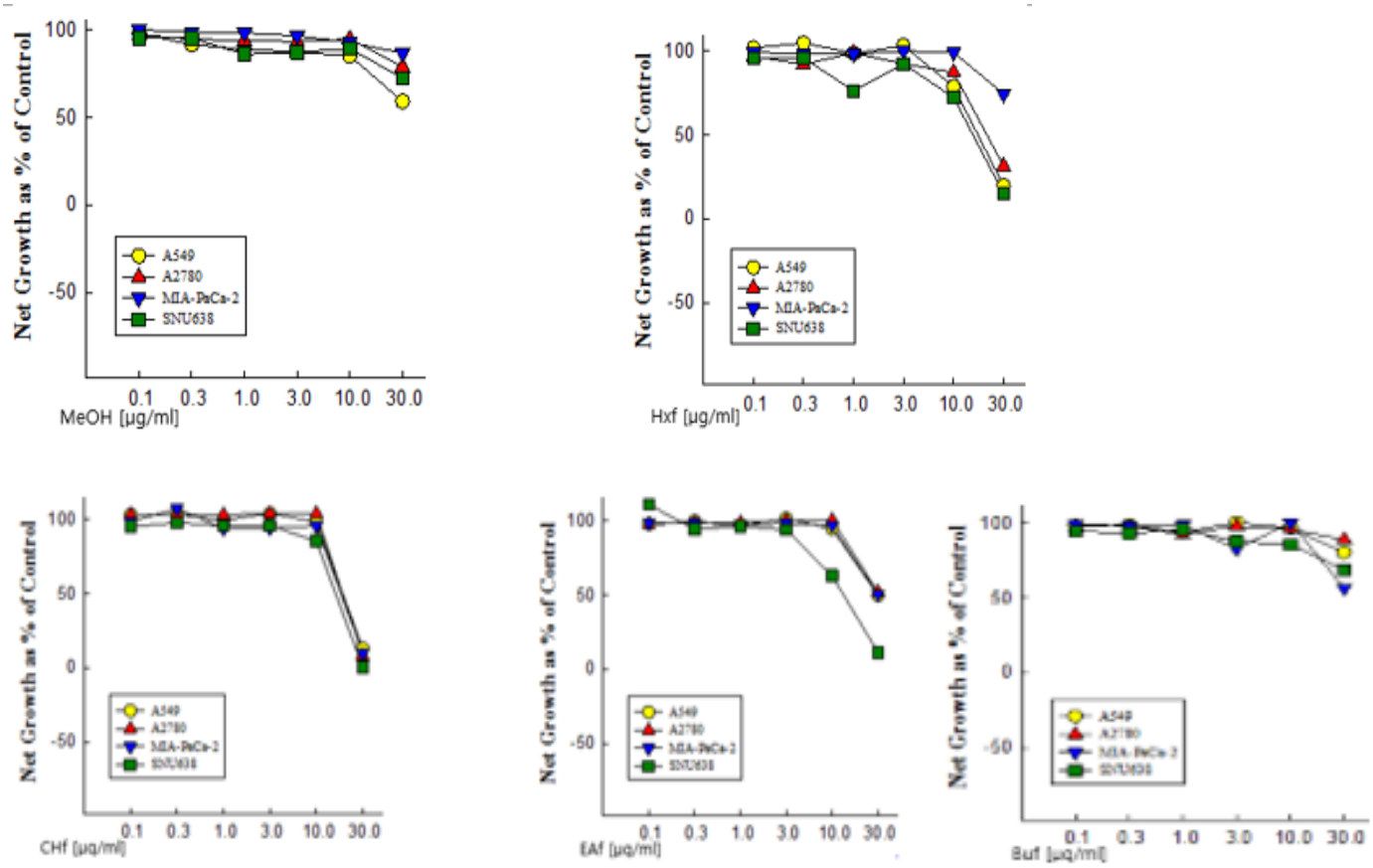


Figure 4.7: Dose response effects of MeOH extract and solvent fractions of *A. pirottae* root on the viability of the cancer cell lines. The results are expressed as the mean of 3 different experiments.

The result presented in Table 4.10 and Figure 4.10 showed that the extract/solvent fractions exhibited anti- MIA-PaCa-2, A2780, SNU-638 and A549 cancer cell lines in vitro with different degrees of potency in dose-dependent manner. The percentage growth inhibition was found to be increasing with increasing concentration of test compounds.

The Hxf, CHf and EAf exhibited strong to moderate cytotoxicity against A549 cell line with IC_{50} value of 17.06, 21.83 and 29.89 µg/ml, respectively. The Hxf and CHf exhibited moderate cytotoxicity against A2780 cell line with IC_{50} value of 21.7 and 25.67 µg/ml, respectively. CHf exhibited strong cytotoxicity against MIA-PaCa-2 cell line with IC_{50} value of 18.96 µg/ml. The EAf, CHf and Hxf exhibited strong cytotoxicity against SNU 638 cell line with IC_{50} value of 12.47, 12.88 and 14.69 µg/ml, respectively. However, MeOH extract was found to be weakly active as cytotoxic agents against all four cancer cell lines with IC_{50} value > 30 µg/ml. The extract and all solvent fractions were less potent and sensitive than etoposide in killing all the cancer cells tested.

The sensitivity of extract/fractions and etoposide in killing A549 and A2780 cell lines decreases in the order: etoposide > Hxf > CHf > EAf > MeOH > Buf. The sensitivity of extract/fractions and etoposide in killing MIA-PaCa-2 cell line decreases in the order: etoposide > CHf > EAf > Buf > Hxf > MeOH. The sensitivity of extract/fractions and etoposide in killing SNU-638 cell line decreases in the order: etoposide > EAf > CHf > Hxf > Buf > MeOH. The sensitivity of cancer cell lines to extract/fractions decreases in the order: SNU 638 > A549 > MIA-PaCa-2 > A2780.

MeOH extract and solvent fractions were less potent than etoposide as a cytotoxic agent in the all cancer cell lines tested. However, extracts/solvent fractions exhibited strong anti-proliferative activity than extract and purified compounds of plants described under 4.2.1.2 and 4.2.1.3. The study revealed that extracts of *A. pirottea* root had high anti-proliferative component(s) than extracts of many medicinal plants and purified compounds in advanced preclincal trals, and had comparable component(s) to many of compounds in advanced clinical trals. The results support that bioactive pure compound (s) isolated from CHf and MeOH extract of *A. pirottea* root could be inhibiting cancerous cell growth more effectively than many of compounds in clinical trials and advanced preclinical trials. Thus purified compounds from these fractions could serve as the basis for the development of a new group of cancer chemotherapeutics and certainly holds great promise towards good active leads.

Based on National Cancer Institute of US reccommandation, all extracts excecpt MeOH extract and Buf were significantly active ($IC_{50} \leq 30\mu g/ml$) [236] against all cancer cell lines tested and considered promising for purification and identification of biologically active substances. Thus, extracst of *A. pirottea* root possesses significant anticancer activity supporting further extensive study to isolate and identify the specific bioactive molecules responsible for the observed activity

4.2.2 Anticancer Activity of Isolated Compounds and Mixtures from Chloroform Fraction of M. *ferrguinea* Seed.

The cytotoxicity of the isolated compounds and mixtures from chloroform fraction of seed of *M. ferrguinea* were evaluated against A2780, MIA-Paca-2 and PANC-1 cancer cell lines by SRB assay to determine percent of growth inhibitory activity and the IC_{50} (50% growth inhibition). Results at different concentrations including 0.1, 0.3, 1, 3, 10, and 30 µg/ml are tabulated in Table 4.11.

Table 4.9: Net Growth as Percent of Control and IC_{50} value of pure compounds and mixtures from chloroform fraction of *M. ferrguinea* seeds against the cancer cell lines.

. Cells		Net Growth as Percent of Control						IC_{50}/ µg/ml	IC_{50}/ µM
	Conc.(µg/ml)	0.1	0.3	1.0	3.0	10.0	30.0		
A2780	CH-01	102.29	32.75	10.89	5.04	5.97	6.62	0.21	0.53
	CH-02	98.06	86.49	69.29	34.84	5.95	3.26	1.87	2.73
	CH-03	4.95	5.82	5.04	4.60	6.00	10.17	<0.1	< 0.12
	CH-04	37.73	7.73	4.30	3.46	3.88	4.79	<0.1	< 0.25
	CH-05	1.87	1.66	1.16	1.46	1.40	2.35	<0.1	< 0.12
	CH-06	14.70	9.36	4.28	2.73	5.74	13.55	<0.1	
	CH-07	73.27	68.19	3.63	2.20	2.67	5.02	0.41	
MIA-Paca-2	CH-01	102.8	94.92	94.95	92.98	-49.64	-27.21	4.27	11.23
	CH-02	97.93	105.83	104.34	85.10	15.55	-8.67	5.64	8.23
	CH-03	96.93	76.45	56.35	31.74	3.92	-12.09	1.33	1.68
	CH-04	97.33	91.19	80.88	77.31	19.48	-80-30	4.59	11.56
	CH-05	90.89	79.39	82.39	12.13	-6.17	-60.75	1.76	2.27
	CH-06	100.50	100.93	75.73	38.18	6.45	-58.04	2.04	
	CH-07	103.69	106.69	81.95	44.37	-15.58	-31.97	2.58	
PANC-1	CH-01	96.34	51.74	38.01	19.59	7.58	13.44	0.34	0.89
	CH-02	106.21	110.91	104.08	74.86	16.09	10.37	5.21	7.60
	CH-03	25.16	25.29	20.04	17.04	8.76	12.97	<0.1	< 0.12
	CH-04	92.47	45.73	32.84	23.26	18.77	14.30	0.28	
	CH-05	42.99	28.88	27.71	20.24	14.38	11.88	<0.1	0.705
	CH-06	62.25	31.89	28.76	6.28	7.45	8.67	0.15	< 0.12
	CH-07	91.32	41.54	32.52	14.36	6.95	13.23	0.26	

All compounds possessed good solubility. The results are expressed as the mean of 3 different experiments.

As per SRB assay (Table 4.11), the results indicated that the treatment of these three cancer cells with the compounds and mixtures significantly inhibited the growth of cancer cells in broad concentration range compared to etoposide. The % growth inhibition was found to be dose-dependent and increasing with increasing concentration of test compounds. All tested compounds exerted significantly high cytotoxicity against tested cell lines with IC_{50} values ranging from < 0. 1 - 5.21 µg/ml (< 0.122 – 11.56 µM). **T**he sensitivity of the cancer cell lines toward the isolates and mixtures decreases in the following order: A2780 > PANC-1 > MIP-PaCa-2.

The results indicated that all compounds exhibited high anti-cancer activity (IC_{50} < 0.10 - 1.87 µg/ml or < 0.12 – 2.73 µM) against A2780 cancer cell lines. All compounds except A10-CH-02

against A2780 cancer cell line exhibited more potency as nearly one and half to five times higher cytotoxicity than that of etoposide (IC_{50} 0.58 µM). In terms of cell growth inhibitions at all the concentrations and value of IC_{50}, the sensitivity of isolates towards A2780 decreases in the order: 10 CH–05 > 10CH-03 > 10 CH-06 > 10CH–04 > 10CH-07 > 10 CH–01 > 10CH–02. All compounds, except 10CH-02, and mixtures showed a significant cell growth inhibitory effects against A2870 cell lines comparable to that observed for purified compounds silvestrol (in clinical trials), clinical use paclitaxel and camptothecin, and advanced preclincal trals such as Neopeltolide, spiruchostatin, Symplocamide A, myricetin (flavonol aglycones) and tangeretin those mentioned above.

The results indicated that all compounds and mixtures exhibited strong inhibitory activity against MIP-PaCa-2 cell line with IC_{50} value ranging from 1.33 -5.64 µg/ml (1.38 – 11.56 µM). Compound 10-CH-03 (mixtures of two isoflavonoid compounds) exhibited a high activity, resulting most potent than etoposide (IC_{50} = 1.48 µM) and CHf of M. *ferrguinea* seed (IC_{50} = 2.53 µg/ml). The highest activity was recorded for 10-CH-03 with IC_{50} values of 1.33 µg/ml (1.38 µM) followed by 10-CH-05 with IC_{50} values of 1.76 µg/ml (2.27 µM). Compound 10CH-03 (mixture of two isoflavonoid) was more potent as nearly one and half to five times higher than that of 10CH-01 - 10CH-06. Compound 10CH-03, with five times higher anticancer activity, has two more methoxyl groups than that of 10CH-02. This probably suggested the importance of number of methoxyl functionality for higher cytotoxicity against cell lines. The activity of 10CH-03 is almost 1.5 times higher than functionally very related 10CH-05 but differ in number of methoxyl functional groups and positions of substituents. 10CH-03 has meta substituted methoxy, groups in one of the compound and one methoxyl groups in other. Whereas 10CH-05 two methoxyl in one of the compound, one on ring A and one on ring B, and on second compound one hydroxyl instead of methoxyl group. This probably suggested the importance of number of methoxyl functionality and position of substitution for higher cytotoxicity of 10CH-03 than 10CH-05. In terms of cell growth inhibitions at all the concentrations and value of IC_{50}, the sensitivity of isolates, mixtures and etoposide towards MIP-PaCa-2 cell line decreases in the order: 10 CH-03 > etoposide > 10CH- 05 > 10 CH-06 > CHf of M. *f* seed > 10CH–0 7 > 10 CH–04 > 10 CH–01 > 10CH–02.

Similarly, the results (Table 4.11) indicate that all compounds exhibited very strong inhibitory activity against PANC-1 cancer cell lines with IC_{50} value ranging from < 0.1 - 5.21 µg/ml (< 0.12

– 7.60 µM). All compounds inhibited cancer cell growth without toxicity to normal cell lines at all the concentration tested and abnormal increase in cell viability at highest concentration tested. 10CH-03 and 10CH-05 compounds exhibited high activity, resulting more potent than etoposide (IC_{50} = 1.28 µM) and CHf of *M. ferrguinea* seed (IC_{50} = 2.53 µg/ml). All the compounds excecpt compound 10CH-02 exhibited more potent inhibitory activities than CHf of *M. ferrguinea* seed. The activity of 10CH-03 and 10CH-05 was found almost fifty-two - fold to one and half–fold greater than that of the rest compounds and mixtures. This result follows the same cytotoxic activity trened as that of MIA-PaCa-2 except in cytotoxic strength. In terms of of % of cell growth inhibitions at all concentrations and value of IC_{50}, the sensitivity of isolates, mixtures and reference to PANC-1 decreases in the order: 10 CH-03 > 10CH–05 > 10CH–06 > 10CH–07 > 10CH–04 > 10CH–01 > etoposide > 10CHf > 10CH–02. In this case, compound 10CH-04 exhibited slightly stronger cytotoxic activity than compound 10CH-01. Both compounds have related structure but differ in number methoxy and methylenedioxy groups. Compound 10CH-01 has a methylenedioxy group at C-3'/4' where as 10CH-04 has two methoxyl group at C- 4'/ 5'. This indicates the importance of the methoxyl group at 4' and 5' than methylenedioxy for the observed activity difference. All compounds, except 10CH-02, showed a significant cell growth inhibitory effects against PANC-1 cell lines comparable to that observed for purified compounds silvestrol (in clinical trials), clinical use paclitaxel and camptothecin [287]. Further more, the fractions were found to be an extremely potent cytotoxin than purified compounds in advanced preclincal trals such as Neopeltolide [288], spiruchostatin B [289], Symplocamide A, myricetin (flavonol aglycones) and tangeretin [291].

Over all, compound 10-CH-03 exhibited the most potent and highest cytotoxic activity against the three cancer cell lines compared to other compounds, mixtures, 10CHf (CHf of *M. ferruginea* seed) and etoposide. Based on National Cancer Institute of US reccommandation, all isolates and mixtures were significantly active ($IC_{50} \leq$ 10µM) [274] against A2780 and PANC-1, and all isolates and mixtures except 10CH-01 and 10CH-04 against MIA-Paca-2 cancer cell lines and were identified as promising anticancer products and should be promising for continued research in these cells and tested for more cancer cell lines. Thus these compounds could serve as the basis for the development of a new group of cancer chemotherapeutics and certainly holds great promise towards good active leads.

4.3 Antiviral Activities

4.3.1 Antiviral Activities Extracts and Solvent Fractions

4.3.1.1 Antiviral Activities of the Methanol Extracts and Solvent Fractions of Plants Against Human Influenza A and B Viruses

The antiviral activity of the methanol extracts and organic solvent fractions of *A. sinana* root and leaves latex, *A. pirottae* root and *M. ferruginea* seed were tested against human influenza A (A/H1N1: PR8, A/Hong Kong/8/68 (H3N2, HK) and B (Lee) viruses at the concentration of 100 µg/ml and 20 µg/ml using a CPE reduction assay. The results are given in Table 4.12. After evaluation of the protection achieved by the extracts/solvent fractions on influenza A and B viruses induced cell destruction, we determined for selected extracts/fractions the reduction in cytopathic effect or CC_{50}, EC_{50} and SI of the virus titer by means of CPE reduction assay. The percent of protection was calculated as follows: Percent protection = [(ODT) V - (ODC) V] / [(ODC) M - (ODC) V] ×100. Where (ODT) V, (ODC) V and (ODC) M indicate absorbance of the sample, the virus-infected control (no compound) and mock-infected control (no virus and no compound), respectively [292]. The results are given in Table 4.13.

Table 4.10: Inhibition by methanol extracts and solvent fractions of influenza PR8, HK and Lee viral replication. Results are expressed as percent of cell protection relative to control (100%)

Plant species	Extract and fractions	20 µg/ml			100 µg/ml		
		PR8	HK	Lee	PR8	HK	Lee
A. sinana root	MeOH Ext	42%	22%	43%	69%	35%	63%
	Hxf	49%	16%	28%	44%	62%	81%
	CHf	40%	67%	49%	-16%	-6%	-5%
	EAf	60%	37%	52%	208%	151%	162%
	BUf	21%	30%	17%	56%	30%	41%
M. ferruginea seed	MeOH E	61%	63%	69%	64%	57%	65%
	Hxf	61%	67%	67%	6%	34%	48%
	CHf	63%	65%	57%	50%	42%	53%
	BUf	5%	52%	3%	43%	9%	4%
A. pirottae root	MeOH Ext	41%	44%	21%	76%	25%	54%
	Hxf	67%	73%	76%	-21%	-9%	-7%
	CHf	117%	60%	97%	17%	24%	33%
	EAf	69%	33%	65%	117%	100%	126%
	BUf	10%	18%	12%	22%	6%	28%
A.sinana leave latex	MeOH Ext	15%	11%	12%	29%	22%	15%
	CHf	43%	18%	31%	144%	94%	123%
	EAf	11%	7%	9%	33%	17%	31%
	BUf	31%	4%	7%	34%	20%	13%

Results are expressed as mean of 3 different experiments.

. The results in Table 4.12 revealed that out of four methanol extracts and fourteen organic solvent fractions, eleven (two methanol extracts and nine organic solvent fractions) showed detectable antiviral activity with greater than 51% to complete cell protection (100% or greater) against influenza A and B viruses induced CPE compared to control wells (Table 4.12). The most effective plant extracts were EAf made from the root of *A. sinana* and *A. pirottea*, and CHf of root of *A. pirottea* and leaves latex of *A. sinana*, which had complete cell protection against influenza A and B viruses induced cytopathic effect compared to control wells. MeOH extracts of the *A. sinana*. root at 100 µg/ml showed strong cell protection against PR8 and Lee induced CPE. CHf at 20 µg/ml and Hxf of the *A. sinana* root at 100 µg/ml showed strong cell protection against HK and Lee induced CPE. Hxf, CHf and Buf of A. pirottea exhibited significantly strong cell protection against influeanza A and B induced CPE with 63% - complete cell protection compared to control wells. The crude MeOH extract, Hxf and CHf of *M. ferrgunia* seed at both concentrations demonstrated strong cell protection against PR8, HK, and Lee viral-induced cytopathic effect with % of cell protection ranged from 61% - 69% compared to control wells. Buf of *M. ferrgunia* seed at 20 µg/ml protected 52% of cell from HK viral-induced cytopathic effect compared to control wells.

It is noteworthy that much of the most effective antiviral activity observed in this study was with EAf which led to the conclusion that the antiviral property of plants resides in medium polar fractions and the active ingredients are medium polar compounds. The extracts made from EAf of *A. sinana* root, CHf of *A. sinana* leaves latex, and EAf of *A. pirottea* root exerted complete cell protection against PR8, HK and Lee, suggesting that these plants have a broader range of strong antiviral activities. Extracts and fractions of *M. ferrgunia* seeds, exerted strong cell protection against PR8, HK and Lee.

4.3.1.2 Antiinfluenza Virus Activities of the Selected Extract and Fractions.

One MeOH extract and six solvent fracrions selected from four parts of three different plant species were investigated *in vitro* for antiviral activities against influenza A and B usig a viral CPE reduction assay to determine CC_{50}, EC_{50} and SI of the samples (Table 4.13). The results were compared with antiviral agents AMT, RBV and OSV-C.

As expected, OSV-C (an NA inhibitor) and RBV (a viral polymerase inhibitor) efficiently inhibited the replication of all viral strains tested, confirming the reliability of the assay. They all showed dose-dependent activity and a higher selectivity value indicates a higher activity. Out of these seven**,** six (one crude extract and five fractios, 85.7%), showed the most acceptable antiviral profile [293] against human influenza A and B viruses with SI values ranged from 3.4 - > 23. 3. In terms of selectivity, crude MeOH extract of *M. ferrguinea* seed, EAf of *A. sinana* root, CHf of *A. sinana* leaf latex and EAf of *A. pirottae* root showed significantly higher anti-influenza A and B activity than reference antiviral reagent RBV and AMT.

Table 4.11: Cytotoxic concentration (CC_{50}), effective concentration (EC_{50}) and selectivity index (SI) of selected fractions against influenza A and B viruses infecting MDCK cells in the CPE reduction assaya.

Plant	Extract/ fraction	Toxicity CC^{d}_{50}(μg/ml)	Antiviral activity (EC^{b}_{50} μg/ml)			Slectivity index		
			Flu A H1N1 PR8	Flu A H3N2 HK	Flu B - Lee	Flu A H1N1 PR8	Flu A H3N2 HK	Flu B - Lee
A, sinana root	EA	> 100.0	22.1	4.3	11.4	> 4.5	> 23.3	> 8.8
M.ferrguinea seed	Me	63.6	9.3	9.2	8.8	6.8	6.9	7.2
	Hx	42.4	28.1	12.4	10.7	1.5	3.4	4.0
A.pirottea root	Hx	7.8	7.9	6.5	6.4	1.0	1.2	1.2
	EA	> 100.0	13.9	5.7	11.7	> 7.2	> 17.5	>8.5
A.sinana latex	CH	> 100.0	13.8	12.5	12.7	> 7.2	> 8.0	> 7.9
	EA	33.3	8.7	7.	6.9	3.8	4.6	4.8
Referance cpds	AMT	> 100.	> 100.	2.1	>100.	ND	> 47.6	ND
	RBV	> 100.0	35.0	18.8	20.0	> 2.9	> 5.3	>5.0
	OSV-C	> 100.0	0.5	<0.005	0.6	>204.1	>20000.0	>175.4

a The data presented are the means ± standard deviations (SD) form three independent experiments. b The EC50 is the 50% effective concentration, which is defined as the compound concentration that reduces the replication of influenza viruses by 50% in the CPE reduction assay. c Selectivity index, CC50/EC50. d The CC_{50} is the 50% cytotoxic concentration, which is defined as the compound concentration that produces cellular toxicity of 50%. E ND not tested. The data presented are the means ± standard deviations (SD) for three to five independent experiments.

A literature survey indicated some extracts of Chinese herbs exhibiting anti-influenza activities *in vitro*, such as *Polygonum chinense* methanol extract inhibited PR8, HK and Lee viruses with SI values of 5.5, 7.8 and 5.4; EAf with SI values of 6.4, 13 and 5.9, respectively [294]. Some purified compounds inhibited PR8, HK and Lee viruses such as epigallocatechinnic-3-gallate [295] gallic acid (SI = 5.1, 6.3 and 6.5), ellagic acid (> 3.7, > 4.8 and > 3.8), β-sitosterol (1.1,1.0 and -1), methyl gallate (16.6, 17.5 and 15.5) and caffeic acid (> 7.9, > 9.4 and > 20.4) [294]. This study revealed that extracts of *M. ferrguinea* seed, EAf of *A. sinana* root, CHf of *A. sinana* leaves latex and EAf of *A. pirottae* root showed higher anti-influenza A and B activity than reference antiviral reagent RBV and AMT, some extracts of Chinese herbs and the active compounds of natural products in above previous reports.

In the US NCI plant screening program, our study suggests that the multi-functional botanical materials of these plant species could be used as a promising antiviral drug resource and merit for continued research in these viruses and screened for more virus types**.**

4.3.1.3 Antiviral activity of the Methanol Extracts and Solvent Fractions of the Plants Against Picornaviruses.

The antiviral activity of the methanol extracts and solvent fractions (Hxf, CHf, EAf and Buf) of *A. sinana* root and leaves latex, *A. pirottae* root and *M. ferruginea* seed were tested in vitro against rhino virus (HRV-B14 and HRV-A16 serotypes) and enterovirus (CB3, PV3, EV68 and EV71(H) serotypes) using a viral cytophatic effect (CPE) reduction assay. The different extracts/fractions tested were independently added and assayed at concentrations of 100 µg/ml and 20 µg/ml and results were given as percentages of cellular viability relative to control and compared to antiviral drug rupintrivir (Table 4.14).

This is the first report of Ethiopian endemic medicinal plant species having anti-picornavirus activity. The results (Table 4.14) of *in vitro* test showed that out of four methanol extracts and fourteen solvent fraction tested, four extracts (22.22%) made from *A. sinana* root and *M. ferruginea* seed at both concentrations exhibited significant strong anti-EV68 and anti-EV71(H) viral activity with values of percentages of cellular viability relative to control is ranging from 65% - complete (100% or greater) against EV68 and EV71 induced CPE. Four fractions (22.22%) made from *A. sinana* root and *A. pirottae* root exhibited moderate anti-EV68 and anti-EV71(H) viral activity that afforded 21% - 39 % cellular viability against EV68 and EV71 induced CPE. EAf of *A. pirottae* root exhibited moderate anti-HRV B14 activity with 30% of cellular viability relative to control against HRV B14 induced CPE. *In vitro*, Hxf of *A. sinana* root exhibited moderate anti-HRV B14 and PV3 activity that afforded 30% of cellular viability against HRV B14 and PV3 induced CPE. All others, extracts and fractions, demonstrated weak and/or no activity against HRV B14, HRV A16, CB3 and PV3 infections.

Table 4.12: Inhibition by methanol extracts and solvent fractions of picornaviruses replication. Results are expressed as percentages of cellular viability relative to control (100%).

Plant	Solvent	HRV B14		HRV A16		CB3		PV3		EV68		EV71(H)	
		100[a]	20[b]	100	20	100	20	199	20	100	20	100	20
A. sinana root	Me	2	1	-1	1	0	-1	0	0	-7	-5	-3	-2
	Hx	12	0	-1	-1.	0	0	31	0	-4	-5	-5	-4
	CH	2	5	2	0	0	0	10	0	39	-11	34	-2
	EA	2	0	-1	0	0	-1	0	-1	-7	-2	32	3
	BU	1	0	0	0	0	-1	0	0	71	0	65	9
M. ferrguinea seed	Me	1	0	-1	-1	-1	-1	0	0	109	103	65	39
	Hx	1	0	-1	-1	-1	-1	-1	-1	87	102	77	28
	CH	1	0	0	-1	-1	-1	0	-1	89	88	69	65
	BU	0	0	0	0	0	-1	0	-1	-2	-2	-4	2
A. pirottea root	Me	0	0	0	0	0	-1	0	-1	-1	-1	-5	-1
	Hx	1	3	-2	0	0	-1	-1	0	21	-6	4	-3
	CH	1	1	-1	0	0	-1	0	-1	-12	-11	26	-5
	EA	30	0	10	1	0	-1	0	-1	-13	-4	21	4
	BU	1	0	0	0	0	-1	-1	-1	-1	1	-2	6
A.sinana latex	Me	0	0	0	0	0	-1	0	0	1	0	-2	3
	CH	14	0	-1	0	-1	-1	1	-1	-11	-1	-8	3
	EA	1	0	-1	0	0	-1	0	-1	-4	-1	-6	3
	BU	0	0	-1	0	0	0	0	0	-6	-1	10	1
Sample		HRV B14		HRV A16		CB3		PV3		EV68		EV71(H)	
Conc.in µM		5	1	5	1	5	1	5	1	5	1	5	1
Rupintrivir		104	100	105	102	107	99	103	10	106	99	73	10

a concentration in µg/ml. Results were expressed as the mean value of three independent experiments. The absorbance of the control group was defined as 100%. Sample results are compared with rupintrivir

The anti- EV 68 activities of MeOH extract and Hxf of *M. ferrguinea* seed were more potent than the reference antiviral compound rupintrivir. The anti- EV71 (H) activities of Hxf of *M. ferrguinea* seed at 100 µg/ml was more potent than the rupintrivir. The anti- EV71(H) activities of MeOH extract and CHf of *M. ferrguinea* seed at 100 µg/ml were comparable to that of rupintrivir. A literature survey indicated some extracts of Chinese herbs exhibiting anti-EV 71(H) activities *in vitro*, such as *Houttuynia cordata* Thunb water extract at a concentration of 125 µg/ml showed 50 % cellular viability against EV71 induced CPE [296]. Geniposide, a primary component of *Fructus gardenia*, protected more than 80 % of cells against EV71 infection at a concentration of 3 mg/ml [297]. Pure compounds, **,**8-dihydroxyflavone, kaempferol, quercetin, hesperetin, and

hesperidin isolated from Chinese herbal medicines *Chrysanthemum morifolium* Ramat reduced 80 % of EV71-induced CPE at a concentration of 50 µM [298].

Overall, this study evaluates that the *n*-butanol fraction of *A. sinana* root and methanol extract, n-hexane and chloroform fractions of *M. ferrgunia* seed have considerable antiviral activity against EV 68 and EV 71(H), indicating the presence of potential anti- EV68 and anti- EV71(H) viral compound (s) in these extracts and fractions. Extracts of these plants exhibited more potent anti-EV71 and Anti-EV 68 activity than some extracts of Chinese herbs and the active compounds of natural products in above previous reports. Further, in the US NCI plant screening programe, these plant species extracts exhibited significantly potent anti-picornavirus activities, and should be promising for continued research in these viral cells and screened for more virus types.

4.3.1.4 Antiviral Activities of the Methanol Extracts and Solvent Fractions of Plants Against Dengue-2 Virus

The antiviral activities of four crude MeOH extracts and fourteen solvent fractions from *A. sinana* root and leaf latex, *A. pirottae* root and *M. ferruginea* seed were assessed in vitro against dengue virus (DENV-2) at the concentrations of 20 µg/ml and 100 µg/ml using an immune fluorescence assay (IFA) in Vero cell to determine % cell viability and % viral inhibition. The resuits of IFA are given in Table 4.15.

Table 4.15: Anti-DENV-2 activities of MeOH extract and solvent fractions. Results are expressed as percentages of cellular viability and viral inhibition relative to control (100%). The results are expressed as the mean of 3 different experiments**.**

Plant species	Extracts and fractions	100 µg/ml		20 µg/ml	
		%cell viability	% inhibition	% cell viability	% inhibition
A. sinana root	MeOH	40	50	75	9
	Hxf	1	100	52	36
	CHf	0	100	23	72
	EAf	49	39	70	14
	Buf	22	100	32	61
M. ferrguinea seed	MeOH	2	100	1	100
	Hxf	1	100	2	100
	CHf	3	100	1	100
	Buf	82	-2	85	-4
A, pirottae root	MeOH	82	-3	89	-9
	Hxf	1	100	58	44
	CHf	0	100	69	16
	EAf	65	22	76	7
	Buf	78	2	95	-16
A sinana latex	MeOH	79	1	91	-11
	CHf	21	98	77	7
	EAf	64	19	75	8
	Buf	56	30	62	24

This is the first report of these endemic medicinal plants to Ethiopian having anti-dengue activity. The results (Table 4.15) of *in vitro* test showed that five extracts (four extracts from *M. ferrguinea* seed and one from *A. pirottae* root) at both concentrations and five extracts (three from *A. sinana* root and two from *A. pirottae* root) at 100 µg/ml exhibited strong inhibition of DENV-2 by exerting complete inhibitory effects on DENV-2 replication. Eight extracts extracts (one from *M. ferrguinea* seed, one from *A. sinana* root, three from *A. sinana* latex and three from *A. pirottae* root) at both concentrations and five extracts from two plant species (two from *A. sinana* root, one from *A. sinana* latex and two from *A. pirottae* root) at 20 µg/ml exhibited strong inhibition of DENV-2 induced CPE with reduction of infectivity ranging from 50% - complete compared to control wells in Vero cell. The result revealed that the percentage viral inhibition was found to be increasing with increasing concentration of test compounds, and percentage of cellular viability increasing with decreasing concentration of test compounds.

In the anti-DENV-2 assay, crude MeOH extract, Hxf, CHf and EAf of *A. sinana* root at 100 µg/ml showed significant inhibitory activity against DENV-2 that afforded complete inhibition of virus infection (Table 4.15). CHf and Buf of *A. sinana* root at 20 µg/ml showed strong inhibitory activity against DENV-2 that afforded 72% and 62% virus inhibition, respectively. MeOH extract, Hxf and EAf of *A. sinana* root at 20 µg/ml showed significant inhibitory activity affording cellular viability 75%, 52% and 70% against DENV-2 induced cytopathic effects, respectively. MeOH extract, CHf, EAf and Buf of *A. sinana* leaves latex at both concentrations exhibited significant inhibitory activity affording cellular viability ranged from 56% to 91% against DENV-2 induced cytopathic effects. CHf of *A. sinana* leaves latex at 20 µg/ml showed significant inhibitory activity against DENV-2 affording 98% virus inhibition. *A. sinana* root extracts showed potent cellular protection against viral cytopathic effects at lower concentration and inhibition of DENV-2 viral production in Vero cell at higher concentration. A. *sinana* leaf latex extracts at both concentrations exhibited potent cellular protection against viral cytopathic effects in Vero cell.

When the cell was treated with 20 µg/ml of MeOH, Hxf, CHf, EAf and Buf of *A. pirrotea* root, infectivity by DENV-2 was reduced by 89%, 58%, 69%, 76% and 95% compared with that in untreated cells, respectively. MeOH, EAf and Buf of *A. pirrotea* root at both concentrations exhibited significant cell protection against the DENV-2 induced cytopathic effect by 82%, 89% and 65%, and 76%,78% and 95%, respectively. Hxf and CHf of *A. pirrotea* root at 100 µg/ml exhibited complete inhibition of DENV-2 replication. In general, the higher concentration was more effective against virus than cells ((treatment mode) and the lower concentration was more effective against virus infection (protective mode).

Crude MeOH extract, Hxf and CHf of *M. ferrguinea* seed at both concentrations exhibited 100 % inhibition of DENV-2 viral production compared to control wells without inducing cytotoxic effect to normal cells. Buf of *M. ferrguinea* seed at both concentrations had afforded 82% and 85% cellular viability, respectively, against DENV-2 viral induced cytopathic effects. *M. ferrguinea* seed extracts demonstrated potent inhibition of DENV-2 viral production and viral induced cytopathic effects.

It is worth to mention that the investigation conducted with *Cladosiphon fucoidan* [299] demonstrated that when the virus was treated with 10 µg/ml of fucoidan, infectivity by dengue virus serotype 2 was reduced by 80% compared with that in untreated cells. Glabranine and 7-O-

methylglabranine showed significant inhibitory activity and presented 70% DENV-2 infection inhibition at 25 µmol/l [300]. The cyclohexenylchalcone derivatives panduran tin A and 4-hydroxypanduratin A inhibited DENV-2 by about 27.1 and 52.0% at 40 µg/ml, 66.7 and 78% at 80 µg/ml, 92.2 And 97.3% at 240 µg/ml, and 99.8 amd 99.6% at 400 µg/ml, respectively. Pinocembrin was inhibiting by about 60% at 20 µg/ml concentration [301]. Methyl gallate which was purified from the methanol extract of *Quercus lusitanica* inhibited 98% of DENV-2 NS2B/3 protease at 0.3 mg/ml [302]. Squalamine discovered in the tissues of the dog fish shark (Squalus acanthias) was evaluated at 40 µg/ml on dengue virus infection of human endothelial cells (HMEC-1) and inhibited dengue infection by 60%. The infection was completely suppressed at 100 µg/ml [303]. This study revealed that extracts of *A. pirottea* root, *M. ferrguinea* seed, and *A. sinana* root and leave latex exhibited higher anti-DENV-2 activity than extracts of many herbs and the active compounds of natural products in above previous reports.

According to our results, MeOH extracts and solvent fractions of plants were concluded to exhibit anti-DENV-2 viral activities in vitro at different degrees of potency by protecting cell from viral infection (protective mode) and/or by potently inhibiting DENV-2 viral production (treatment mode). In the US NCI plant screening program, all the plants extract and fractions could be potential therapeutic agent against DENV-2 infection and should be promising for continued research in this virus and screened for more virus types.

4.3.2 Antipicorna virus activity of pure compounds A10CH-01 to A10CH-07 from chloroform fraction of *M. ferrguinea* seed.

The study evaluated the cytotoxicity achieved by the isolated compounds and mixtures from CHf of *M. ferrguinea* seed on picornaviruses: rhino virus (HRV-B14, HRV-A21 and HRV-A7**1** serotypes) and enterovirus [Cox-B1, Cox-B3, PV3, EV-68, EV-71(H) and EV-71(BrCr) serotypes] induced cell destruction. The different compounds tested were independently added and assayed at concentrations of 0.16, 0.8, 4, 20 and 100 µg/ml using a viral cytopathic effect (CPE) reduction assay. The results were compared with antiviral compound rupintrivir and 10CHF of *M. ferrguinea* seed. Further more, results are presented herein based on the recommendation of the National Cancer Institute (NCI, USA) plant screening program that pure compound is generally considered to have *in vitro* cytotoxic activity and considered promising for further study, if the SI value of ≥ 4µM [304]. Further more, the activity rating and recommendation for further study is based on

comparision with the 10CHF and antiviral reagent rupintrivin that evaluated under the same condition with that of isolated compounds.

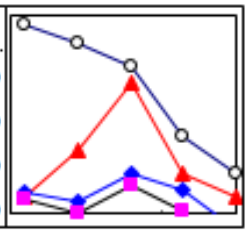
4.3.2.1 Antiviral activity of isolated compounds and mixtures against EV68, EV71(H) and EV71(BrCr) viruses.

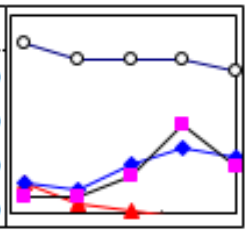
Isolated compounds, mixtures and A10-CHf were screened in vitro for their antiviral potential against enteroviruses using a viral CPE assay to determine 50% cytotoxic concentration (CC_{50}), and 50% effective concentration (EC_{50}) of isolates, mixtures and A10-CHf on EV68, EV71(H) and EV71(BrCr). The results are tabulated in Table 4.16 and graphically represented in Figure 4.11.

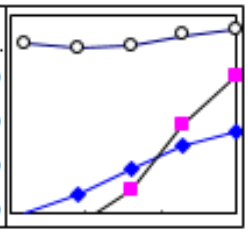
Table 4.16: The CC50, EC50 and SI values of different concentrations of isolates, mixtures and A10-CHf on EV68, EV71(H) and EV71(BrCr) in the CPE reduction assay[d].

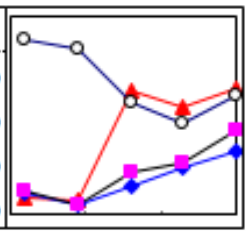
Compoud	CC_{50}[b] /µg/ml	EC_{5a}[a] /µg/ml)			Selectivity index [c](SI)		
	Mock	EV68	EV71(H)	EV71(BrCr)	EV68	EV71(H)	EV71(BrCr)
10CH-01	11.9	1.9	> 11.9	> 11.9	6.3	< 1.0	< 1.0
10CH-02	> 100	0.9	> 100	> 100	> 111.1	n. d.	n..d.
10CH-03	> 100	> 100	> 100	17	n. d.	n. d.	> 5.9
10CH-04	37.8	> 37.8	> 37.8	> 37.8	< 1.0	< 1.0	< 1.0
10CH-05	> 100	> 100	> 100	28	n. d.	n. d.	> 3.6
10CH-06	> 100	2.4	> 100	> 100	> 41.7	n. d.	n. d.
10CH-07	> 100	2.2	> 100	100	> 45.5	n. d.	> 1.0
10CHF	> 100	3.3	> 100	> 100	> 30.3	n. d.	n. d.

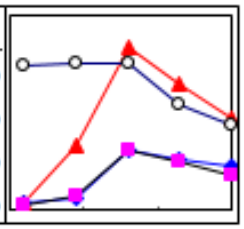
[a]The EC_{50} is the 50% effective concentration, which is defined as the compound concentration that reduces the replication of influenza viruses by 50% in the CPE reduction assay. [b]The CC_{50} is the 50% cytotoxic concentration, which is defined as the compound concentration that produces cellular toxicity of 50%. Selectivity index, CC_{50}/EC_{50}. n, d not tested. [d] Results were expressed as the mean value of three independent experiments. The absorbance of the control group was defined as 100%. Sample results are compared with rupintrivir.

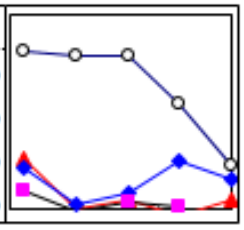
17EV10 a1		CC_{50} & EC_{50}	100	20	4	0.8	0.16	
A10-CH-01	EV68[▲]	**1.9**	8%	20%	65%	32%	8%	
	EV71(H)[◆]	**>11.9**	-8%	11%	20%	5%	10%	
	EV71(BrCr)[■]	**>11.9**	-24%	1%	14%	0%	6%	
	Mock [○]	**11.9**	19%	39%	73%	85%	95%	

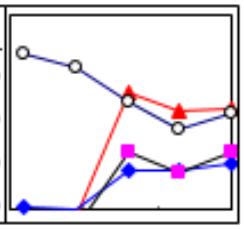
17EV10 a2		CC_{50} & EC_{50}	100	20	4	0.8	0.16	
A10-CH-03	EV68[▲]	**>100**	-4%	-3%	1%	5%	18%	
	EV71(H)[◆]	**>100**	33%	39%	29%	14%	18%	
	EV71(BrCr)[■]	**17**	28%	53%	23%	10%	9%	
	Mock [○]	**>100**	85%	93%	93%	93%	102%	

17EV10 a3		CC_{50} & EC_{50}	100	20	4	0.8	0.16	
A10-CH-05	EV68[▲]	**>100**	-4%	-9%	-6%	-2%	-4%	
	EV71(H)[◆]	**>100**	41%	34%	22%	9%	0%	
	EV71(BrCr)[■]	**28**	69%	45%	11%	-6%	-9%	
	Mock [○]	**>100**	92%	90%	84%	83%	85%	

17EV10 a4		CC_{50} & EC_{50}	100	20	4	0.8	0.16	
A10-CH-07	EV68[▲]	**2.2**	76%	64%	74%	8%	10%	
	EV71(H)[◆]	**>100**	38%	27%	17%	6%	12%	
	EV71(BrCr)[■]	**100**	50%	31%	25%	5%	13%	
	Mock [○]	**>100**	71%	55%	67%	100%	105%	

17EV10 a1		CC_{50} & EC_{50}	100	20	4	0.8	0.16	
A10-CH-02	EV68[▲]	**0.9**	66%	90%	116%	46%	5%	
	EV71(H)[◆]	**>100**	31%	35%	42%	8%	5%	
	EV71(BrCr)[■]	**>100**	24%	33%	42%	10%	3%	
	Mock [○]	**>100**	60%	76%	105%	104%	104%	

17EV10 a2		CC_{50} & EC_{50}	100	20	4	0.8	0.16	
A10-CH-04	EV68[▲]	**>37.8**	5%	-5%	5%	-1%	31%	
	EV71(H)[◆]	**>37.8**	19%	29%	9%	2%	25%	
	EV71(BrCr)[■]	**>37.8**	-21%	2%	3%	-2%	11%	
	Mock [○]	**37.8**	27%	65%	94%	94%	96%	

17EV10 a3		CC_{50} & EC_{50}	100	20	4	0.8	0.16	
A10-CH-06	EV68[▲]	**2.4**	62%	60%	72%	-5%	-3%	
	EV71(H)[◆]	**>100**	28%	24%	23%	-1%	1%	
	EV71(BrCr)[■]	**>100**	35%	22%	34%	-12%	-8%	
	Mock [○]	**>100**	58%	49%	66%	87%	95%	

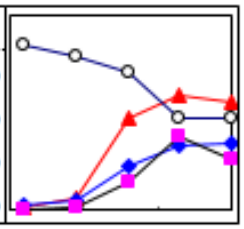
17EV10 a4		CC_{50} & EC_{50}	100	20	4	0.8	0.16	
A10-CH	EV68[▲]	**3.3**	66%	70%	56%	6%	1%	
	EV71(H)[◆]	**>100**	40%	40%	27%	6%	3%	
	EV71(BrCr)[■]	**>100**	31%	45%	17%	2%	0%	
	Mock [○]	**>100**	56%	57%	84%	95%	102%	

Figure 4.8: Dose response effects of compounds, mixtures and A10CHf of *M. ferrguinea* seed on EV68, EV71(H) and EV71(BrCr) viruses.

In the anti-enteroviruses assay, the results showed that 10CH-01, 10CH-02, 10CH-06, 10CH-7 and 10-CHF exhibited potent antiviral activity against EV68 strain with selectivity index (SI) value of 6.3, > 111.1, > 41.7, > 45.5. and > 30.3, respectively, without cytotoxicity or an abnormally increase in cell viability at all concentrations treated. In terms of selectivity, 10CH-2 showed the most potent antiviral activity with EC_{50} value of 0.9 µg/ml (1.314 µM) and relatively high SI value of > 111.1 without cytotoxicity or an abnormally increase in cell viability at all concentrations treated. 10CH-7 also showed potent antiviral activity with EC_{50} values 2.2 µg/ml and SI values of 45.5. 10CH-01, 10CH-06 and reference 10-CHF were active against EV-68 strain, even though 10CH-01 showed cytotoxicity with CC_{50} of 11.9 µg/ml (Table 4.16). The antiviral activity profile

of compounds against EV-68 decreased in the order: 10CH-02 > 10CH-07 > 10CH-06 > 10-CHF > 10CH-01.

Compounds 10CH-3 and 10CH-5 exhibited acceptable antiviral activity against EV71(BrCr) (Table4.16 and Fig.4.11). Compound 10CH-05 showed moderate antiviral activity against EV71(BrCr) with EC_{50} value of 28.0 µg/ml (36.12 µM) and SI value of >3.6 without cytotoxicity or an abnormally increase in cell viability at all concentrations treated. Where as compound 10CH-03 showed moderate antiviral activity against EV71(BrCr) with EC_{50} value of 17 µg/ml (21.59 µM) and relatively high SI value of > 5.9 without cytotoxicity or an abnormally increase in cell viability at all concentrations treated. The rest compounds exhibited no or marginal anti-EV71(BrCr) activity. Compounds 10CH-4 and 10CH-7 displayed marginal or no anti- EV68, EV71(H) and EV71(BrCr) viral activity with a CC_{50} value of 37.8 µg/ml and SI value of < 1.0. Compounds 10CH-2, 10CH-7 and 10CHF had no detectable antiviral activity. No compounds exhibited acceptable antiviral activity against EV71(H) virus.

Many active compounds of natural products have been identified as potential anti-EV71/ CA16 agents [305], including galangin (EC_{50} = 1030 µM, SI = 14.36) and quercetin (EC_{50} = 7.39 µM, SI = 20.03) which were identified from 400 natural compounds as the most potent inhibitory effect on EV71 and CA16 infection.gallic acid (EC_{50} = 4.47 µM, SI = 99.57l) [306], allophycocyanin (IC_{50} = 0.056 µM, SI = 36.7) [307], chrysosplenetin (IC_{50} = 0.2 µM, SI= 107.5), penduletin (IC_{50} = 0.2 Mm, SI = > 100) [308], apigenin (EC_{50} =25.5 µM, SI=8.7) [309], chrysin (EC_{50} = 10 µM, SI = 20) [310], chrysin phosphate ester (EC_{50} = 6 µM, SI = 33) [310], luteolin (EC_{50} = 31 µM, SI = 9.35) [311], formononetin (EC_{50} = 3.98 µM, SI = 43.07) [312], ursolic acid (EC_{50} = 1.1 µM, SI= 200 [313], linalool (EC_{50} = 273 µM, SI = 4.2) [309], GLTA ((EC_{50} < 0.16 µg/ml) [314], hederasaponin B (EC_{50} = 24.7 µM, SI = 2.02) [315], geraniin (EC_{50} = 10.5, SI = 20) [316], epigallocatechin gallate (EGCG) (EC_{50} = 25 µM) [3173], chebulagic acid (EC_{50} = 13.1 µM, SI = 16) [318], anemarrhenasaponin II (EC_{50} = 22.2 µM, SI = 3.8) [319], timosaponin G (EC_{50} = 0.1 µM, SI = 2.3) [319] and resveratrol (EC_{50} = 20.2 mM, SI = 15.2) [320].

This study revealed that compounds 10CH-02 exhibited a high activity against EV68, resulting more potent than almost all of the active compounds of natural products in above previous reports. 10CH-07, 10CH-06 and CHf of *M. ferrguinea* seed exhibited strong anti-EV68 activity comparable to majority of the active compounds of natural products in above previous reports.

Compounds 10CH-03 and 10CH-05 exhibited moderate anti-EV71(BrCr) activity comparable to some of the active compounds of natural products in above previous reports. More over, 10CH-01, 10CH-02, 10CH-06, 10CH-7 and 10-CHf exhibited higher antiviral activity against EV68 than antiviral drug rupintrivin. Compounds 10CH-03 and 10CH-05 exhibited higher antiviral activity against EV71(BrCr) than antiviral drug rupintrivin. Further more, based on National Cancer Institute of US reccommandation, 10CH-01, 10CH-02, 10CH-06, 10CH-07 and 10CHf against EV68, and 10CH-03 and 10CH-05 against EV71(BrCr) exhibited significantly strong antiviral activity (SI $\geq$ 4) and considered promising for further biological study on many other virus types *in vitro* and *in vivo* along with exact mechanism(s) underlying behind their activity to be an antiviral agent. Thus, these compounds and mixtures could serve as the basis for the development of a new group of enteroviruses chemotherapeutics and certainly holds great promise towards good active leads.

4.3.2.2 Antiviral Activity of Isolated Compounds and Mixtures against Rhinoviruses HRV-B14, HRV-A21 and HRV-A71.

Compounds, mixtures and A10CHf of *M. feerguinea* seed were screened *in vitro* for their antiviral potential against HRV B14, HRV A21 and HRV A71 viruses to determine 50% cytotoxic concentration (CC_{50}), 50% effective concentration (EC_{50}) and SI (CC_{50} /EC_{50}). The results are given in Table 4.17 and Fig. 4.12

Table 4.17: The CC50, EC50 and SI values of different concentrations of isolates, mixtures and 10CHf on HRV B14, HRV A21 and HRV A71.

Compound	CC_{50}(ug/ml)	EC_{50} (ug/ml)			Selectivity index (SI)		
	Mock	HRV B14	HRV A21	HRV A71	HRV B14	HRV A21	HRV A71
A10-CH-01	<0.16	<0.16	<0.16	<0.16	n.d.	n.d.	n.d.
A10-CH-02	2	>2.0	<0.16	<0.16	<1.0	>12.5	>12.5
A10-CH-03	<0.16	<0.16	<0.16	<0.16	n.d.	n.d.	n.d.
A10-CH-04	<0.16	<0.16	<0.16	<0.16	n.d.	n.d.	n.d.
A10-CH-05	<0.16	<0.16	<0.16	<0.16	n.d.	n.d.	n.d.
A10-CH-06	<0.16	<0.16	<0.16	<0.16	n.d.	n.d.	n.d.
A10-CH-07	<0.16	<0.16	<0.16	<0.16	n.d.	n.d.	n.d.
A10-CH	<0.16	<0.16	<0.16	<0.16	n.d.	n.d.	n.d.

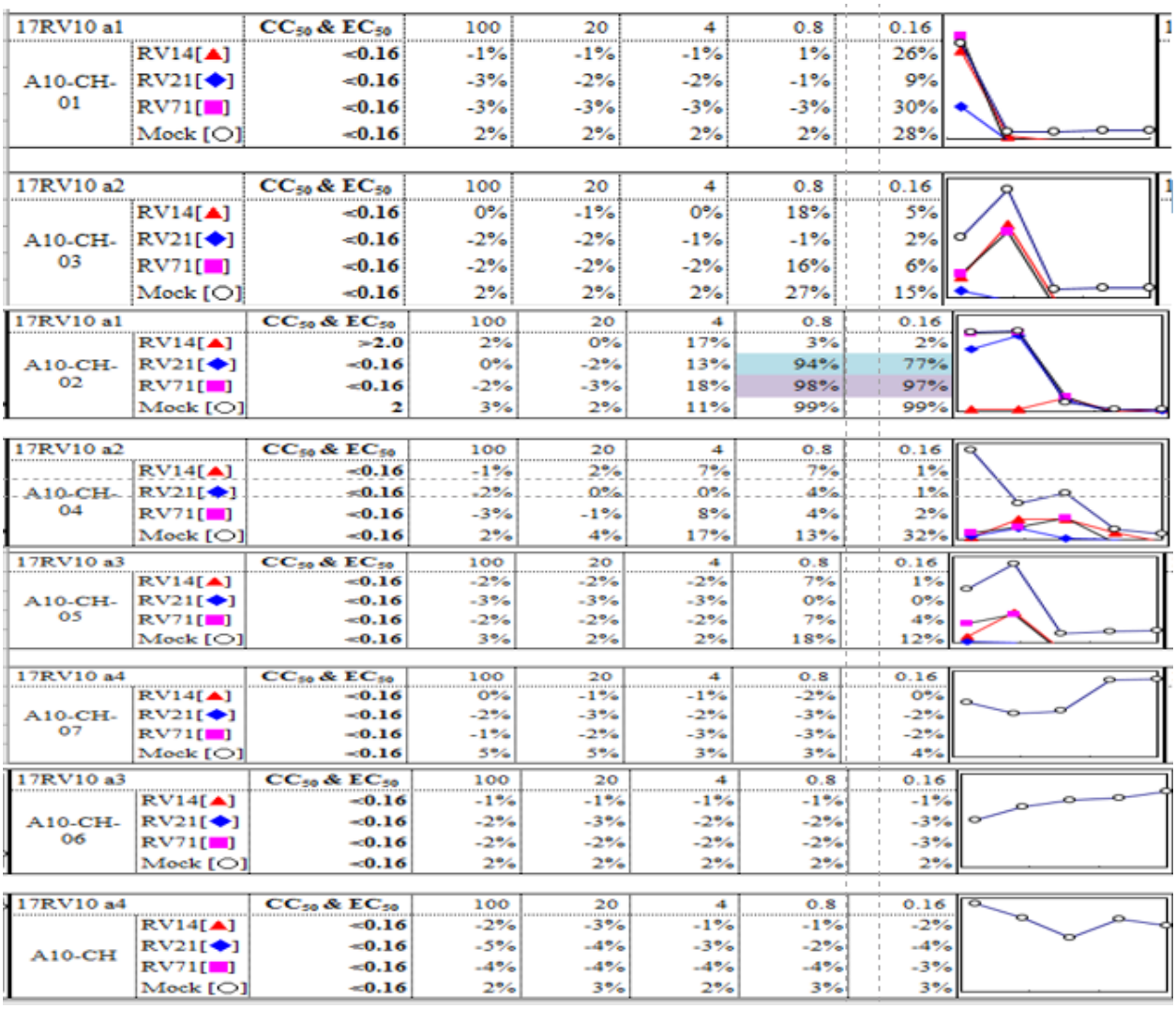


Figure 4.9: Dose response effects of compounds, mixtures and A10CHf of M. ferrguinea seed on the HRV B14, HRV A21 and HRV A71.viruses.

In the anti-HRV assay, among the tested samples, compound A10-CH-02 showed the most potent inhibitory effect on HRV A21 and HRV A71 with EC_{50} value of < 0.16 µg/ml and < 0.16 µg/ml with SI value of 12.5 and 12.5, respectively. Compound 10CH-2 showed strong antiviral activity with relatively high S.I. values than all the isolates and 10-CHF. All tested samples did not produce significant effect on all the rhino viruses used in this study.

In the US NCI plant screening program, Compound 10CH-2 was identified as promising antiviral products and should be promising for continued research in these viruses and screened for more virus types.

4.3.2.3 Antiviral activity of isolated compounds and mixtures against coxsackie Cox B1, Cox B3 and PV3 viruses.

Compounds, mixtures and A10CHf of *M. feerguinea* seed were screened in vitro for their antiviral potential against Cox B1, Cox B3 and PV3 to determine CC_{50}, EC_{50} and SI values of compounds, mixtures and 10-CHF. The results are tabulated in Table 4.18.

Table 4.18: The CC50, EC50 and SI values of different concentrations of compounds, mixtures and A10CHf on Cox-B1, Cox-B3 and PV3 viruses.

Compound	CC50(ug/ml)	EC50 (ug/ml)			Selectivity index (SI)		
	Mock	Cox B1	Cox B3	PV3	Cox B1	Cox B3	PV3
A10-CH-01	<0.16	<0.16	<0.16	<0.16	n.d.	n.d.	n.d.
A10-CH-02	1.8	>1.8	>1.8	>1.8	<1.0	<1.0	<1.0
A10-CH-03	<0.16	<0.16	<0.16	<0.16	n.d.	n.d.	n.d.
A10-CH-04	0.4	>0.4	>0.4	>0.4	<1.0	<1.0	<1.0
A10-CH-05	<0.16	<0.16	<0.16	<0.16	n.d.	n.d.	n.d.
A10-CH-06	<0.16	<0.16	<0.16	<0.16	n.d.	n.d.	n.d.
A10-CH-07	<0.16	<0.16	<0.16	<0.16	n.d.	n.d.	n.d.
A10-CH	<0.16	<0.16	<0.16	<0.16	n.d.	n.d.	n.d.

In the anti- Cox B1, anti-Cox B3 and anti-PV3 assay, the results showed that all samples displayed very weak or no antiviral effect on Cox B1, Cox B3 and PV3 replication. The strong cytotoxicity of compounds prevented from evaluating exact antiviral activity and determining the SI value of isolates.

CHAPTER V

5. CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

Extracts from four parts of three endemic plant species to Ethopia were studied in a search for anticancer and antiviral activities. CHF of the seed of *M. ferrguinea* was further fractionated to yield one new isoflavonoid and seven known isoflavonoid compounds. The structures of these compounds were elucidated using 1-D NMR, LC- mass spectrometry and litreture data.

5.1.1 Anticancer Activity of Methanol Extracts and Solvent Fractions

In the course of our studies, it was found that the cell growth inhibition on all the human cancer cell lines used in this study strengthed with increasing concentration of tested compounds. Fractionation revealed that semi polar compound may play vital role, as chloroform fractions of all the plants were endowed with highest cytotoxic activity against all the cancer cell lines. The activity of extract and fractions towards all the cancer cell lines nearly decreased in the order: CHf > EAf > MeOH > Hxf > Buf. The cytotoxicity strength of the extract and fractions of plants against A549, A2780 and SNU-638 cell lines decreased in the order: *M. ferrguinea* seeds > *A. sinana* root > *A. pirottea* root >*A. sinana* leaf latex. The strength of cytotoxicity of the plants extract and fractions on MIA-PaCa-2 cancer cell line decreased in the following order: *M. ferrguinea* seeds > *A. pirottea* root > *A. sinana* root > *A. sinana* leaf latex. MeOH extract and CHf of *M. ferrguinea* seed were more potent and sensitive as nearly two-fold to eighteen-fold cytotoxic IC_{50} values than reference standard anticancer drug etoposide in killing A549, A2780 and SNU-638 cancer cells. MeOH extract and CHf were found to be an extremely potent cytotoxin than purified compounds in advanced preclincal trals such as neopeltolide, spiruchostatin B, symplocamide A, myricetin (flavonol aglycones) and tangeretin. Towards A549, A2780, MIP-PaCa-2 and SNU-638 cancer cell lines, the sensitivity of extract and solvent fractions of the *M. ferrguinea* seed decreased in the following order: CHf > MeOH > etoposide > Hxf > Buf. The sensitivity of cells to the extract and fractions of *M. ferrguinea* seed was in the following order: A549 > SNU-638 > A2780 > MIP-PaCa-2. The sensitivity of cells to the extract and fractions of the *A. pirottea* root, *A. sinana* root and leave latex decreased in the following order: SNU-638 > A549 > A2780 > MIP-PaCa-2.

The present study provided evidence that the *M. ferrguinea* seed, *A. pirottea* root, *A. sinana* root and leave latex possess remarkable anticancer activities supporting the folkloric assertion of the

plants. In many reported study, the active principles and their derivatives have proven more active than their crude extracts for example artemisinin and derivatives from Chinese *Artemisia annua*. Thus reported data support that bioactive principles from these species could be more active than their crude extracts and could exhibit higher anti-A549, A2780, MIP-PaCa-2 and SNU-638 cancer cell lines and inhibiting cancerous cell growth more effectively than standard anticancer compound etoposide and many of compounds in clinical use, clinical trials and advanced preclinical trials. Therefore, purified compounds and/ or their derivatives from these three plants could serve as the basis for the development of a new group of cancer chemotherapeutics and certainly holds great promise towards good active leads. Further more, in the US NCI plant screening program, the extracts of plants were identified as promising anticancer products and should be promising for continued research in these cells and screened for more cancer cell lines.

5.1.2 Anticancer Activity of Isolated Compounds and Mixtures

In the course of our studies, it was found that the cytotoxic effect of isolates steadily strengthens with increases in the concentration of isolates. All compounds, except A10-CH-2, demonstrated higher cytotoxicity against A2780 and PANC-1 cancer cell lines compared to reference 10CHf and standard anticancer drug etoposide, and many compounds in clinical use, clinical trials and advanced preclinical trials. Compound 10CH-03 exhibited a high activity against MIP-PaCa-2 and PANC-1 cancer cell lines resulting more potent than etoposide, 10CHf, and many of compounds in clinical use, clinical trials and advanced preclinical trials. A10CH-05 and A10CH-03 exhibited the most potent cytotoxic activity than other compounds, 10CHf and anticancer drug etoposide against A2780 cancer cell lines. The sensitivity of compounds and mixtures in killing A2780 and PANC-1 cancer cells decreases in the following order 10CH-03 > 10CH-05 > 10CH-06 > 10CH-0 4 > 10CH-07 > 10CH-01 > etoposide > 10CH–02 > 10CHf. The sensitivity of compounds and mixtures in killing MAI- PaCa-2 cancer cells decreases in the following order of 10CH–03 > etoposide > 10CH-05 > 10CH-06 > 10CHf > 10CH-0 7 > 10CH-01 > 10CH-04 > 10CH-02. In the US NCI plant screening program, our study suggests that the multi-functional botanical materials of *M. ferrguinea* seed could be promising inhibitors of A2780, PANC-1 and MIP-PaCa-2 cancer cell lines and may be applied to development of a novel herbal medicine, and should be promising for continued research in these cells and screened for more cancer cell lines.

5.1.3 Antiviral Activity Methanol Extracts and Solvent Fractions

In the antiinflueanza A and B assay, the extracts made from EAf of *A. sinana* root, CHf of *A. sinana* leaf latex and EAf of *A. pirottea* root exerted complete cell protection against PR8, HK and Lee, suggesting that these plants have significantly strong antiviral activities. Extract and fractions of *M. ferrguinea* seeds exerted between 50% and 89% cell protection against PR8, HK and Lee, suggesting that it has strong antiviral activities. EAf of *A. sinana* root and CHf of *A. sinana* leaf latex were more potent and sensitive than reference antiinfluenza reagents RBV and AMT.

In the antipicornaviruses assay, the methanol extract and Hxf of *M. ferrguinea* seed were more potent and sensitive than antipicornaviruses drug rupintrivin in inhibiting EV68 and EV71(H) viruses. EAf and Buf of *A. sinana* root and CHf of *M. ferrguinea* seeds exerted antiEV 68 and anti-EV 71 (H) virus activity comparable to rupintrivin. It was found that extracts and their fractions of *A. sinana* and *A. pirottea* root showed no significant antiviral ativity against tested picornaviruses. For all the plants tested, no extracts and fractions showed reasonable antivial activity against rhinovirus HRV-B-14, HRV-A-21, Coxsackie Cox-B1, Cox-B3 and polio PV3 viruses.

In the anti-DENV-2 assay, all extracts, except Buf, of *M. ferrguinea* seeds exerted 100% inhibition of DENV-2 viral production. All extracts of *A. pirottea* root and all extracts except CHf at 100 µg/ml of *A. sinana* latex provided 55% to 100% viable cell against DENV-2 viral induced CPE. Ingeneral, the extracts and its fractions of all plants showed decreasing protective effect against virus infection and increasing viral inhibition with increasing concentration of test compounds. That is, the lower concentration was effective to protect cells from DENV-2 infection (protective mode) and the higher concentration was effective to inhibit cytopatic effect of DENV-2 (treatment mode). *M. ferrguinea* seed shows more potent inhibitory activitity than protective activitity. *A. pirottea* root and *A. sinana* leaf latex exhibit more significantly protecting cell from viral infection than inhibiting virus replication in Vero cells.

According to our results, extracts and fractions of all these plants, commonly used in Ethiopia as traditional medicine for various ailment including viral infection, were concluded to exhibit anti-influenza A and B, antipicorna and anti-DENV-2 activities in vitro at different degrees of potency by protecting cell from viral infection (protective mode) and/or by potently inhibiting viral production (treatment mode). In the US NCI plant screening program, plants extracts exhibit

potential therapeutic agent against currently circulating influenza A and B, picorna and DENV-2 viruses including drug-resistant viruses to warrant further analysis in vitro, along with purification of the extracts and chemical characterization of isolated compounds followed by toxicity and clinical tests *in vivo* and the exact mechanism(s) underlying behind their activity,which should permit identification of those compounds possessing specific activities which may be useful in the development of new and effective antiviral agents.

5.1.4 Antipicornaviruses Viral Activiy of Compounds

In the antipicornaviruses assay, compounds 10CH-01 and 10CH-02, and mixtures 10CH-06 and 10CH-7 exhibited strong antiviral activity against EV68 without cytotoxicity or an abnormally increase in cell viability at the maximum concentration treated (100.0 µg/ml). The EC_{50} values of 10CH-01, 10CH-02, 10CH-06 and 10CH-07 were 1.9, 0.9, 2.4 and 3.2 µg/ml and therapeutic index (SI) values of 6.3, > 111.1. 41.7 and 45.5, repectively. In terms of EC_{50} and selectivity index, 10CH-02 showed the most potent anti-EV68 activity. In terms of selectivity index, the anti-EV68 viral activity of 10CH-02 almost eighteen, three, four times higher than that of 10CH-1, 10CH-06 and 10CH7, and 10CHF, resyectively. The anti-EV68 viral activity profile of the compounds and mixtures decreased in the following order: 10CH-02 > 10CH-07 > 10CH-06 > 10CHf > 10CH-01. Compounds 10CH-03 and 10CH-05 exhibited moderate activity against EV71(BrCr). A10-CH-02 showed strong anti- HRV A21 and HRV A71 viruses activity with EC_{50} value of < 0.16 µg/ml and SI value of >12.5 for both viruses. Our study suggests that the isolates may provide a therapeutic option for the treatment of EV 68, EV71(BrCr), HRV A21 and HRV A71 infection; furthermore, the compounds could potentially be effective against picornaviridae viruses in general and applied to development of a novel herbal medicine. Therefore, further studies are required to explore the detailed antiviral mechanisms of compounds as well as to assess *in vivo* antiviral activity.

5.2 RECOMMENDATIONS

I. Further phytochemical investigation of the *M. ferrguinea* seed extract of CHf and other active extracts should be carried out in order to determine fully all the major bioactive compounds and evaluated *in vitro* and *in vivo* against many other cancer cell lines and viral serotypes including the studied cancer cell lines and viral serotypes.

II. Extensive studies for these three endemic medicinal plant species of Ethiopia should be conducted for their anticancer and antiviral activity on other cancer cell lines and viruses both in vitro and in vivo followed by toxicity and clinical tests along with identification, characterization and evaluation of their active components which may be useful in the development of new and effective anticancer and antiviral agents.

III. Extracts of the seed of M. *ferrguinea*are very active and have broad activities to spark the interest of scientists to develop new drugs, further study should be done *in vitro* and *in vivo* on, their bioactivity, mechanism of action, pharmacotherapeutics and toxicity for isolated and purified bioactive markers against many other cancer cells and viral types.

IV. For isolates, therapeutic window should be optimized in vivo, these compounds or itheir chemically modified versions could be tested as a single or combination therapy for the treatment of A2780, MIA-PaCa-2 and PANC-1, including drug-resistant cancers and many other cancer cell lines, and influeanza A and B virses , picornavirses and DENV-2 virses, drug-resistant viruses and many other virses *in vitro* and *in vivo* on its bioactivity, mechanism of action, pharmaco therapeutics and toxicity to develop more effective new anticancer and antiviral agents.

V. Plants and their chemical compounds are considered the main natural product used actually in pharmaceutical industries and therefore, it is very important to report the preliminary screening of their activities and safety to establish a good data base which may help for their better use.

VI. As tropical rainforest plants offer considerable chemical diversity in terms of the secondary metabolites, Ethiopia contains many and diversified naturally grown endemic and indegeneous tropical rainforest medcinal plants. These are unexploited and should be evaluated and exploited for therapeutic applications against genetically and functionally diverse cancer cell lines and viruses' families such as retroviridae, hepadnaviridae and Herpesviridae.

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APPENDICES

I. List of Publications

1. **Akalu Terfa**, Hailemicahel Tesso, Young Sik Jung, Anuradha. G, Sung Un Cher, Sang Ho Lee and J. Sreekantha Kumar Antiviral activity of Aloe pirottea A. Berger root extract against influenza A and B viruses, picornaviruses and dengue virus: an endemic plant species of Ethiopia. Springer publication.EAI-ICAST-2020.

2. **Akalu Terfa**, Hailemicahel Tesso, Young Sik Jung, Anuradha. G, Sung Un Cher, Sang Ho Lee and J. Sreekantha Kumar. Screening of antiviral activity studies from root and leaf latex extracts of Aloe sinana Reynolds: an endemic plant species of Ethiopia. Int. J Clinical and Diagnostic Res, Vol.8, Issue 1, Jan-Feb 2020.

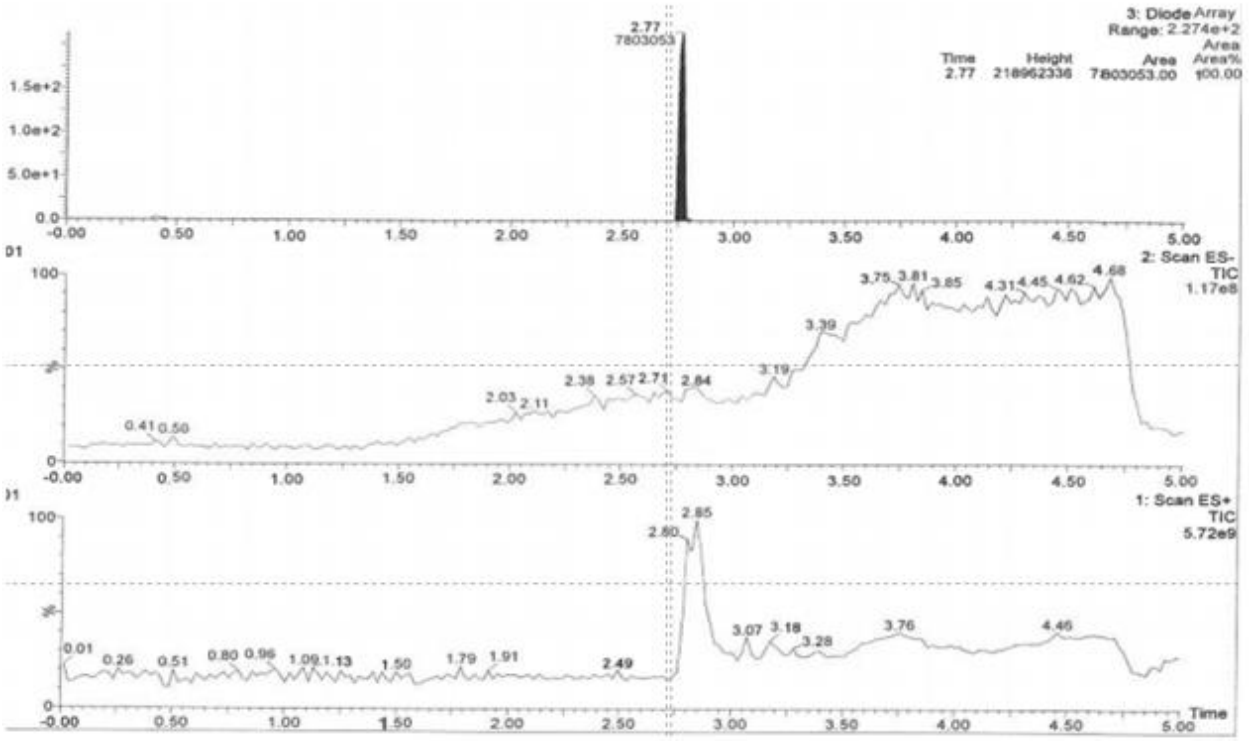
3. **Akalu Terfa**, Hailemicahel Tesso, Anuradha. G, J. Sreekanth kumar & Young-Sik Jung. Anticancer activity studies of root extract of Aloe pirottae A. Berger Endemic plant species of Ethiopia. Int J. Pharm. Drug. Anal, Vol: 7, Issue: 6, 2019; 49 – 53.

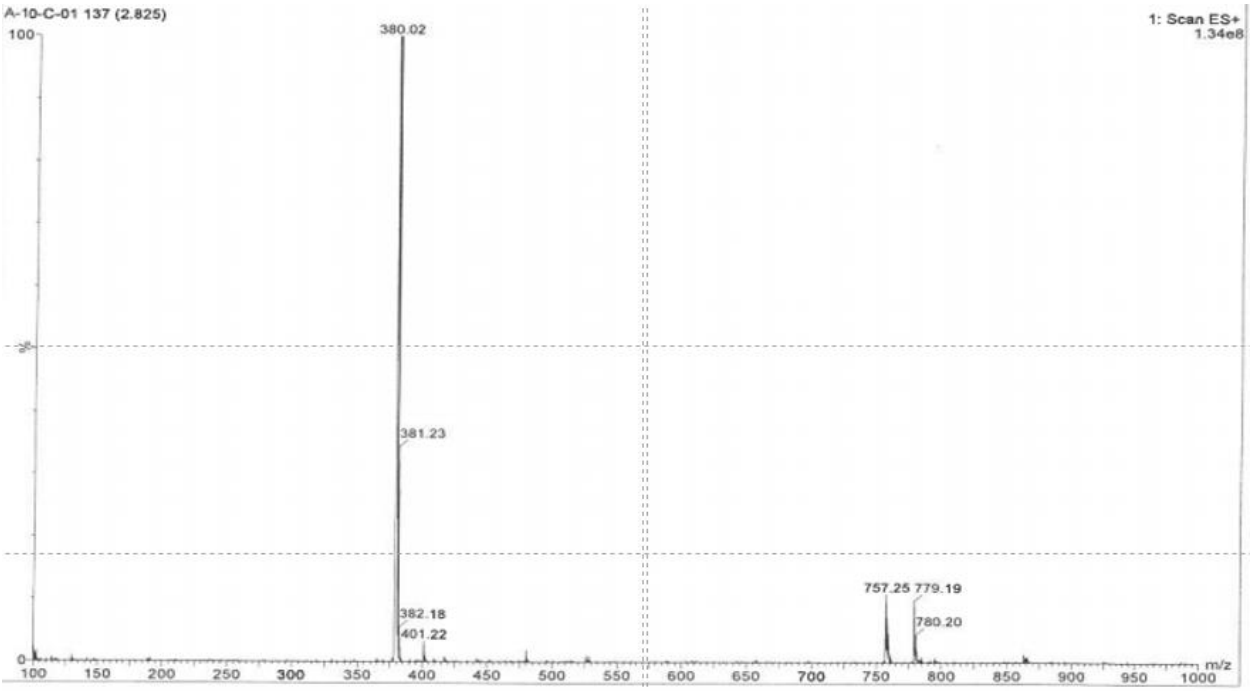
4. Yeonjoong Yong, Hailemichael Tesso**, Akalu Terfa**, Aman Dekebo, Worku Dinku, Young Han Lee, Soon Young Shin & Yoongho Lim. Biological evaluation of the diterpenes from Croton macrostachyus. Appl Biol Chem (2017) 60(6):615–621.

5. Soon Young Shin, Aman Dekebo, Worku Dinku, **Akalu Terfa**, Young Han Lee, Yoongho Lim & Yeonjoong Yong. Identification of an anticancer compound contained in seeds of Maesa lanceolata, a medicinal plant in Ethiopia. J Korean Soc Appl Biol Chem (2014) 57(4), 519−522.

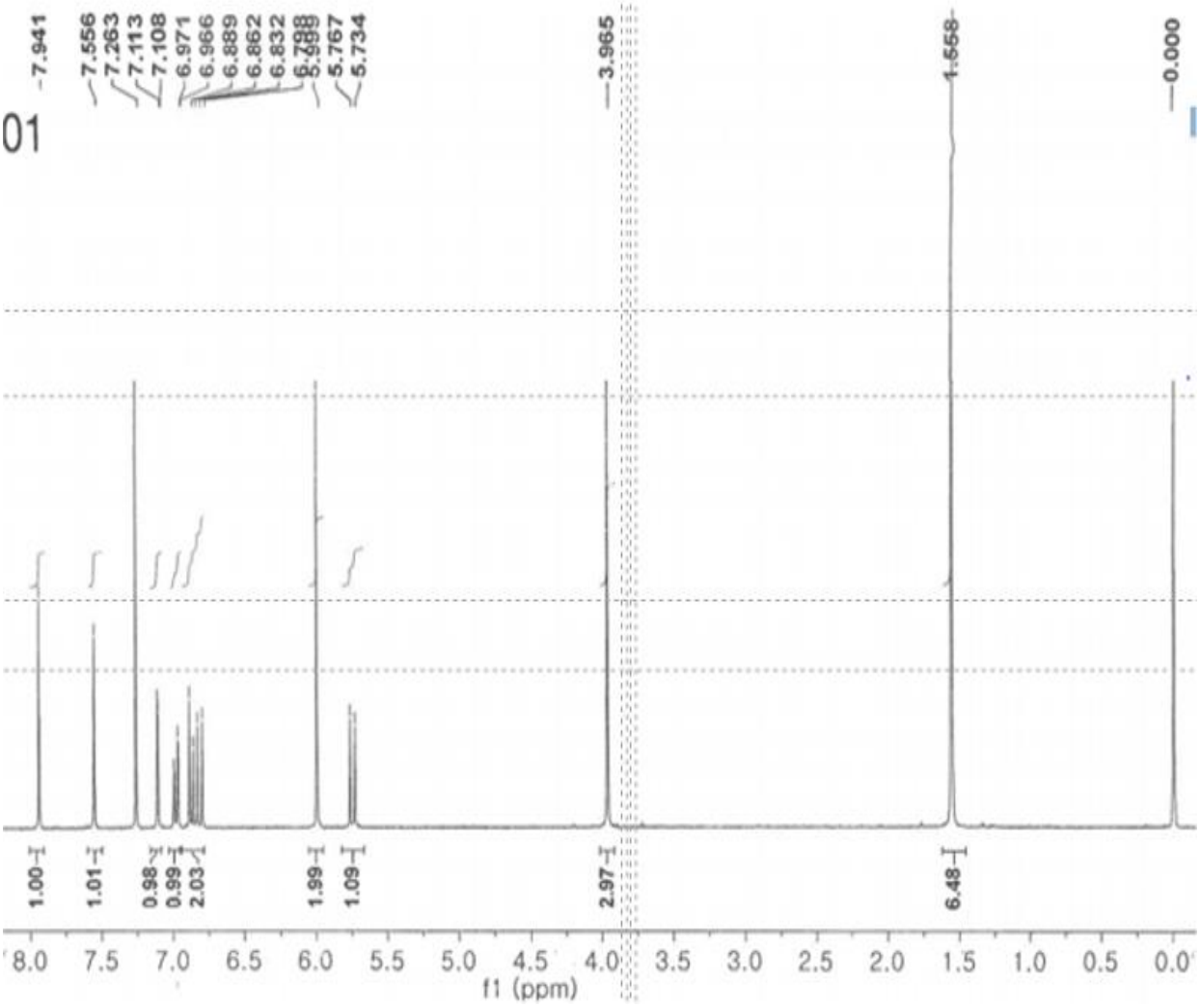
II. Supplimentary Figures

Appendix 1A : ESIMS spectrum of compound 10CH-01

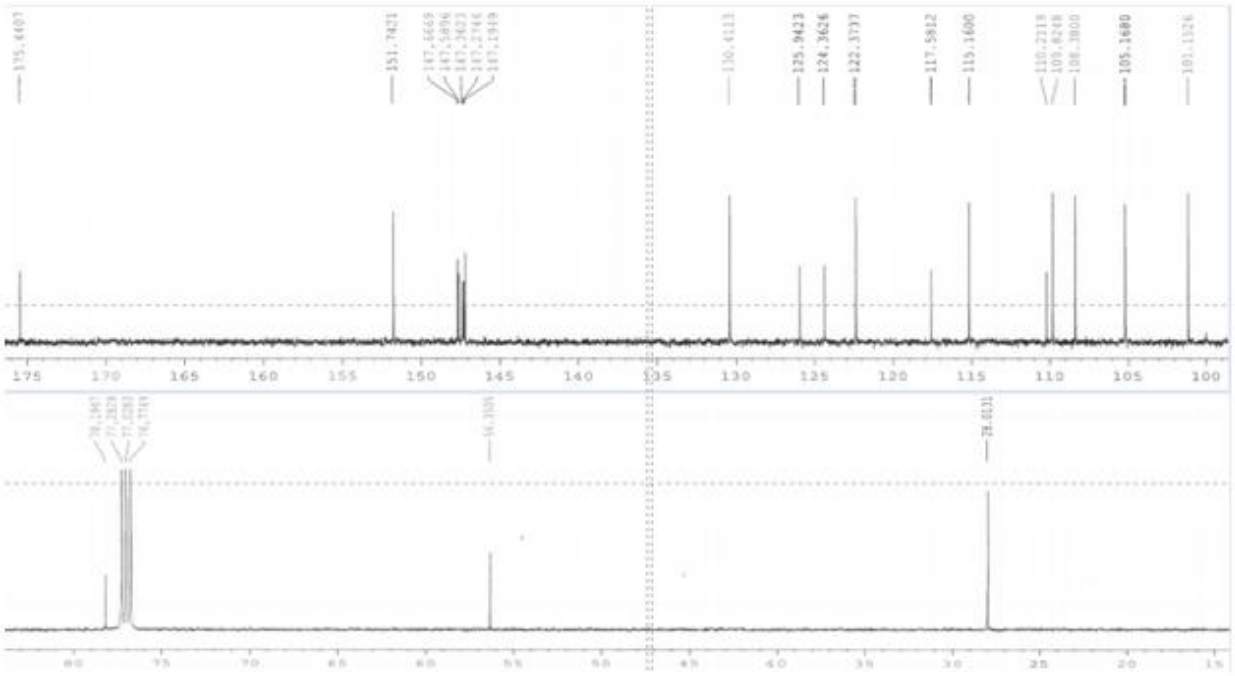




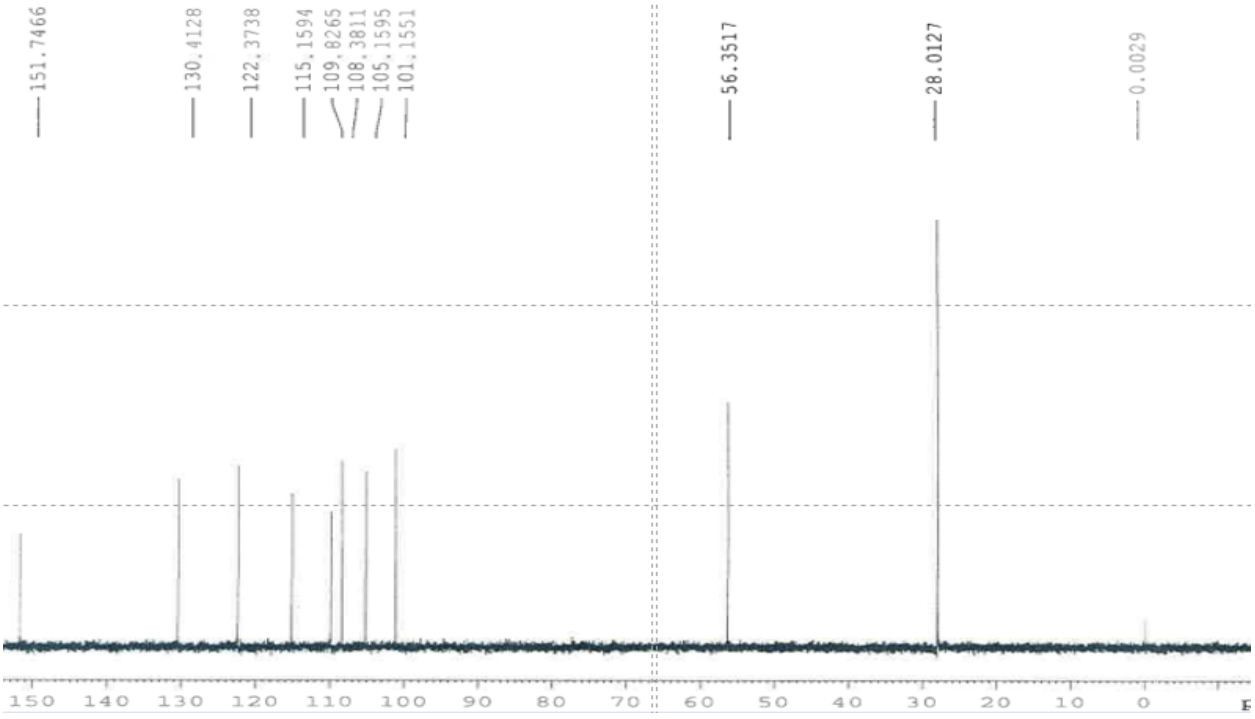
Appendix 1B: ^{1}H NMR (500.87 MHz) spectrum of compound 10CH-01



Appendix 1C: (I) ^{13}C NMR (125.77 MHz) (II) Dept-45 spectrum of compound 10CH-01

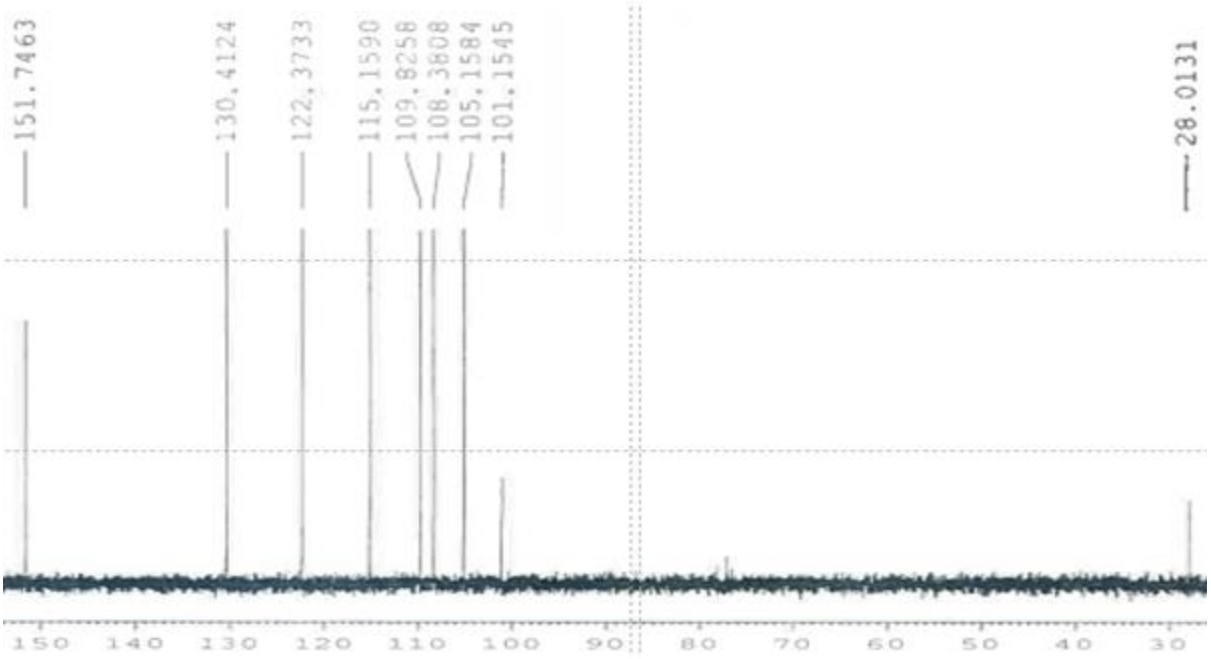


(I) ^{13}C NMR

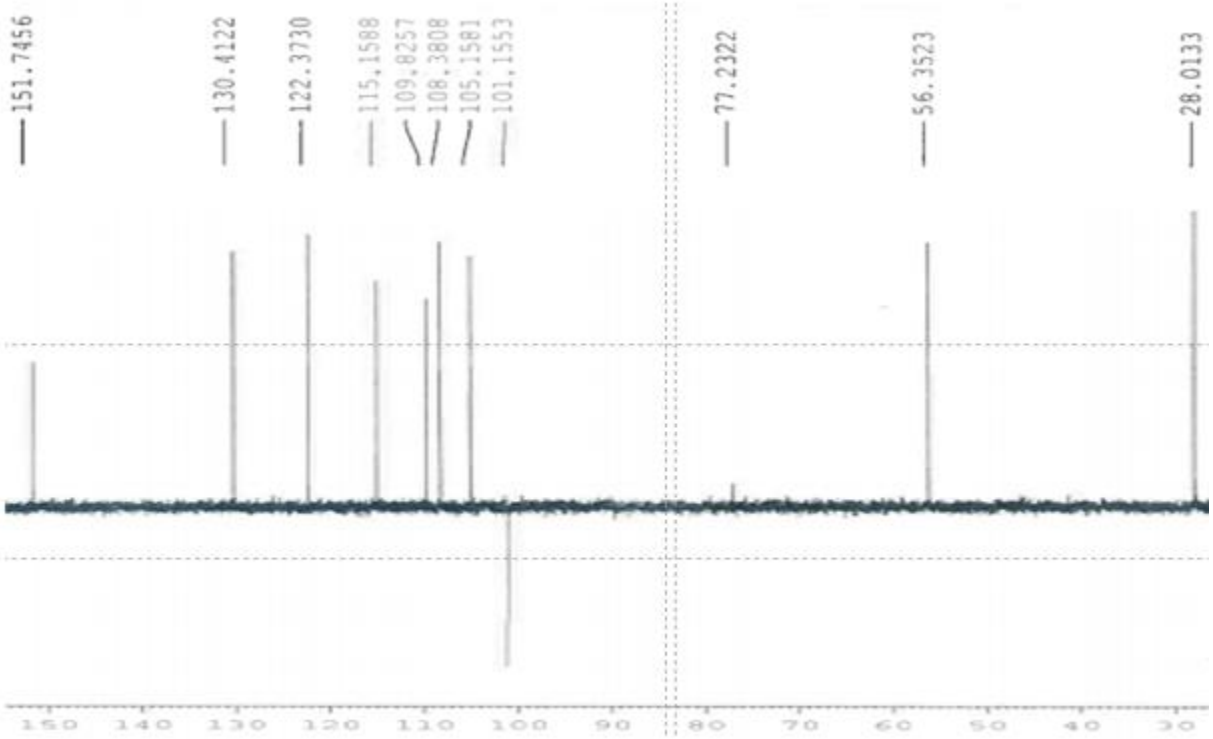


(II) Dept-45

Appendix 1D : (I) Dept-90 (II) Dept-135 spectrum of compound 10CH-01

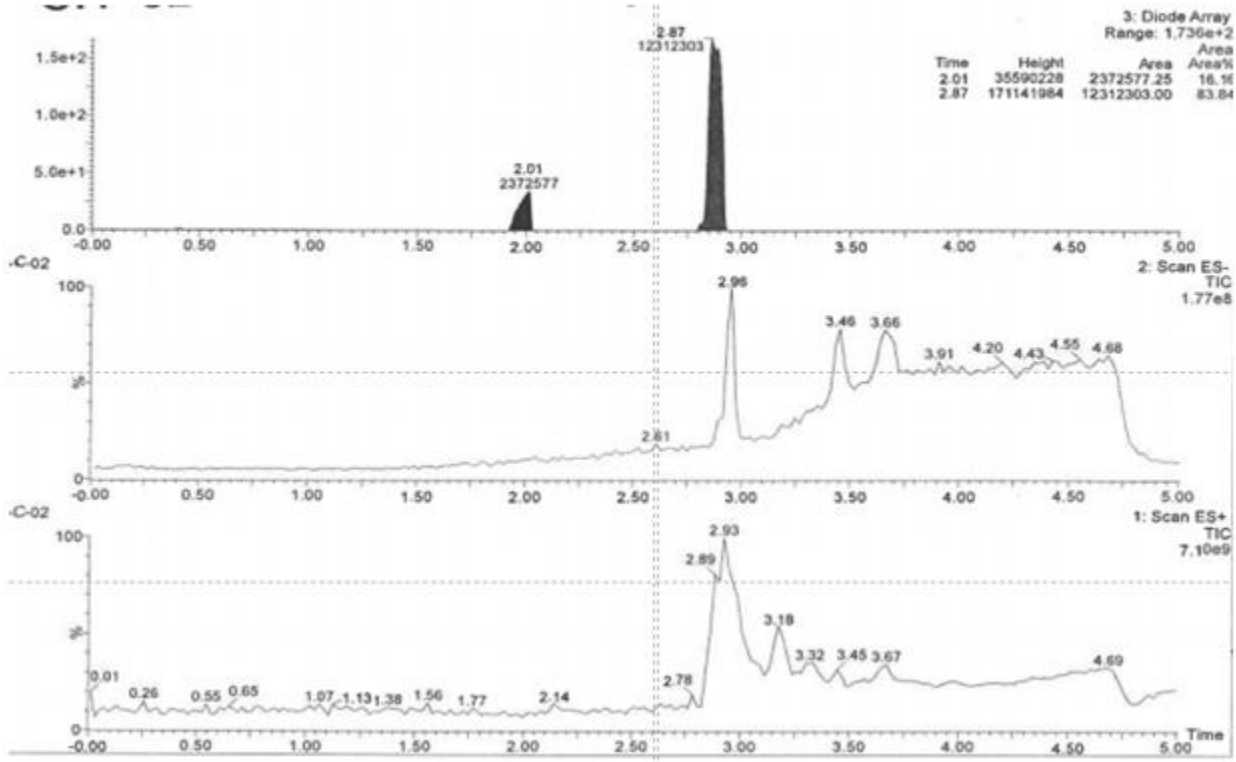


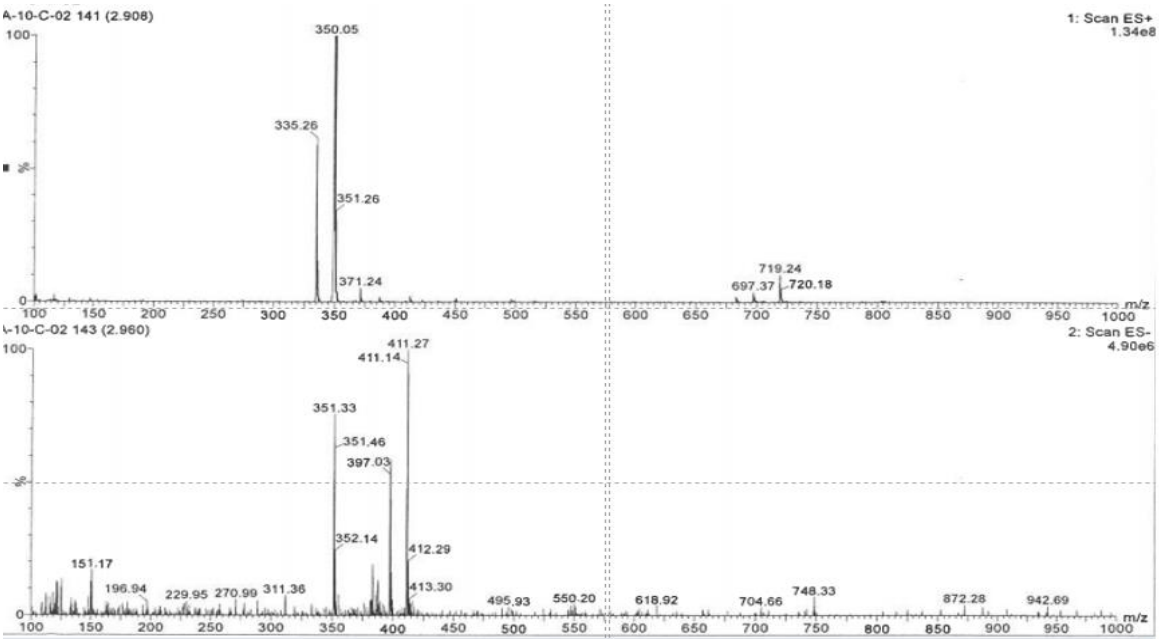
: (I) Dept-90



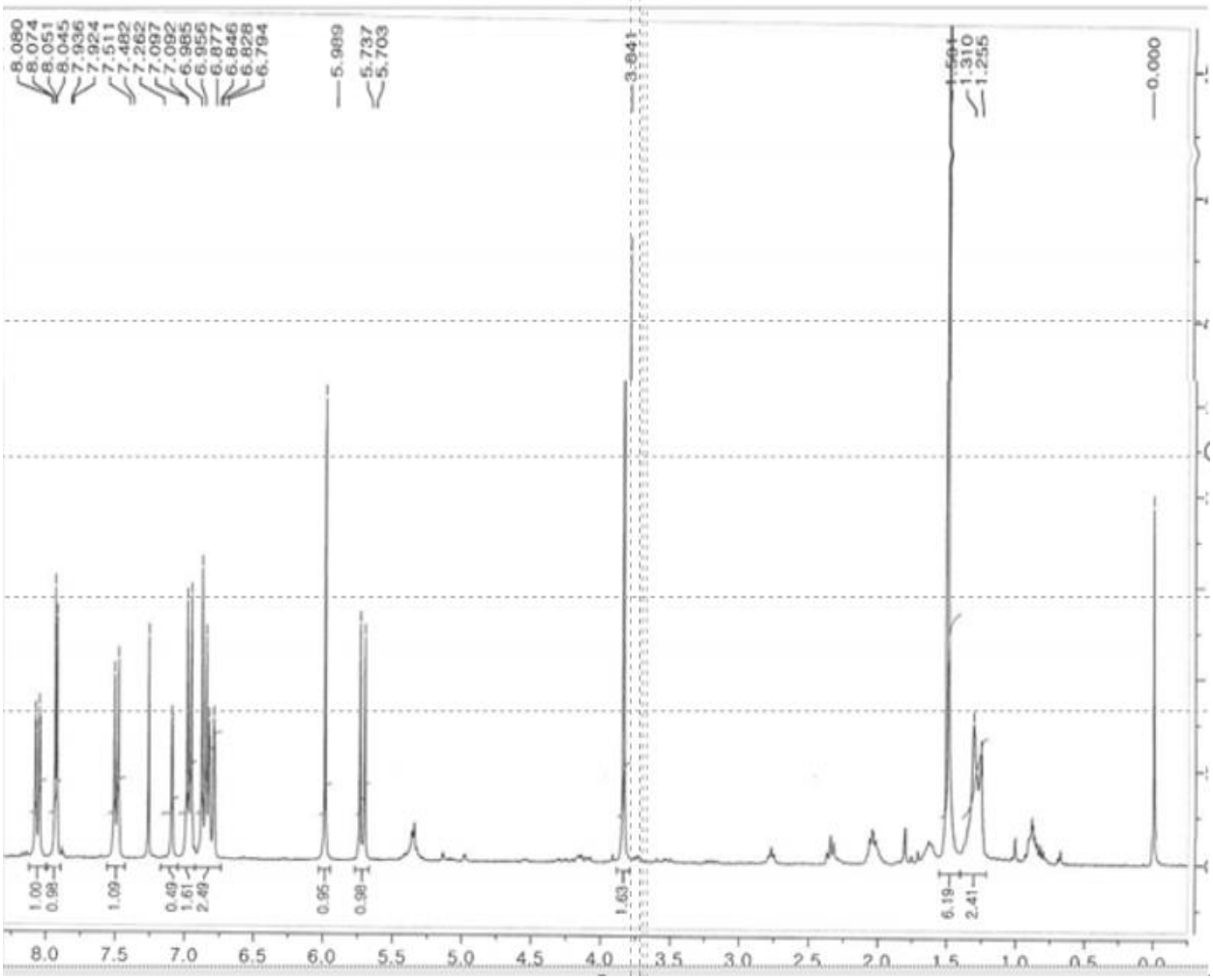
(II) Dept-135

Appendix 2A : ESIMS spectrum of compound 10CH-02

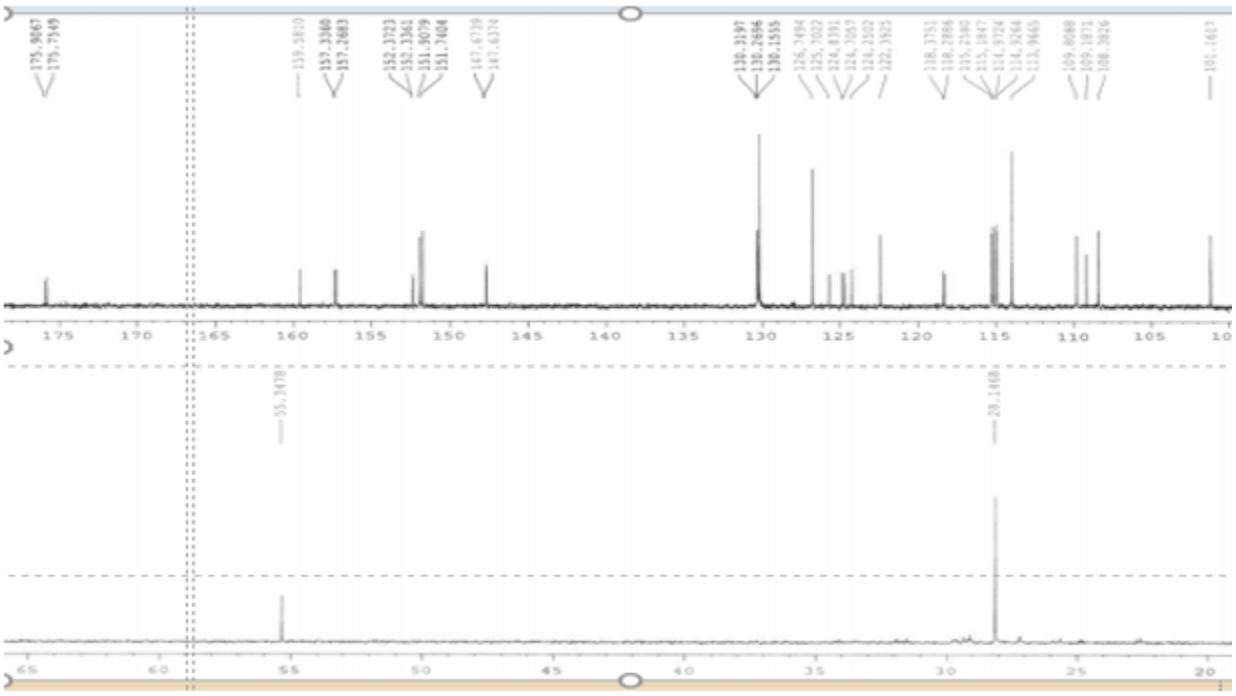




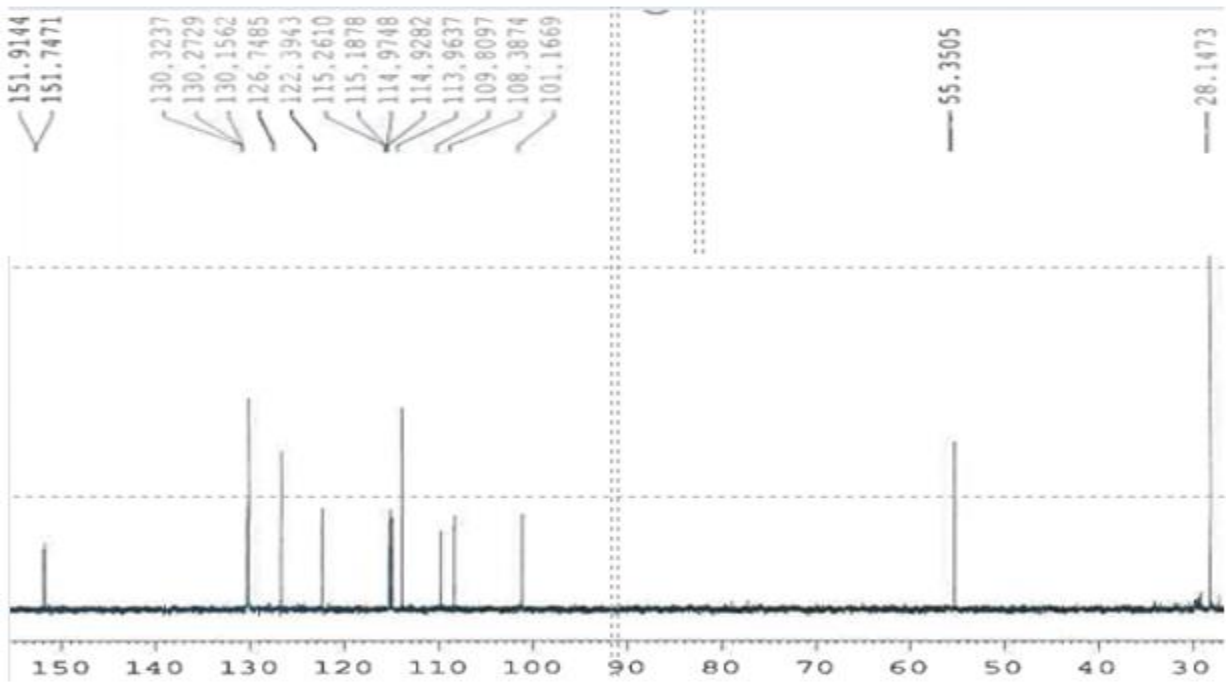
Appendix 2B: ^{1}H NMR (500.87 MHz) spectrum of compound 10CH-02



Appendix 2C: (I) ^{13}C NMR (125.77 MHz) (II) Dept-45 spectrum of compound 10CH-02

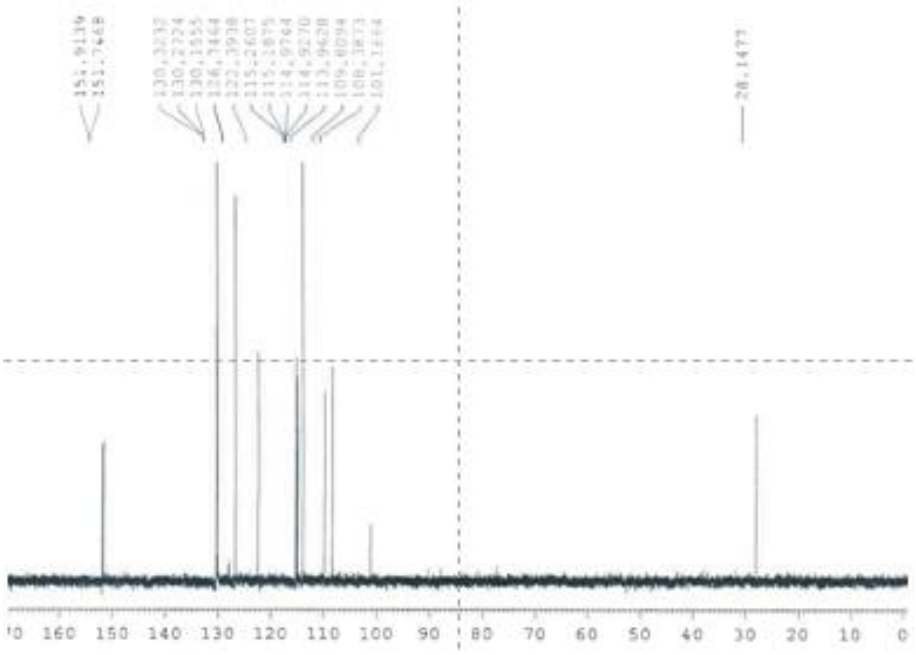


(I) ^{13}C NMR (125.77 MHz)

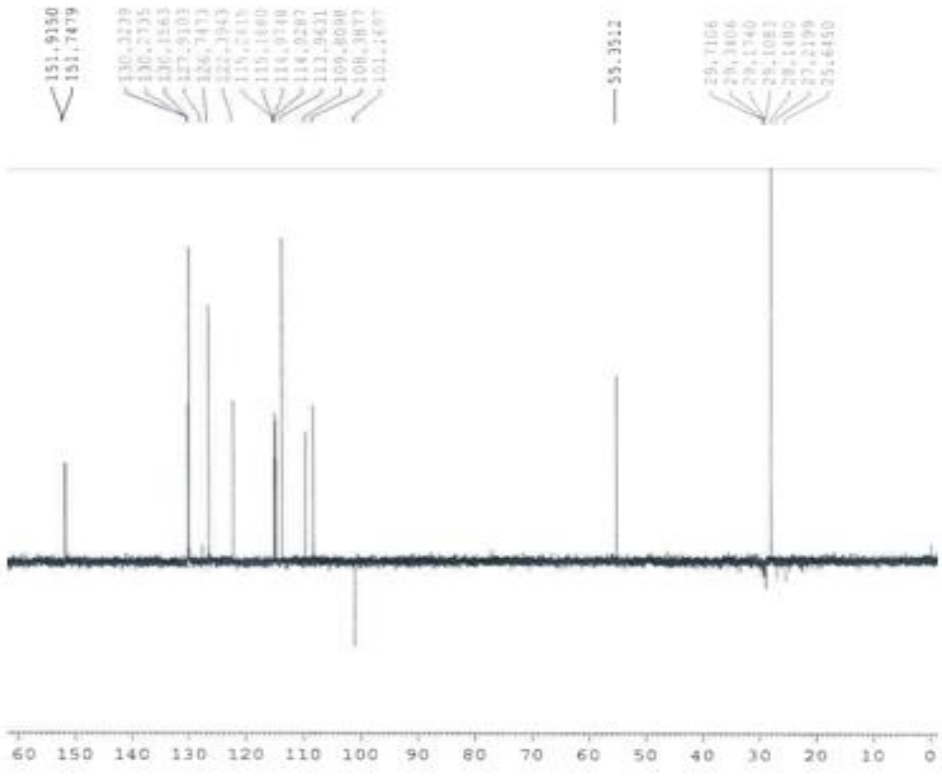


(II) Dept-45

Appendix 2D : (I) Dept-90 (II) Dept-135 spectrum of compound 10CH-02

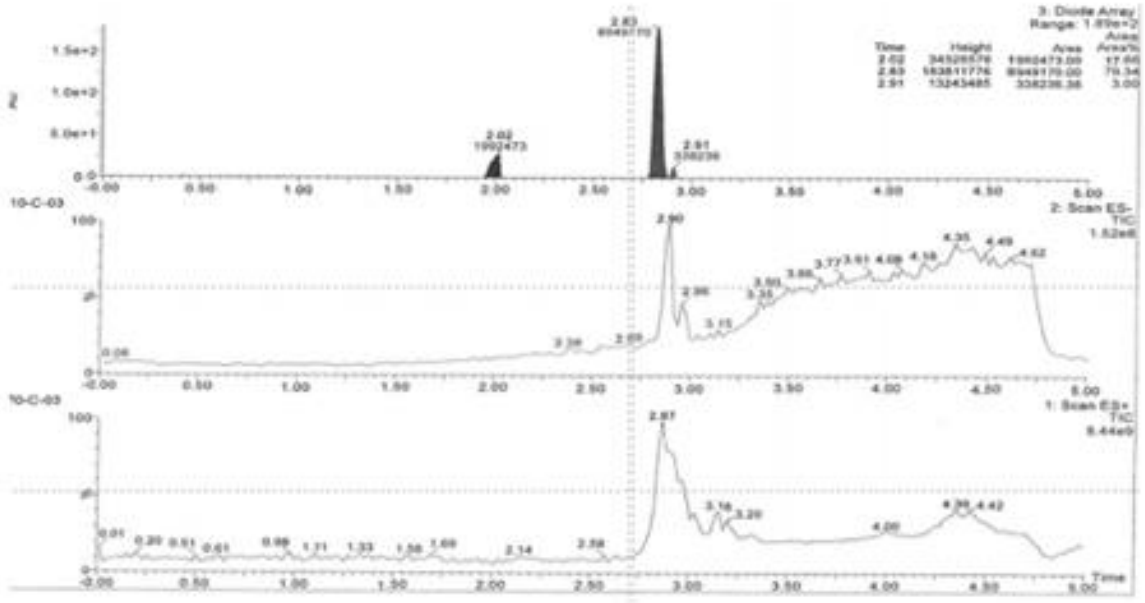


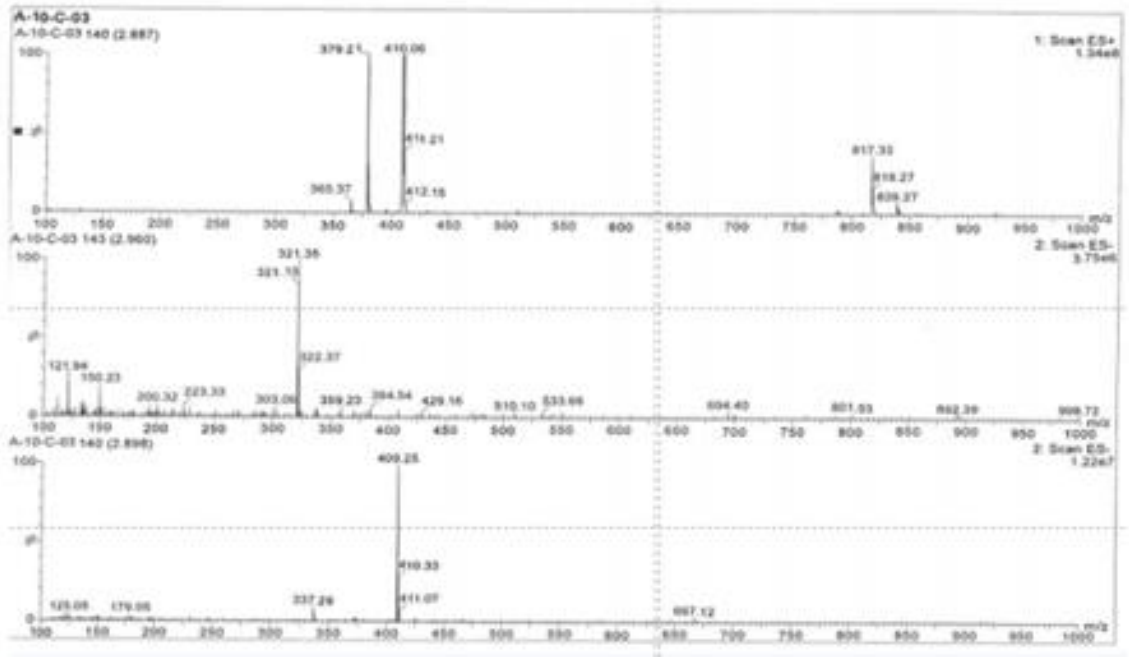
DEPT-90



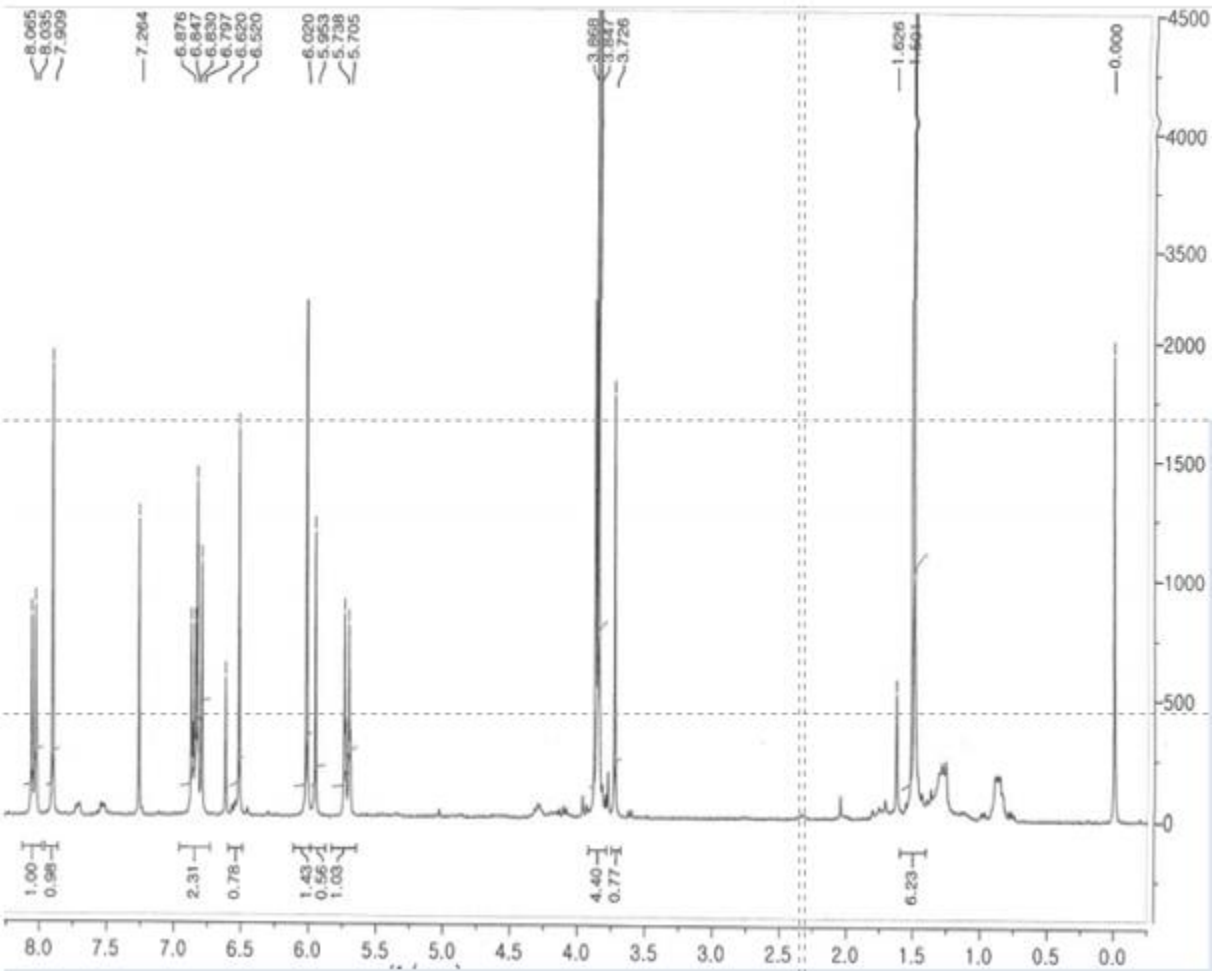
DEPT-135

Appendix 3A: ESIMS spectrum of compound 10CH-03

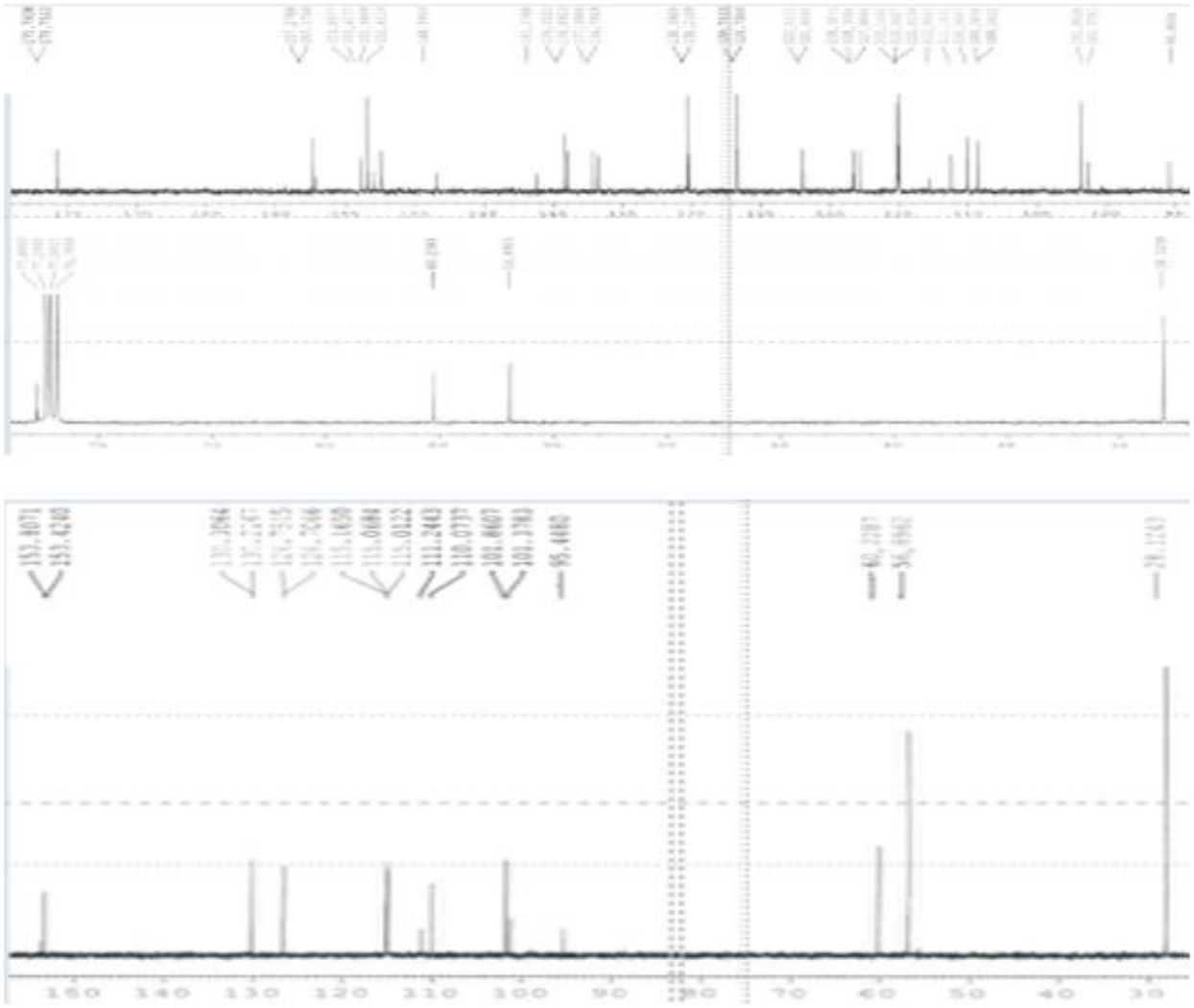




Appendix 3B: ^{1}H NMR (500.87 MHz) spectrum of compound 10CH-03

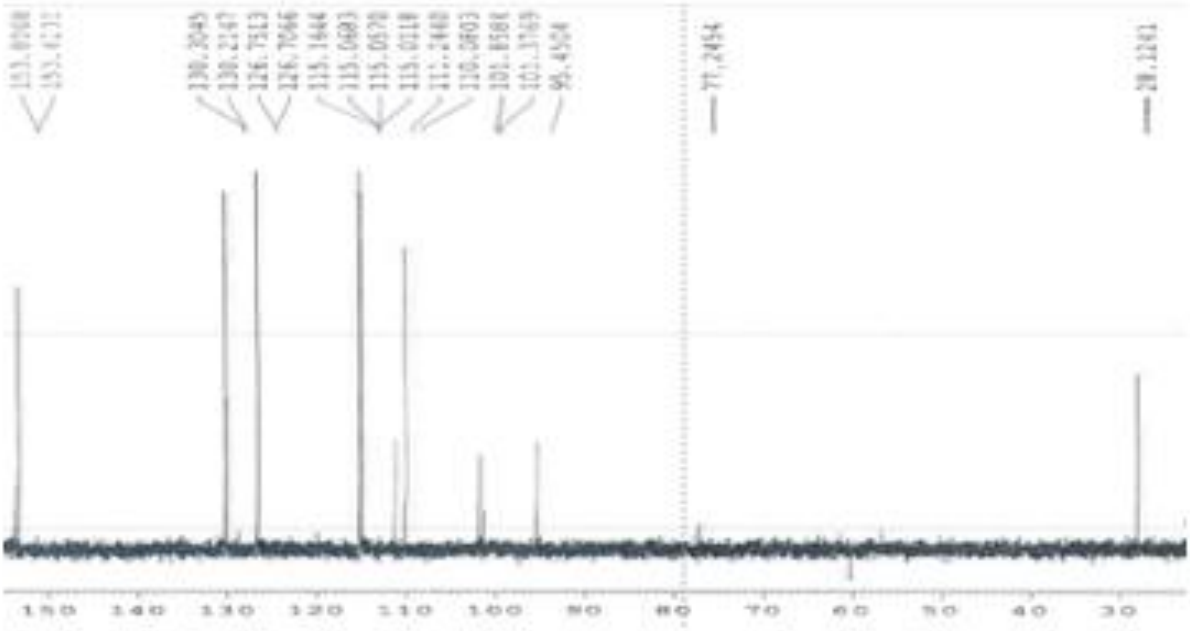


Appendix 3C: (I) ^{13}C NMR (125.77 MHz) (II) Dept-45 spectrum of compound 10CH-03

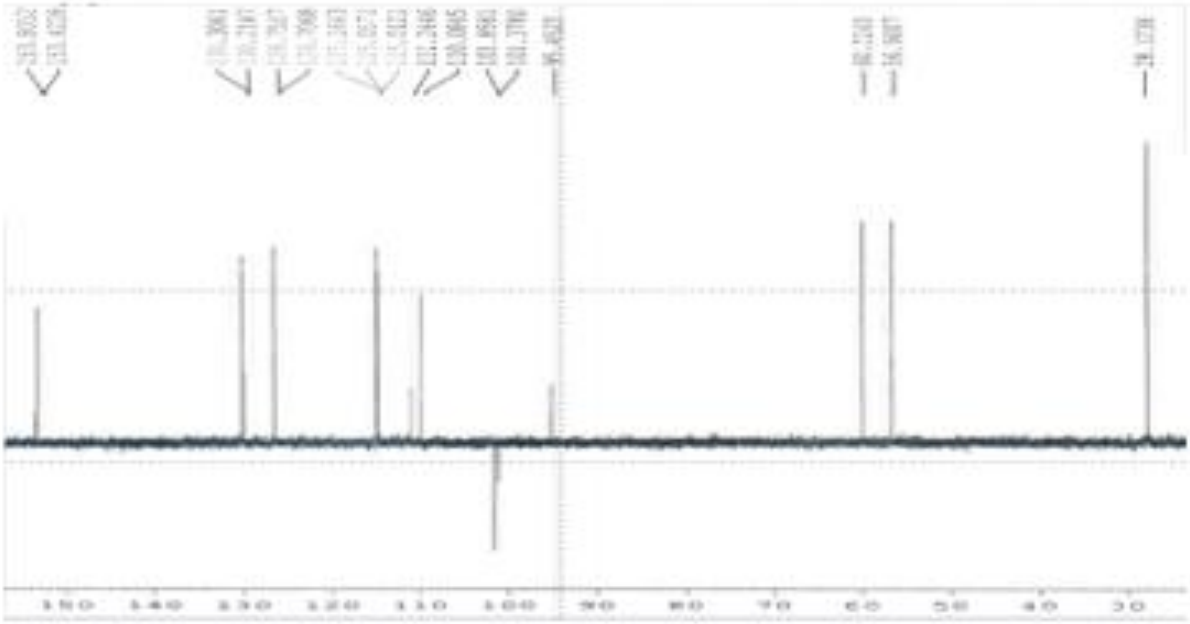


10-C-03 Dept-45

Appendix 3D : (I) Dept-90 (II) Dept-135 spectrum of compound 10CH-03

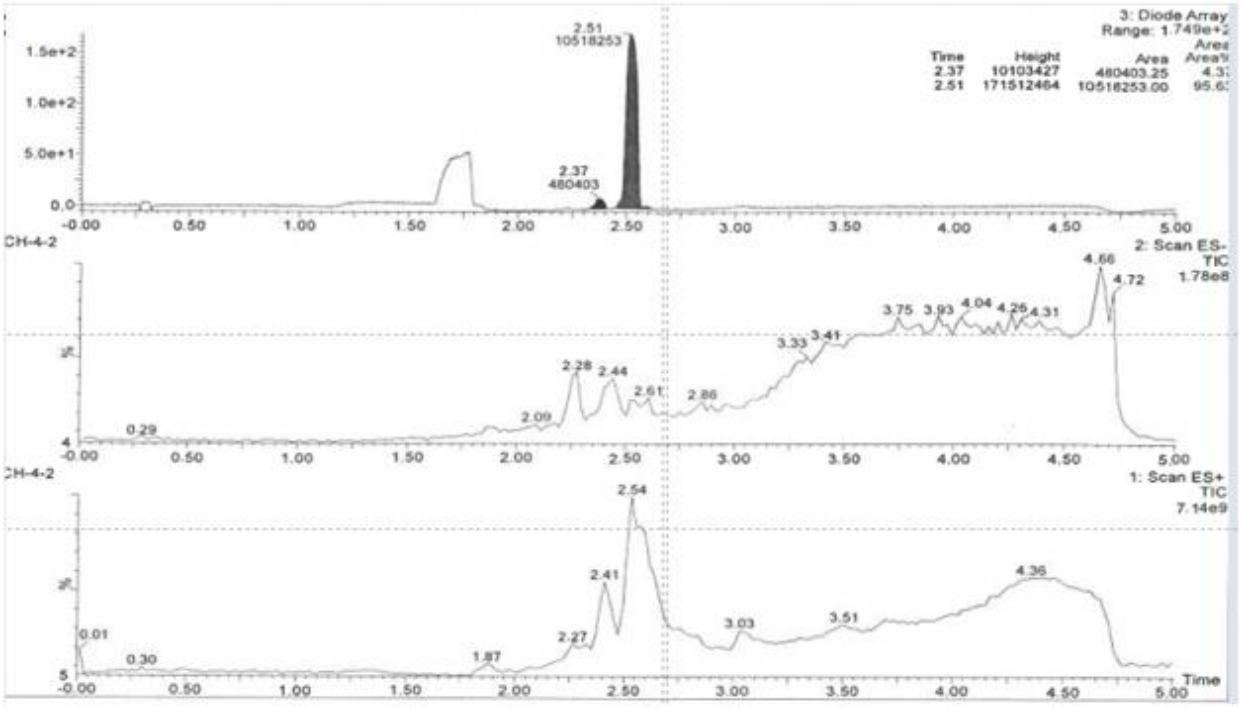


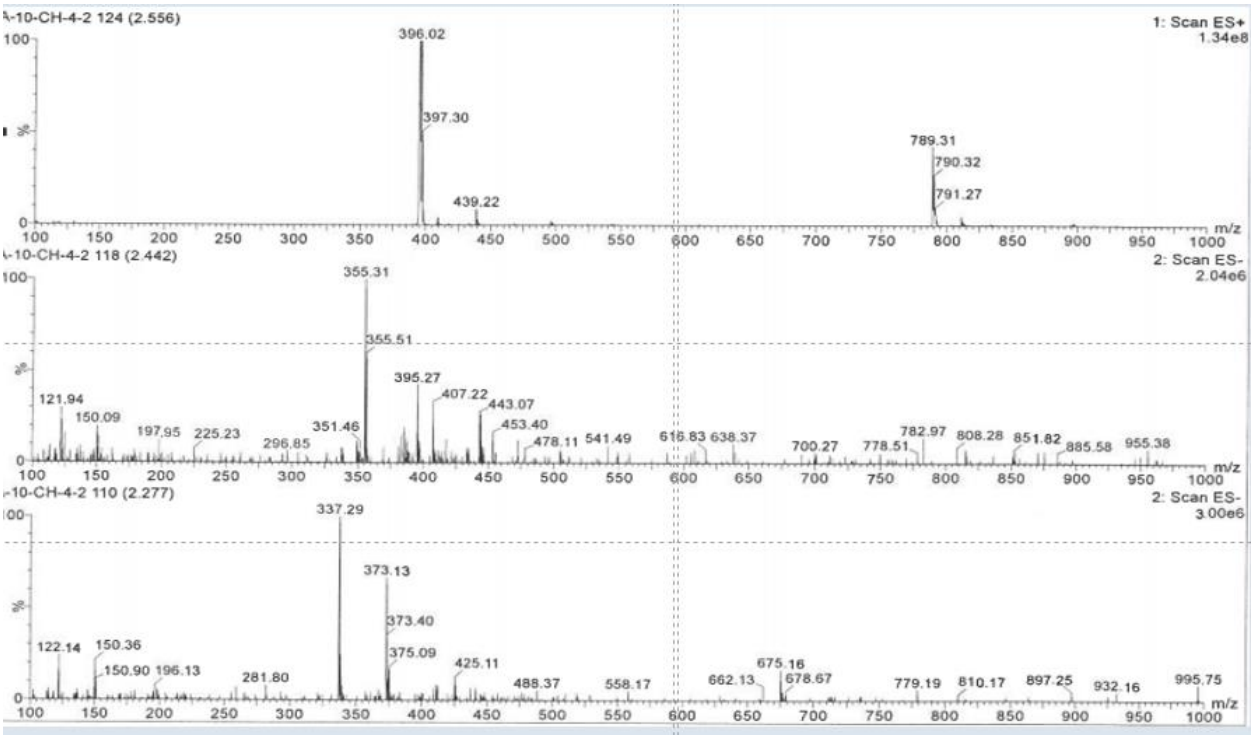
(I) Dept-90



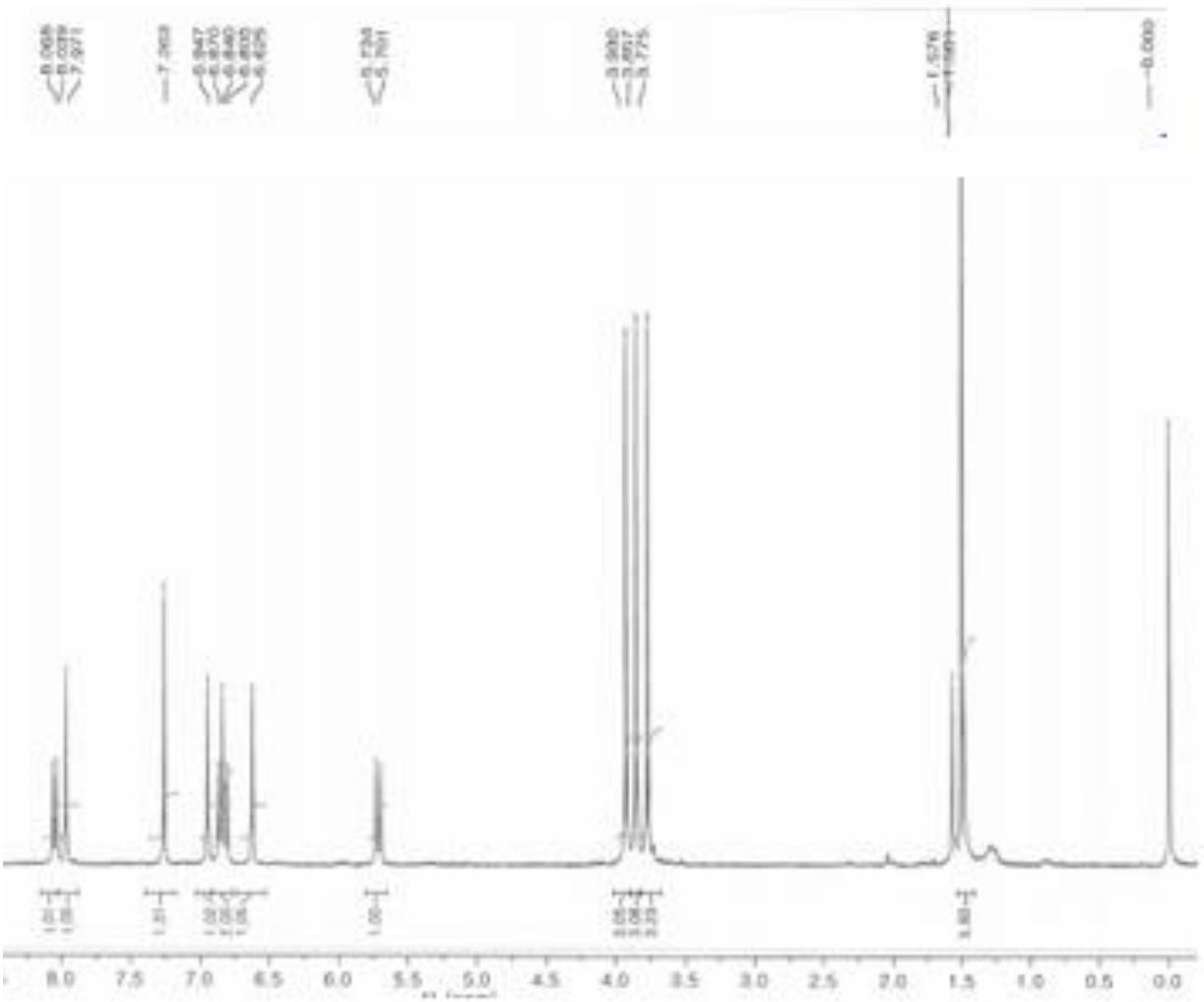
(II) Dept-135

Appendix 4A : ESIMS spectrum of compound 10CH-04

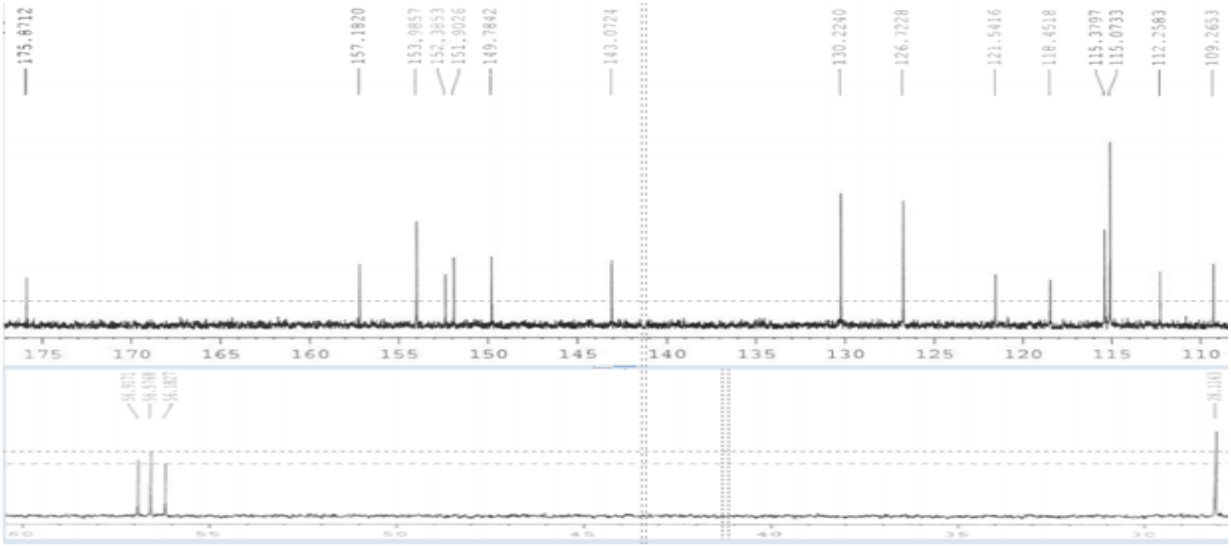




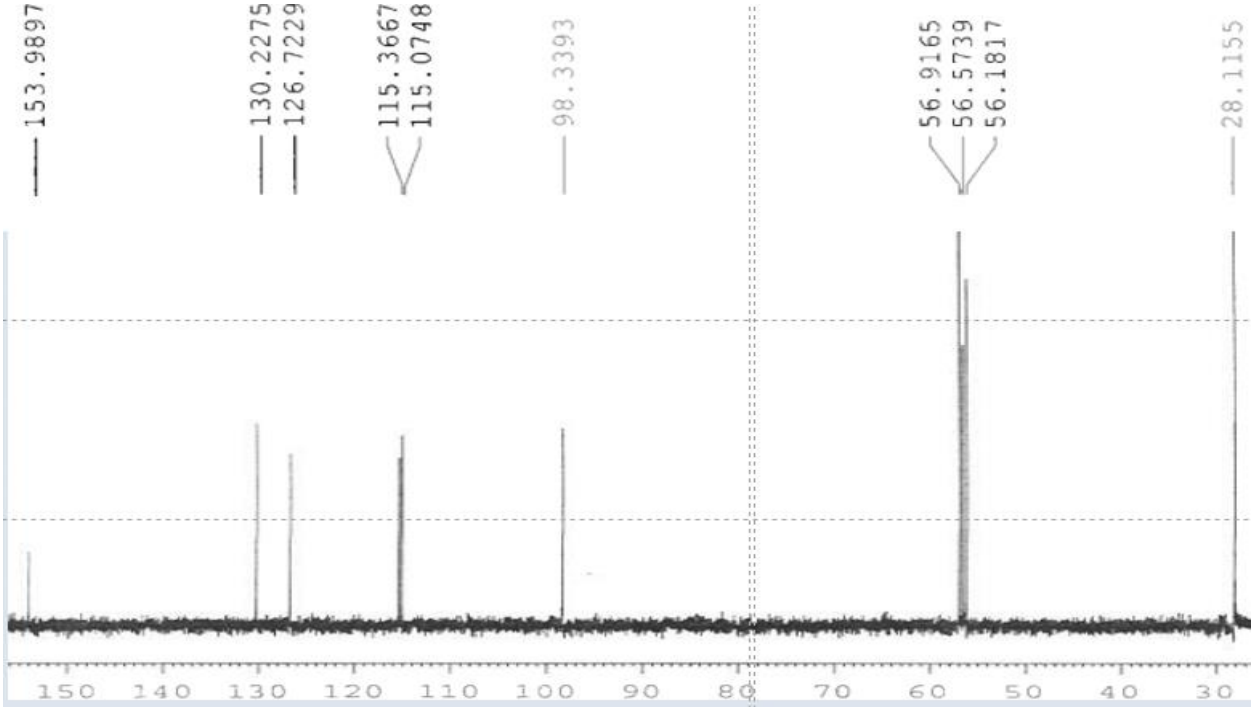
Appendix 4B: ^{1}H ($CDCl_3$, 500.87 MHz) NMR data of compound 10CH-04



Appendix 4C: (I) ^{13}C NMR (125.77 MHz) (II) Dept-45 spectrum of compound 10CH-04

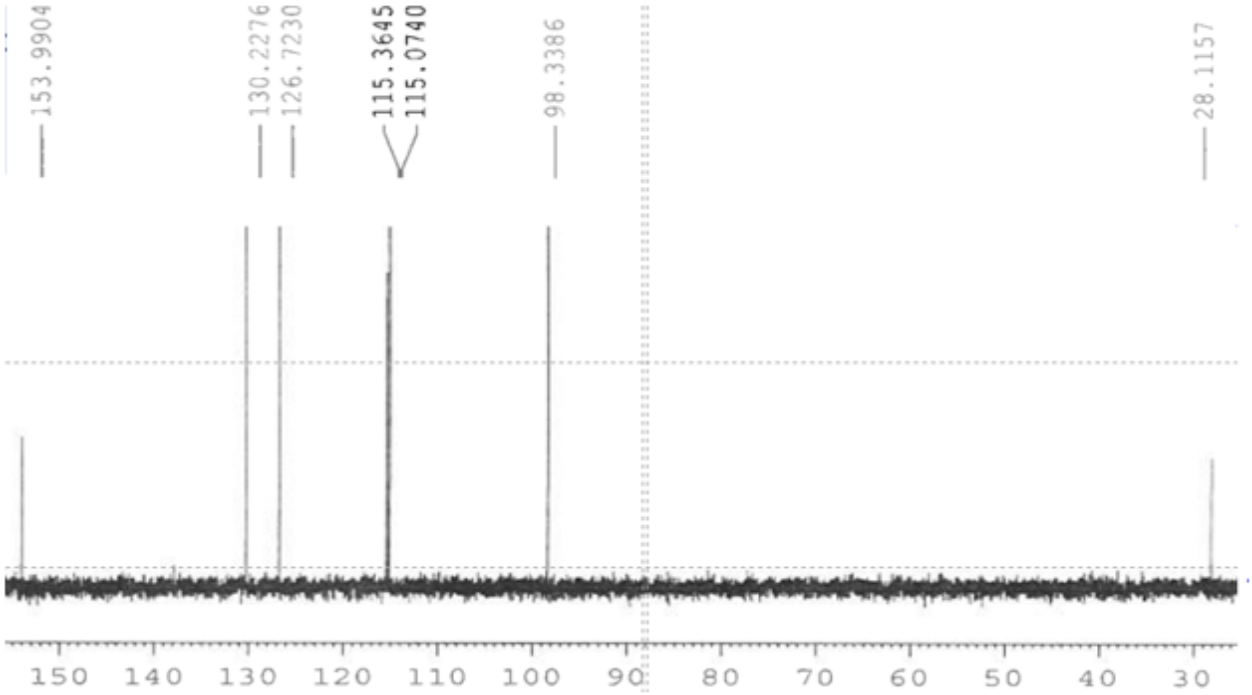


:(I) ^{13}C NMR

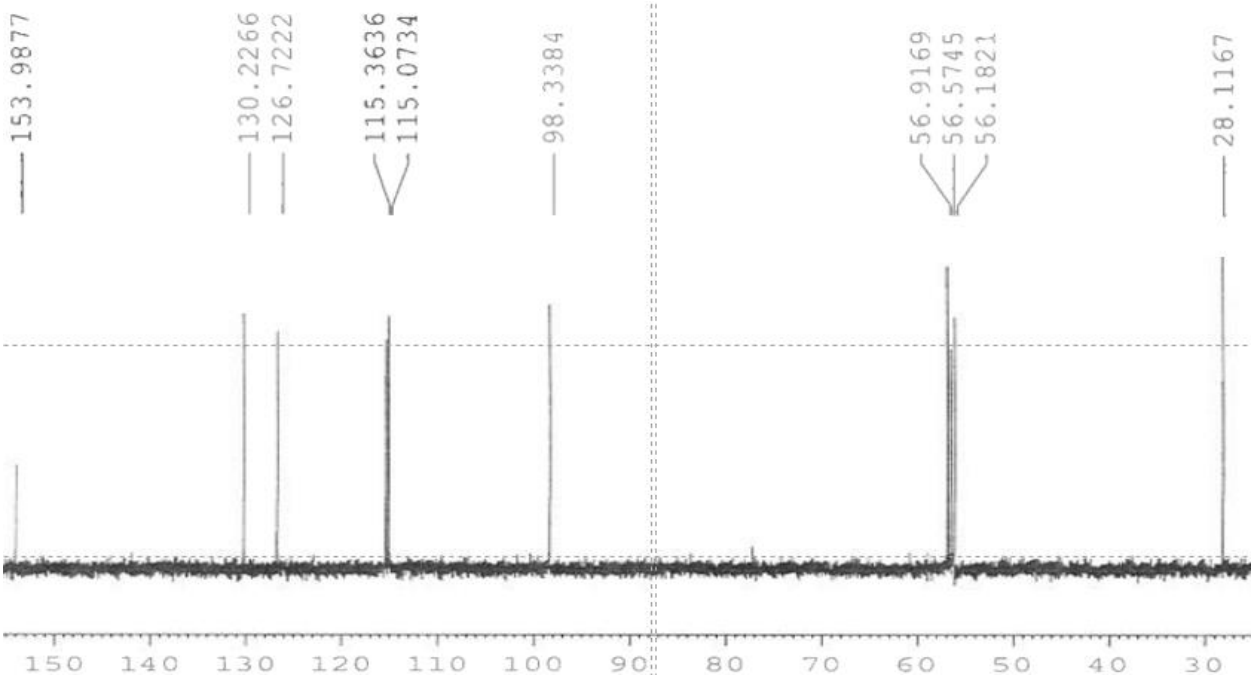


(II) Dept-45

Appendix 4D: (I) Dept-90 (II) Dept-135 spectrum of compound 10CH-04

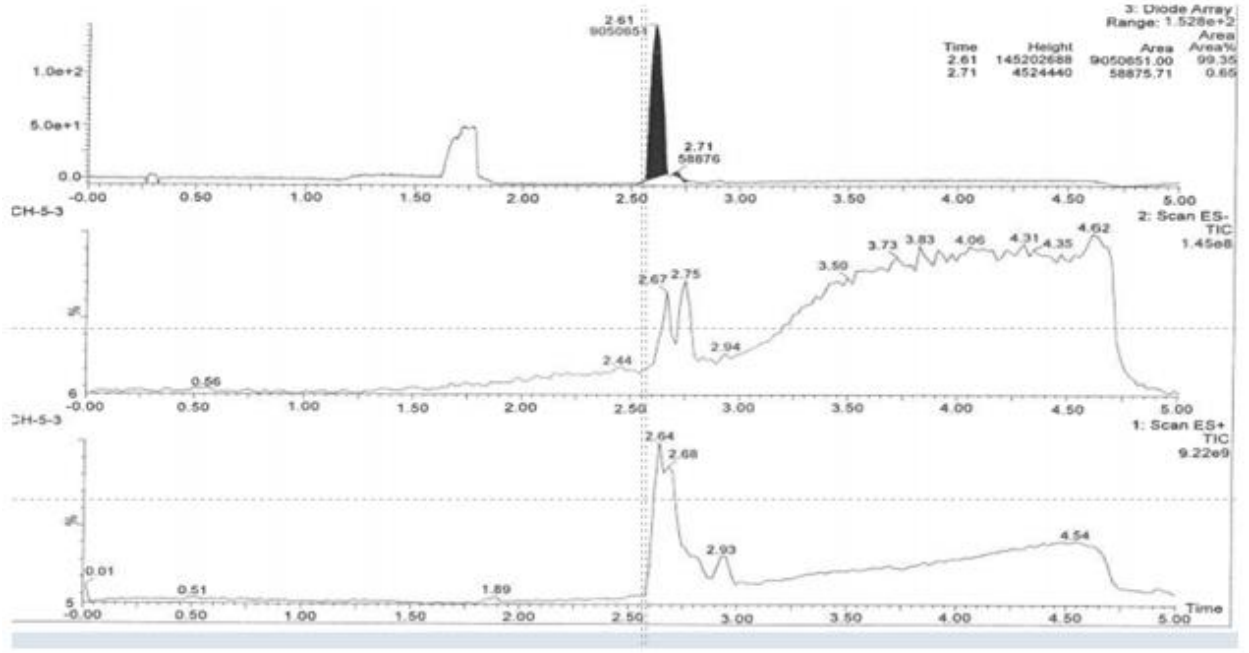


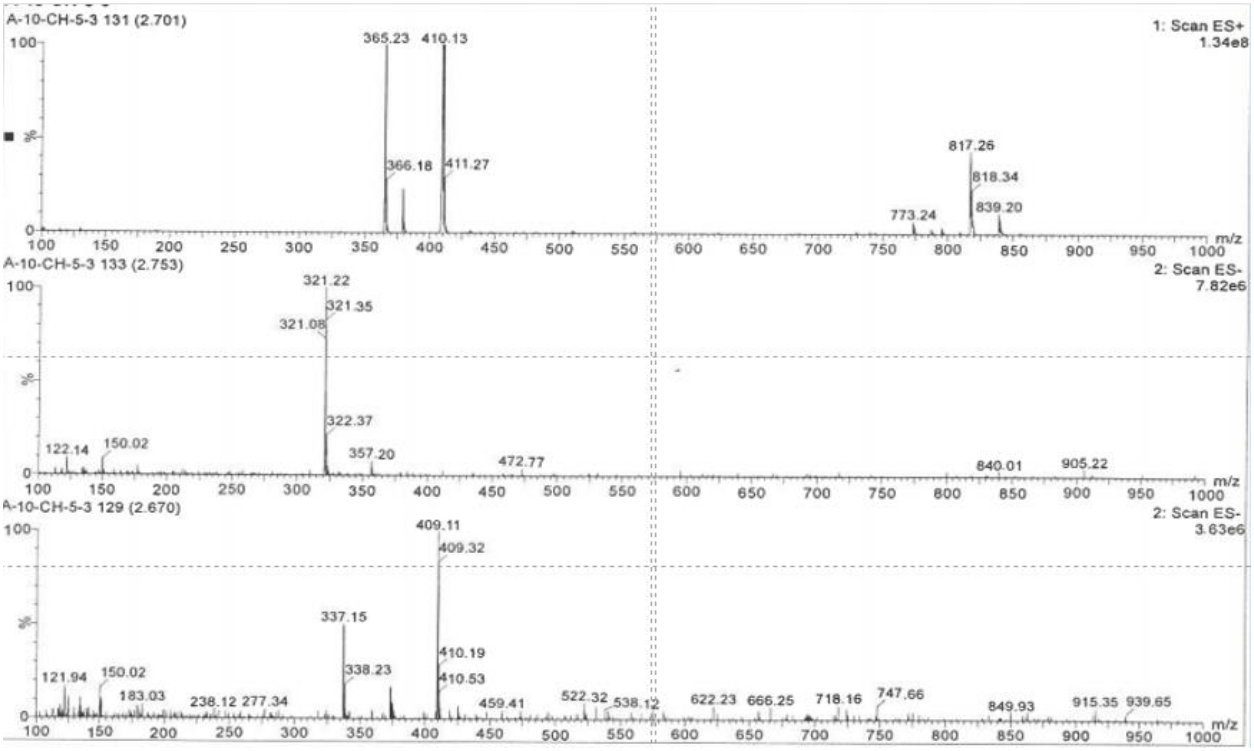
(I) Dept-90



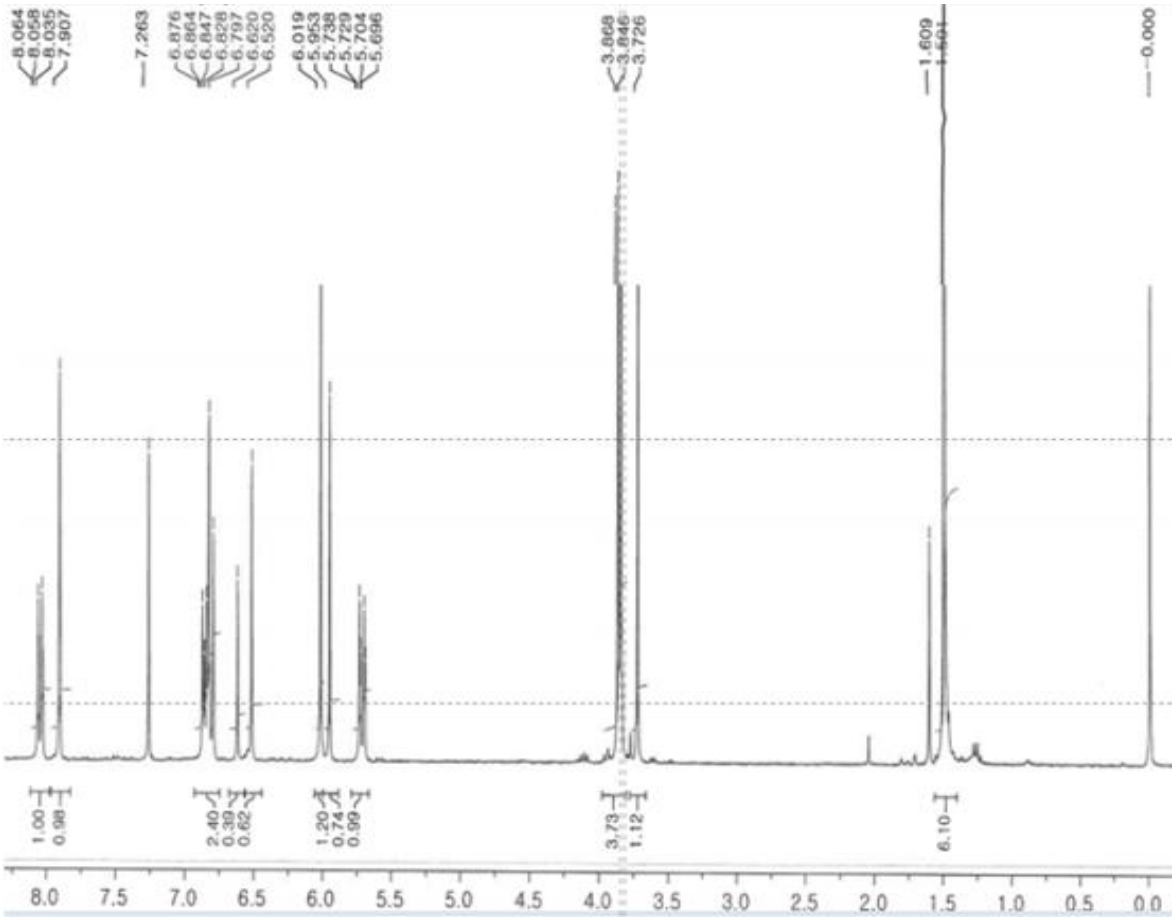
(II) Dept-135

Appendix 5A: ESIMS spectrum of compound 10-CH-05.

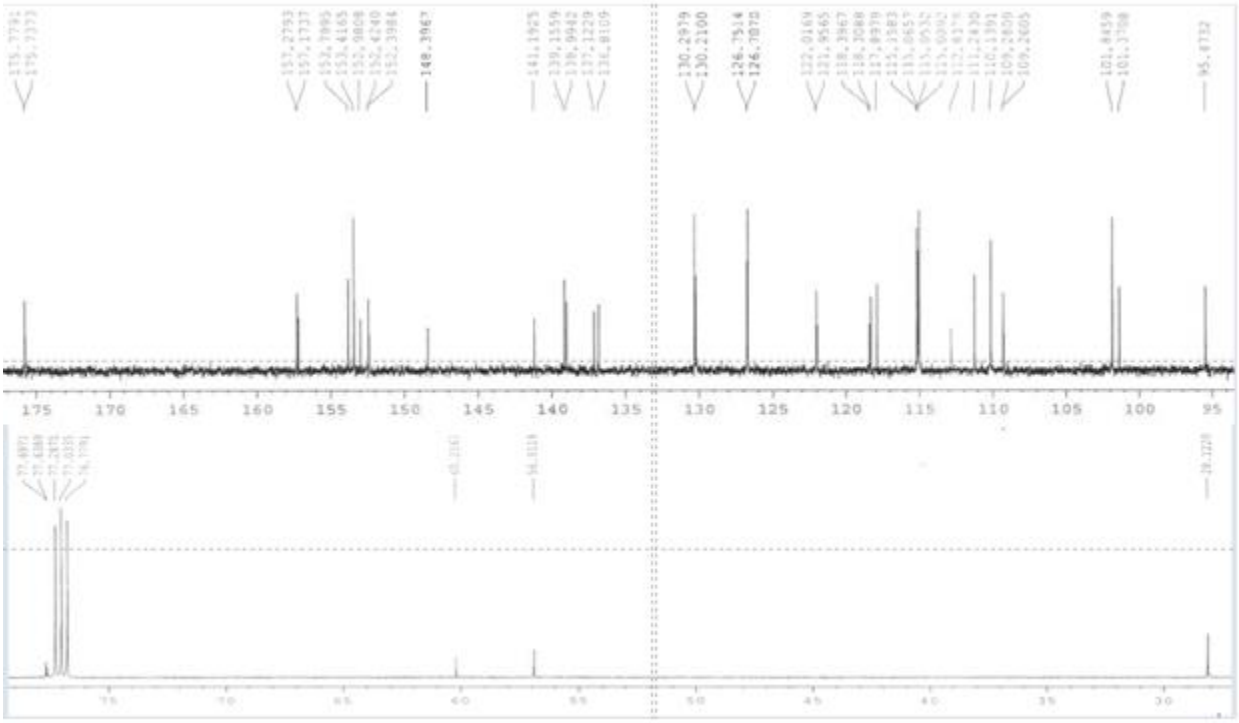




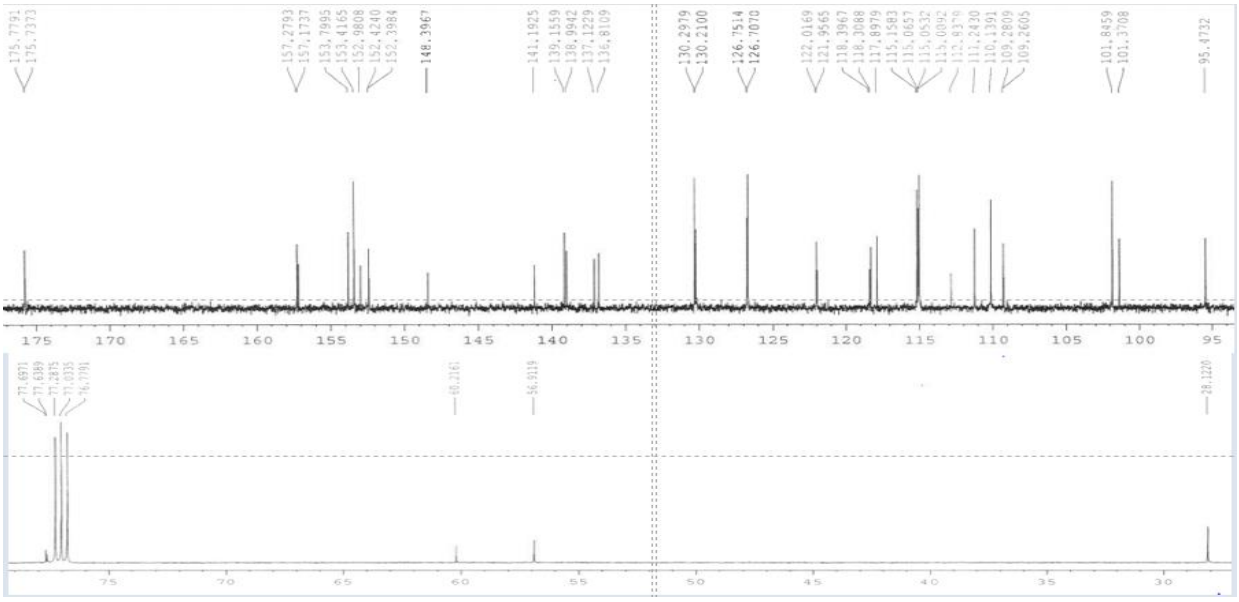
Appendix 5B: ^{1}H ($CDCl_3$, 500.87 MHz) NMR data of compound 10CH-05



Appendix 5C: (I) ^{13}C NMR (125.77 MHz) (II) Dept-45 spectrum of compound 10CH-05

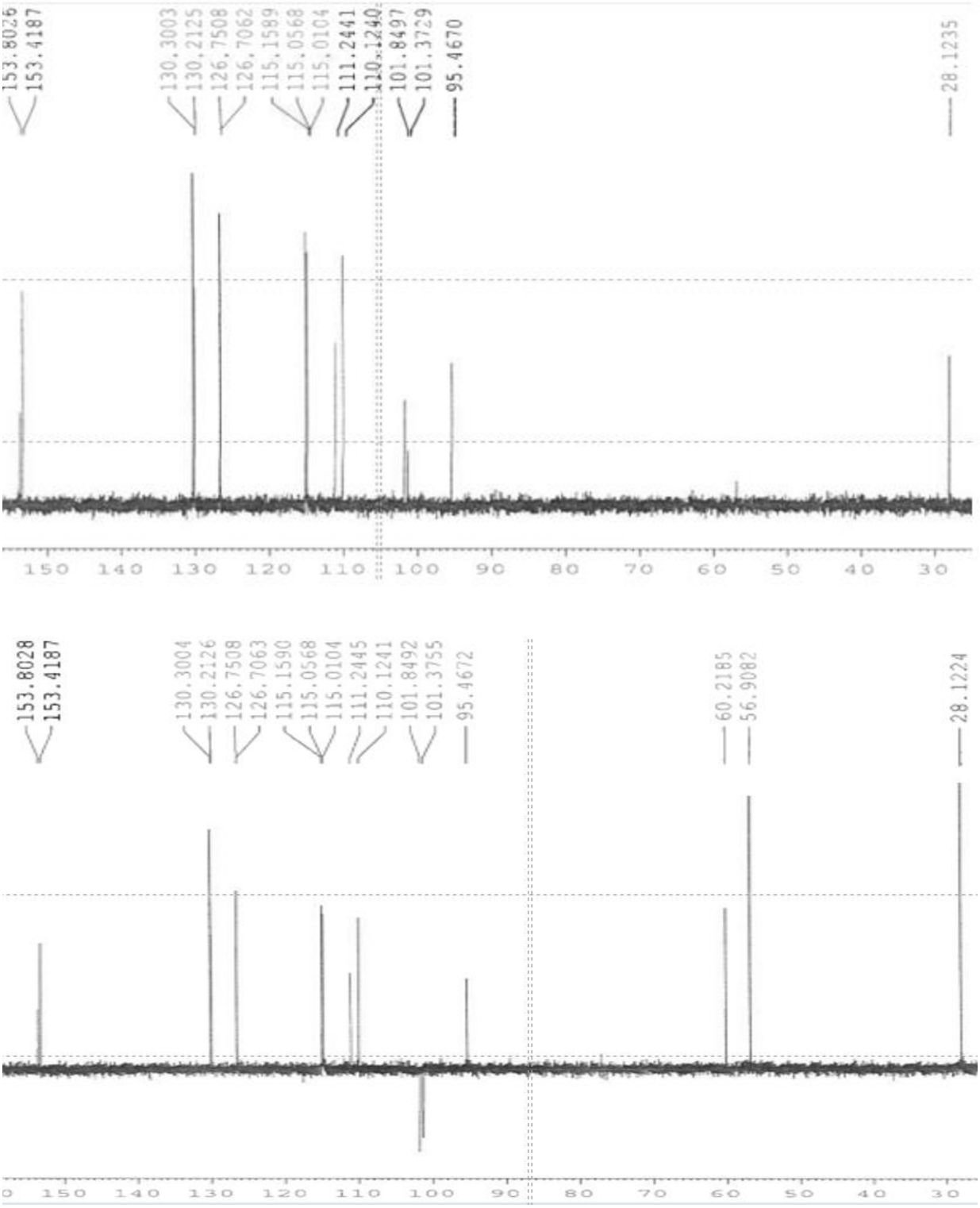


(I) ^{13}C NMR

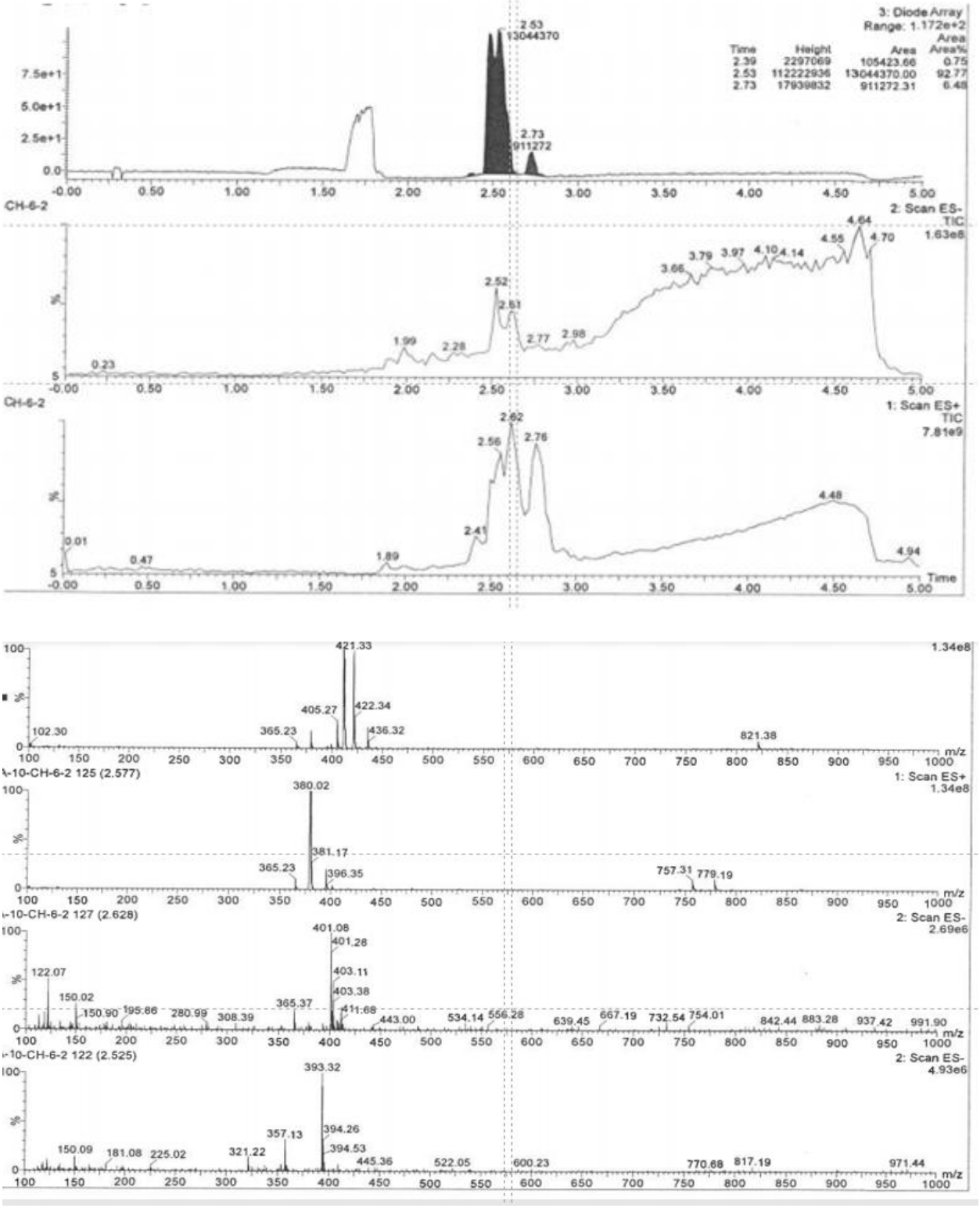


(II) Dept-45

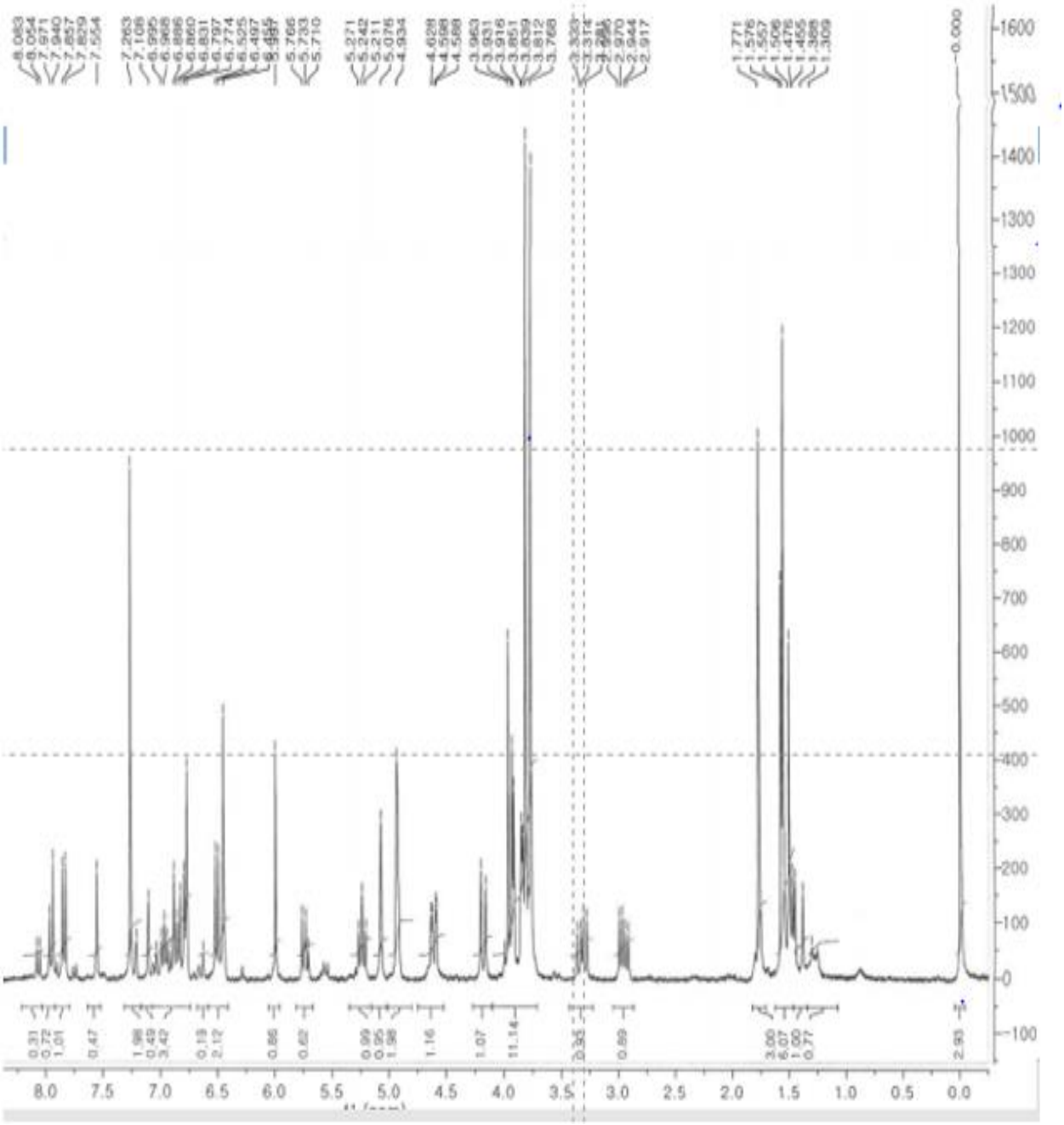
Appendix 5D: (I) Dept-90 (II) Dept-135 spectrum of compound 10CH-05



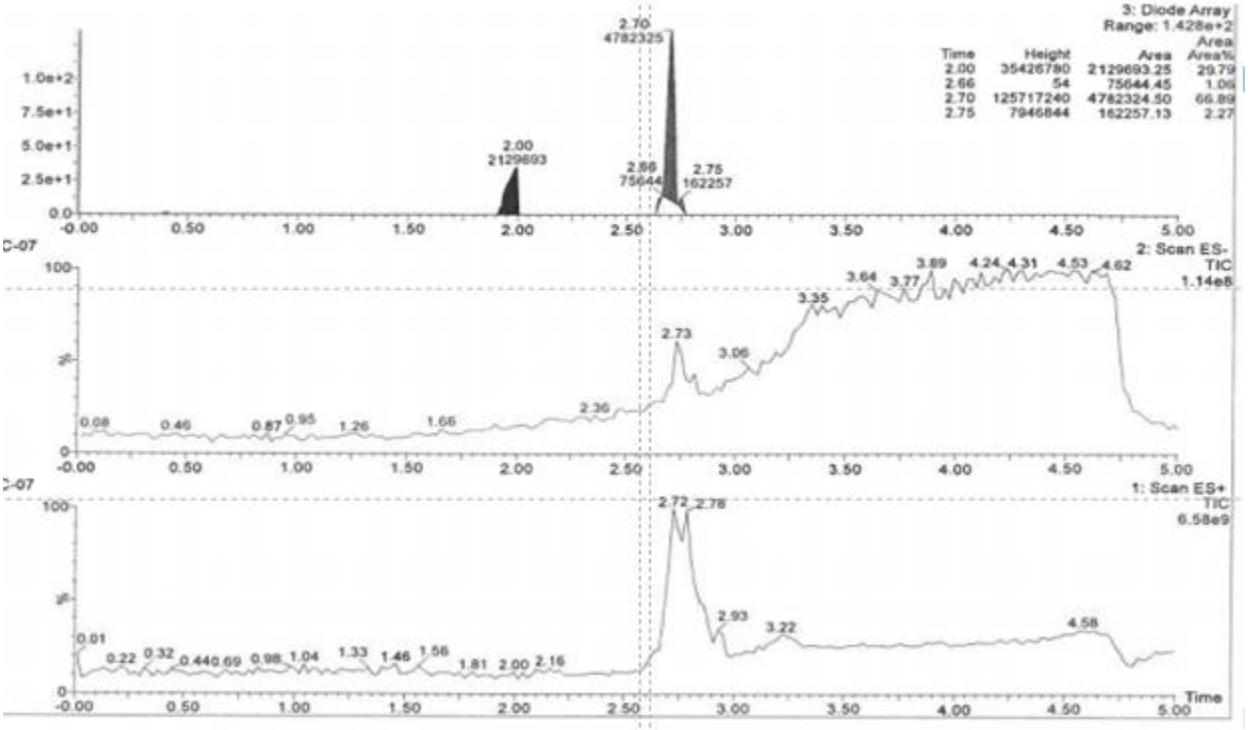
Appendix 6A: ESIMS spectrum of compound 10-CH-06

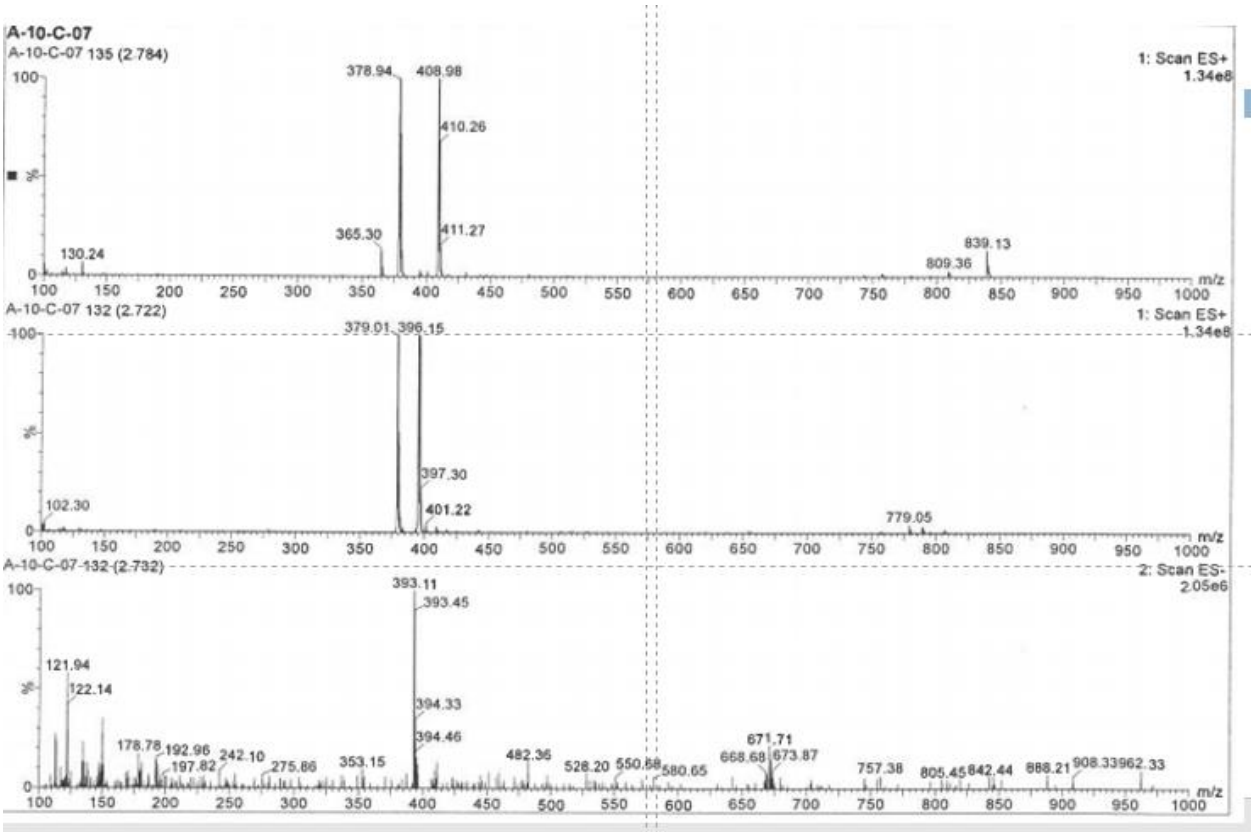


Appendix 6B: ^{1}H NMR (500.87 MHz) spectrum of compound 10CH-06



Appendix 7A: ESIMS spectrum of compound 10-CH-07.





Appendix 7B: ^{1}H NMR (500.87 MHz) spectrum of compound 10CH-07