INVESTIGATION OF ANTI-CANCER POTENTIAL OF *PLEIOCARPA PYCNANTHA* LEAVES

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BY

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A thesis submitted in partial fulfillment of the requirements for the degree of Doctor Philosophiae in the Department of Chemistry, University of the Western Cape.

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ABSTRACT

The Apocynaceae family is well known for its potential anticancer activity. Pleiocarpamine isolated from the Apocynaceae family and a constituent of *Pleiocarpa pycnantha* has been reported for anti-cancer activity. Prompted by a general growing interest in the pharmacology of Apocynaceae species, most importantly their anticancer potential together with the fact that there is scanty literature on the pharmacology of *P. pycnantha*, we explored the anticancer potential of the ethanolic extract of *P. pycnantha* leaves and constituents. Three known triterpenoids, ursolic acid C**1**, 27-*E* and 27-*Z* p-coumaric esters of ursolic acid C**2**, **C3** together with a new triterpene 2,3-seco-taraxer-14-en-2,3-lactone (pycanocarpine **C5**) were isolated from an ethanolic extract of *P. pycnantha* leaves. The structure of **C5** was unambiguously assigned using NMR, HREIMS and X-ray crystallography. The cytotoxic activities of the compounds were evaluated against HeLa, MCF-7, KMST-6 and HT-29 cells using the WST-1 assay. Ursolic acid C**1** displayed potent cytotoxic activity against HeLa, HT-29 and MCF-7 cells with IC_{50} values of 5.06, 5.12 and 9.51 µg/ml respectively. The new compound **C5** and its hydrolysed open-chain derivative **C6** were selectively cytotoxic to the breast cancer cell line, MCF-7 with IC_{50} values 10.99 and 5.46 µg/ml respectively. We further investigated the mechanism of action of the isolated compounds using specific markers of apoptosis. Exposure of **C1**-**C6** (12.5 µg/ml) to HeLa cells showed a significant increase in reactive oxygen species (ROS) production with the exception of **C5**. On HT-29, **C1**, **C4**, **C5** and **C6** at 25 µg/ml increased ROS production while on MCF-7 using the same dose, only **C5** and **C6** caused a significant increase in ROS production compared with a control at $P< 0.05$. The result on caspase 3/7 activation showed that **C1** and **C2** (50 µg/ml) caused a marked increase in caspase 3/7 activity between 6-24 h on HeLa cells while only **C1** (50 µg/ml) showed a significant increased caspase 3/7 activity on both HT-29 and MCF-7 cell

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lines when compared with the control, P< 0.05. Some selected compounds were further investigated for their dose-response on caspase 3/7 activity on HeLa and MCF-7 cells. Compounds **C2** and **C3** activated caspase 3/7 at 12.5 and 25 µg/ml respectively, while on MCF-7only **C6** significantly increased caspase 3/7 activity within 24 h of treatment when compared with an untreated control. The result of time -dependent caspase 9 activity showed that **C1**, **C2** and **C3** caused an increased activity on HeLa cells between 6-12 h, while only **C1** activated caspase 9 on HT-29 cells (3-24 h) and MCF-7 (6-24 h). The dose-response caspase 9 activity showed a significant increase in activation for **C6** (12 and 25 µg/ml) on HeLa and **C5** (25 µg/ml) on HT-29 cells. All isolated compounds inhibited Topoisomerase I when compared with Camptothecin. Compounds **C1-C6** could induce apoptosis on cancer cell lines through an intrinsic pathway and topoisomerase 1 inhibition. This is the first report on the isolation of a 2,3-seco-taraxerene derivative from Apocynaceae family and the anticancer activity of *Pleiocarpa pycnantha* constituents.

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KEYWORDS

Apoptosis

Apocynaceae

Apo-percentage

Cancer

Caspases

CM-H_2DCFDA (5-(and-6)-chloromethyl-2', 7'-dichlorodihydrofluorescein diacetate, acetyl ester)

Cytotoxicity

Flow cytometry

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WST-1, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazolium monosodium salt)

Lactone

Phytochemistry

Pleiocarpa pycnantha

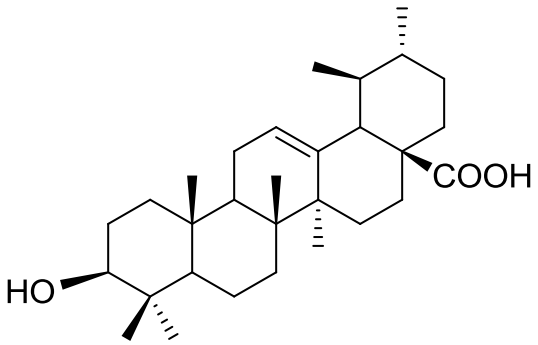
Reactive oxygen specie

Topoisomerases

Triterpenes

Ursolic acid

LIST OF ISOLATED COMPOUNDS



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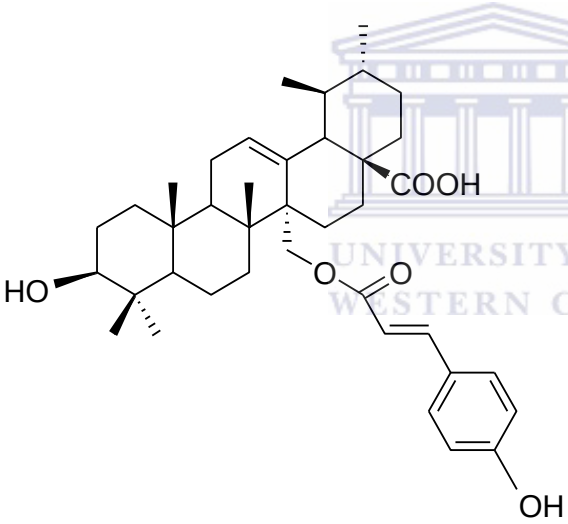

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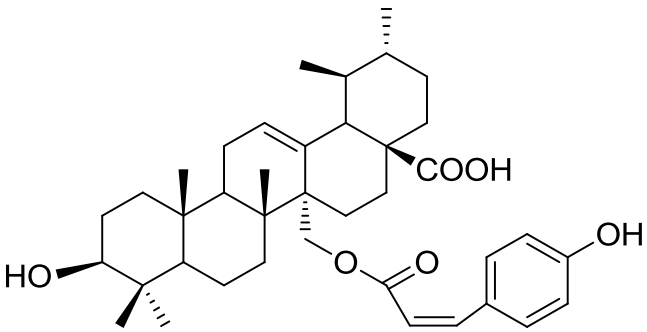

27-p-*Z*-coumaroloxyursolic acid

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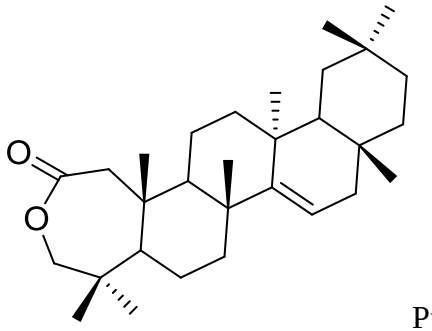
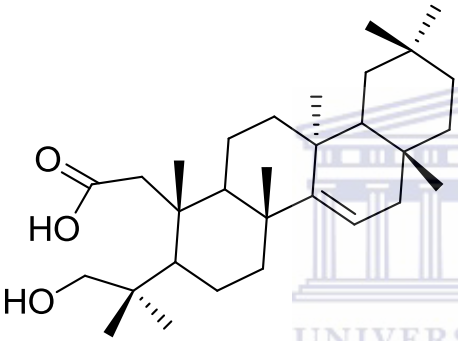


Pycanocarpine A (New compound)



Pycanocarpene A (New compound)

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DECLARATION

I declare that "The Investigation of anti-cancer potential of *Pleiocarpa pycnantha* leaves" is my own work, that it has not been submitted before for any degree or assessment in any other University, and that all the sources I have used or quoted have been indicated and acknowledged by means of complete references.

Olubunmi Adenike Omoyeni December 2013

Signed……

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To God be the glory, great things He hath done. I give all the glory, honor and adoration to God for the strength, wisdom, knowledge and understanding throughout my study period. I want to thank Him specially for seeing me through my dark moments and the grace to be victorious at last. You deserve all the glory and praise my Lord.

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My deepest gratitude to my darling husband, Mr. Samuel Abayomi Akinwumi, for the love and support you have so faithfully given me especially during the course of this work and for holding forth at the home front, thank you so much.

DEDICATION

This project is dedicated to:

The **Omnipotent God** who has all powers in heaven and the earth for seeing me through in this great journey.

To my parents: Late Elder Adebayo Emmanuel Omoyeni and Mrs Mary Mobolaji Omoyeni for their endless love, support and care.

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GLOSSARY OF ABBREVIATIONS

APAF1 Apoptotic protease-activating factor 1

ATP Adenosine triphosphate

^{13}C-NMR Carbon nuclear magnetic resonance

CAD Caspase-activated deoxyribonuclease

CARD Caspase activated recruitment domains

1D-NMR One-dimensional nuclear magnetic resonance

^{1}H-NMR Proton nuclear magnetic resonance

2D-NMR Two-dimensional nuclear magnetic resonance

br s Broad singlet

CC Column chromatography

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CI Chemical ionization

COSY Correlation spectroscopy

DCM Dichloromethane

DD Death domain

DEDs Death effector domains

DISC Death inducing signaling complex

DNA Deoxyribonucleic acid

d Doublet

dd Doublet of doublet

DEPT Destortionless enhancement by polarization transfer

dm Doublet of multiplet

DMSO Dimethyl sulfoxide

DSBs Double-strand breaks

EtOAc Ethyl acetate

FACS Flourescent Activated Cell Sorting

FADD Fas-activated Death Domain

e.g. For example

EI Electron impact

ESI Electrospray ionization

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ETOH Ethanol

etc. et cetera

FAB Fast atom bombardment

GC-MS Gas chromatography-Mass spectrometry

h Hour

Hz Hertz

HMBC Heteronuclear multiple bond correlation

HSQC Heteronuclear single quantum correlation

Hex Hexane

HPLC High performance liquid chromatography

HRMS High Resolution Mass Spectrum

IR Infra red

IAPs Inhibitors of Apoptosis proteins

IC_{50} 50% Inhibitory concentration

J Coupling constant in Hz

LC-MS Liquid chromatography-Mass spectrometry

m Multiplet

MeOH Methanol

NMR Nuclear Magnetic Resonance

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min Minute

MS Mass spectrometry

OD Optical Density

PBS Phosphate buffer saline

PTLC Preparative thin-layer chromatography

qq Quartet of quartet

ROS Reactive Oxygen Species

ROESY Rotating-frame overhauser enhancement Spectroscopy

s Singlet

TLC Thin-layer chromatography

TMS Tetramethysilane

TNFR Tumor necrosis factor receptor

UV Ultra violet

WST-1 Tetrazolium salt (4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate)

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LIST OF TABLES

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LIST OF PROPOSED PUBLICATIONS

1. Olubunmi A. Omoyeni, Mervin Meyer, Emmanuel Iwuoha, Ivan Green, Ahmed A. Hussein. An unusual 2, 3-secotaraxerene and other cytotoxic triterpenoids from *Pleiocarpa pycnantha* (Apocynacae) leaves collected from Nigeria. *Molecules* **2014**, *19*, 3389-3400; doi:10.3390/molecules19033389.
2. Olubunmi A. Omoyeni, Ahmed A. Hussein, Ivan Green, Emmanuel Iwuoha, Mervin Meyer. Novel Triterpenes from *Pleiocarpa pycnantha* leaves induce apoptosis on MCF-7 breast cancer cell lines. Manuscript in preparation.
3. Olubunmi A. Omoyeni, Ahmed A. Hussein, Ivan Green, Mervin Meyer, Emmanuel Iwuoha. Investigation of anticancer potential of ethanol extract from *Pleiocarpa pycnantha* leaves and constituents. Manuscript in preparation.
4. Olubunmi A. Omoyeni, Ahmed A. Hussein, Emmanuel Iwuoha, Ivan Green. The review of the ethnomedicinal uses, pharmacology and phytochemistry of *Pleiocarpa* genus. Manuscript in preparation.

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CHAPTER 1

1.0 INTRODUCTION

1.1. General overview of the use of plants

Plants have been serving the animal kingdom as its main source of energy (food, fuel) as well as its means of shelter and sustenance since the very beginning of life on earth. Over 250,000 species of flowering plants have so far been recognized. In addition to provision of food, fuel and shelter, each of these plants have also been great sources of medicinal agents since they possess chemical constituents such as phenolic compounds, alkaloids, terpenes, glycosides, steroids and a host of others commonly referred to as secondary metabolites. The secondary metabolites are present in low concentrations and are sometimes species specific in terms of their production, in some cases, specific metabolites may be found in only one part of a plant (Trease and Evans, 1996).

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In early times and in the present day traditional medicine as practised in many countries, medicinal plants are taken in as their crude forms and prepared in the form of decoctions, infusions or powders. The isolation of defined chemical substances from plants started in the nineteenth century. At the beginning, structural elucidation of isolated compounds were limited by the technology available at that time in which case, it was done by classical and exhaustive degradative methods and the structures that were proposed were confirmed through synthesis and in most cases followed by biological activity determinations. One of the most common activities tested with isolated compounds was antibacterial activity since this can easily be detected even from an unpurified crude extract while scientists avoided other biological activities. The in vivo experiments conducted were cumbersome, requiring large numbers of animals and crude

extracts. In more recent times developments of *in vitro* and biochemical screening methods have renewed interest in the screening of plants for biological activity since the methodologies do not require pure material and very small quantities can be evaluated quite easily and rapidly.

Currently, the isolation of chemical compounds from plant sources in drug discovery is bioassay guided involving selective and sensitive screening procedures with low concentrations (nanomole/litre) of compounds which are sufficient for the detection of a specific response.
The structure of an isolated compound is elucidated with only a few milligrams of pure material using sophisticated instrumentation such as HPLC, NMR (1D and 2D), COSY, HMQC, HMBC and HREMS. Many new compounds (flavonoids, alkaloids, terpenoids, peptides and phytosterols to name a few) are generated each year through the combination of sensitive screens and modern structural elucidation methods. Some of these compounds are antimicrobial, anti-inflammatory, anthelmintics, anticancer, antimalarial, enzyme inhibitor and some are known to bind to specific receptors of pharmacological interest. However, there are some compounds isolated through chemically guided isolation that have proved to be biologically active.

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After the isolation and identification of a compound, larger quantities are normally required for further and more comprehensive evaluation and this is achieved through either chemical synthesis or cultivation and collection of large quantities of plant material for extraction and purification by large scale chromatographic means. Nigeria and South Africa have abundant flora and fauna which are yet to be fully explored for potential drugs. Traditional medicines from plants are cheap and are the main sources for herbal drugs for citizens to be used against diseases endemic to the tropical and non tropical countries.

Cancer is one of the classes of disease that is a major public burden and a threat to both developed and the developing nation. It is characterized by uncontrolled growth and the consequent spread of abnormal cells and if the spread is not controlled, it usually results in death. It is caused by both external factors (tobacco, chemicals, radiation, and infectious organisms) and internal factors (inherited mutations, hormones, immune conditions and mutations that occur from metabolism). These causal factors may act together or in sequence to initiate or promote carcinogenesis. Worldwide, one in eight deaths is due to cancer which additionally causes more deaths than AIDS, tuberculosis and malaria combined. When countries are grouped according to economic development, it is the leading cause of death in developed countries and the second leading cause of death in developing countries (following heart diseases) (ACS, 2011). According to the World Health Organization (WHO) projections in 2007, cancer will have replaced ischemic heart disease as the overall leading cause of death worldwide in 2010 (WHO, 2007).

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The estimates from the International Agency for Research on Cancer (IARC) proposed 12.7 million new cancer cases in 2008 worldwide, of which 5.6 million occurred in economically developed countries and 7.1 million in economically developing countries. The corresponding estimates for total cancer deaths in 2008 were 7.6 million (about 21,000 cancer deaths per day), 2.8 million in economically developed countries and 4.8 million in economically developing countries. By 2030, the global burden is expected to grow to 21.4 million new cancer cases and 13.2 million cancer deaths simply due to the growth and aging of the population, as well as reductions in childhood mortality and deaths from infectious diseases in developing countries (Ferlay et al., 2010; Jemal et al., 2011).

The global cancer statistics released by the American Cancer Society reads "The total number of deaths in 2007 was 7.6 million, or about 20,000 deaths per day, with 38% in developed countries and 62% in developing countries. By 2050, 27 million new cancer cases and 17.5 million cancer deaths are projected to occur in the world" (Dunham, 2007; ACS, 2007).

Cancer is an emerging public health problem in Africa, about 681,000 new cancer cases and 512,400 cancer deaths occurred in 2008. These numbers are projected to nearly double (1.28 million new cancer cases and 970,000 cancer deaths) by 2030 simply due to the aging and growth of the population with the potential to be even higher because of the adoption of behaviors associated with western lifestyles, such as smoking, unhealthy diet and physical inactivity (Ferlay et al., 2008; WHO, 2007; 2008). Cancers related to these factors, such as lung, breast, and colorectal cancers are increasing in economically transitioning countries. Rates of cancers common in western countries will continue to rise in developing countries if preventive measures are not widely applied. In economically developed countries, the three most commonly diagnosed cancers were prostate, lung / bronchus and colorectal among men and in women; breast, colorectal and lung / bronchus. In economically developing countries, the three most commonly diagnosed cancers were lung / bronchus, stomach and liver in men while in women were breast, cervix uteri and stomach. In both economically developed and developing countries, the three most common cancer sites were also the three leading causes of cancer deaths (ACS, 2007).

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According to the new cancer statistics released by GLOBOCAN (2012), an estimated 14.1 million new cancer cases and 8.2 million cancer-related deaths occurred in 2012, compared with 12.7 million and 7.6 million, respectively in 2008. The most commonly diagnosed cancers

worldwide were those of the lung (1.8 million, 13.0% of the total), breast (1.7 million, 11.9%), and colorectum (1.4 million, 9.7%). The projections based on the GLOBOCAN (2012) estimates has also predicted a substantive increase to 19.3 million new cancer cases per year by 2025 due to growth and ageing of the global population. More than half of all cancers (56.8%) and cancer deaths (64.9%) in 2012 occurred in less developed regions of the world, and these proportions will increase further by 2025 (Ferlay et al., 2013).

Breast cancer is the most common cause of cancer death among women (522 000 deaths in 2012), the most frequently diagnosed cancer among women in 140 of 184 countries worldwide, it now represents one in four of all cancers in women and also a leading cause of cancer death in the less developed countries of the world. In 2012, 1.7 million women were diagnosed with breast cancer, since the 2008 estimates, breast cancer incidence has increased by more than 20%, while mortality has increased by 14% (Ferlay et al., 2013). Based on the recent cancer statistics, cervical cancer has been reported to be the fourth most common cancer affecting women worldwide after breast, colorectal and lung cancers; it is most notable in the lower-resource countries of sub-Saharan Africa. It is also the fourth most common cause of cancer death (266 000 deaths in 2012) in women worldwide. Almost 70% of the global burden falls in areas with lower levels of development and more than one fifth of all new cases are diagnosed in India.

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Cervical cancer was estimated to have about 528 000 new cases every year. In sub-Saharan Africa, 34.8 new cases of cervical cancer are diagnosed per 100 000 women annually and 22.5 per 100 000 women die from the disease. These figures compare with 6.6 and 2.5 per 100 000 women, respectively in North America. The drastic differences might be the lack of access to

effective screening and to services that facilitate early detection and treatment in the developing countries (Ferlay et al., 2013).

Despite this growing burden, cancer unfortunately continues to receive low public health priority in Africa, largely because of limited resources and other pressing public health problems, including communicable diseases such as acquired immune deficiency syndrome (AIDS)/human immunodeficiency virus (HIV) infection, malaria and tuberculosis. It may also be partly due to a lack of awareness about the magnitude of the current and future cancer burden among policy-makers, the general public and international private or public health agencies interested in global health. Although the knowledge of cancer in this region is now improving, better surveillance of cancer incidence, mortality, and prevalence of risk factors is urgently needed to monitor the development of the cancer epidemic, formulate appropriate cancer-control strategies, and assess the outcomes of these strategies (Jemal et al., 2012).

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Since cancer is one of the most common devastating diseases affecting millions of people per year and also the second leading cause of death in humans, there has been an intense search on various biological sources to develop a novel anti-cancer drug to combat this disease. The use of natural products as anticancer agents has a long history that began with folk medicine and through the years has been incorporated into traditional and allopathic medicine. To date, more than 3000 plant species have been described as displaying evidence of previous use for treating cancer (Hartwell, 1982). Several drugs currently used in chemotherapy were isolated from plant species or derived from a natural prototype. They include the *Vinca* alkaloids, vinblastine and vincristine, isolated from *Catharanthus roseus*, etoposide and teniposide, the semisynthetic derivatives of epipodophyllotoxin, isolated from species of the genus *Podophyllum*, the naturally

derived taxanes isolated from species of the genus *Taxus*, the semisynthetic derivatives of camptothecin, irinotecan and topotecan, isolated from *Camptotheca acuminata* and several others (Cragg and Newmann, 2000; Chabner et al., 2005; DeVita et al., 2008).

A recent analysis of the anticancer drug market in North America, Europe and Japan during the period 1981-2006 showed that 47.1% of a total of 155 clinically approved anticancer drugs was either unmodified natural products or their semi-synthetic derivatives, or synthesized molecules based on natural product pharmacophores (Newman and Crag, 2007). Medicinal plants would assist in the fight against cancer by reducing or minimizing the associated toxic side effects of chemotherapy and radiation treatment regimes.

1.2. Recent progress in drug discovery from medicinal plants

Plants, either as the whole plant or its leaves, stem bark and even the roots, have been utilized as medicines for thousands of years (Samuelsson, 2004) and natural products have been the single most productive source of lead compounds for the development of drugs (Harvey, 2008).

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Current research in drug discovery from medicinal plants involves an all round approach combining botanical, phytochemical, biological, and molecular techniques. Medicinal plant drug discovery continues to provide new and important leads against various diseases including cancer, HIV/AIDS, Alzheimer's, malaria and pain. Several natural product drugs of plant origin have either recently been introduced to the United States market, including arteether, galantamine, nitisinone, tiotropium, elliptinium and huperzine or are currently involved in late-phase clinical trials (Newman and Cragg, 2007; Butler, 2008; Chin et al., 2006). Although drug discovery from medicinal plants continues to provide an important source of new drug leads, numerous challenges are encountered including the procurement of plant materials, the selection

and implementation of appropriate high-throughput screening bioassays, and the scale-up of either synthesizing or isolating active compounds (Balunas and Kinghorn, 2005).

The use of plants as a source for medicines involved the isolation of active compounds, beginning with the isolation of morphine from opium in the early 19th century (Kinghorn, 2001; Samuelsson, 2004). Others are cocaine, codeine, digitoxin and quinine, of which some are currently still in use (Newman et al., 2000; Butler, 2004; Samuelsson, 2004). Isolation and characterization of pharmacologically active compounds from medicinal plants still is an on-going exercise and continues to date. More recently, drug discovery techniques have been applied to the standardization of herbal medicines and to elucidate analytical marker compounds. Numerous methods have been utilized to acquire compounds for drug discovery including isolation from plants and other natural sources, synthetic chemistry, combinatorial chemistry and molecular modeling (Ley and Baxendale, 2002; Geysen et al., 2003; Lombardino and Lowe, 2004). Despite the recent interest in the above stated methods; natural products, particularly medicinal plants, remain an important source of new drugs, new drug leads, and new chemical entities (NCEs) (Newman et al., 2000, 2003; Butler, 2004). Between 2001 and 2002, approximately one quarter of the best selling drugs worldwide were natural products or derived from natural products (Butler, 2004).

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Some new medicinal plant-derived drugs that have been recently introduced to the U.S. market include; Arteether (**1**, trade name Artemotil) which is a potent antimalarial drug derived from artemisinin, a sesquiterpene lactone isolated from *Artemisia annua L*. (Asteraceae) and a plant used in traditional Chinese medicine (TCM) (van Agtmael et al., 1999; Graul, 2001) (See Fig **1.1**

for the chemical structures of some of these new drugs.). Other derivatives of artemisinin are in various stages of use or clinical trials as anti-malarial drugs in Europe (van Agtmael et al., 1999). Galantamine (**2**, also known as galanthamine, trade name Reminyl) is a natural product discovered through an ethno botanical lead and first isolated from *Galanthus woronowii* Losinsk (Amaryllidaceae) in Russia in the early 1950s. It is used for the treatment of Alzheimer's disease (Heinrich and Teoh, 2004; Pirttila et al., 2004).

Nitisinone (**3**, trade name Orfadin) is a newly released medicinal plant-derived drug that works on the rare inherited disease, tyrosinaemia, demonstrating the usefulness of natural products as lead structures (Frantz and Smith, 2003). It is a modification of mesotrione, an herbicide based on the natural product leptospermone, a constituent of *Callistemon citrinus* Stapf. (Myrtaceae) (Hall et al., 2001; Mitchell et al., 2001). It inhibits HPPD enzyme in humans preventing tyrosine catabolism and the accumulation of toxic bioproducts in the liver and kidneys (Hall et al., 2001).

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Tiotropium (**4**, trade name Spiriva) is employed for treating chronic obstructive pulmonary disease (COPD) (Mundy and Kirkpatrick, 2004; Frantz, 2005). It is an inhaled anticholinergic bronchodilator, based on ipratropium, a derivative of atropine that has been isolated from *Atropa belladonna* L. (Solanaceae) and other members of the Solanaceae family (Barnes et al., 1995; Dewick, 2002; Mundy and Kirkpatrick, 2004). Tiotropium has shown increased efficacy and longer lasting effects when compared with other available COPD medications (Barnes, 2002; Mundy and Kirkpatrick, 2004).

Morphine-6-glucuronide or M6G (**5**) is a metabolite of morphine isolated from *Papaver somniferum* L. (Papaveraceae) and is used as an alternate pain medication with fewer side effects to that of morphine (Lotsch and Geisslinger, 2001).

Vinflunine (**6**) is a modification of vinblastine isolated from *Catharanthus roseus* (L.) G. Don (Apocynaceae) for use as an anticancer agent with improved efficacy (Bonfil et al., 2002; Okouneva et al., 2003).

Exatecan (**7**) is an analog of camptothecin isolated from *Camptotheca acuminata* Decne. (Nyssaceae) and is being developed as an anticancer agent (Butler, 2004; Cragg and Newman, 2004). Modifications of existing natural products exemplify the importance of drug discovery from medicinal plants as new chemical entities and as possible new drug leads.

Calanolide A (**8**) is a dipyranocoumarin natural product isolated from *Calophyllum lanigerum* var. austrocoriaceum (Whitmore) P.F. Stevens (Clusiaceae), a Malaysian rainforest tree (Kashman et al., 1992; Yang et al., 2001; Yu et al., 2003). Calanolide A (**8**) is an anti-HIV drug with a unique and specific mechanism of action behaving as a non-nucleoside reverse transcriptase inhibitor (NNRTI) of type-1 HIV and is effective against AZT-resistant strains of HIV (Currens et al., 1996; Buckheit et al., 1999; Yu et al., 2003). It is currently undergoing Phase II clinical trials (Creagh et al., 2001).

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Huperzine A **(9)** is a naturally occurring sesquiterpene alkaloid found in the firmoss *Huperzia serrata (*Kozikowski et al., 1999) and also found in varying quantities in other Huperzia species including *H.elmeri*, *H. carinat* and *H. aqualupian* (Lim et al., 2010). It is an acetyl cholinesterase inhibitor which has a mechanism of action similar to donepezil, rivastigmine and galantamine. Its pro drug form huperzine A (ZT-1) is under development as a treatment for Alzheimer's disease (Scalfaro, 2003). Huperzine A (**9**) is sold as a dietary supplement for memory support in the US, while it is used in China for the treatment of swelling, fever and blood disorders.

Clinical trials in China have shown it to be effective in improving cognitive performance in patients with Alzheimer's disease and enhancing memory in students (Wang et al., 2009; Sun et al., 1999).

The search for phytochemicals effective against the human immunodeficiency virus (HIV), the causative agent of Acquired Immune Deficiency Syndrome (AIDS) has resulted in the isolation of several promising compounds, including the tetrahydroxyindolizidine alkaloid castanospermine (**10**) from *Castanospermum australe* (Leguminosae) and the phorbol ester prostratin (**11**) from *Homalanthus nutans* (Euphorbiaceae) (Cox and Balick, 1994).

Natural products have played an important role as new chemical entities (NCEs) - approximately 28% of NCEs discovered between 1981 and 2002 were natural products or natural product-derived (Newman et al., 2003). Another 20% of NCEs discovered during this period were considered natural product mimics, meaning that the synthetic compound was derived from the study of natural products (Newman et al., 2003). Combining these categories, research on natural products accounts for approximately 48% of the NCEs reported from 1981–2002.

Newman and Cragg (2007) have also reported that inspection of NCE approvals demonstrated that the natural product field is still producing or its involved in ~ 50% of all small molecules in the year 2000-2006. Many structural features common to natural products (e.g., chiral centers, aromatic rings, complex ring systems, degree of molecule saturation, and number / ratio of heteroatoms) have been shown to be highly relevant to drug discovery efforts (Clardy and Walsh, 2004; Piggott and Karuso, 2004; Koehn and Carter, 2005). Drugs derived from medicinal plants can serve not only as new drugs themselves but also as drug leads suitable for optimization by medicinal and synthetic chemists (Balunas and Kinghorn, 2005). Even when

new chemical structures are not found during drug discovery from medicinal plants, known compounds with new biological activity can provide further important drug leads.

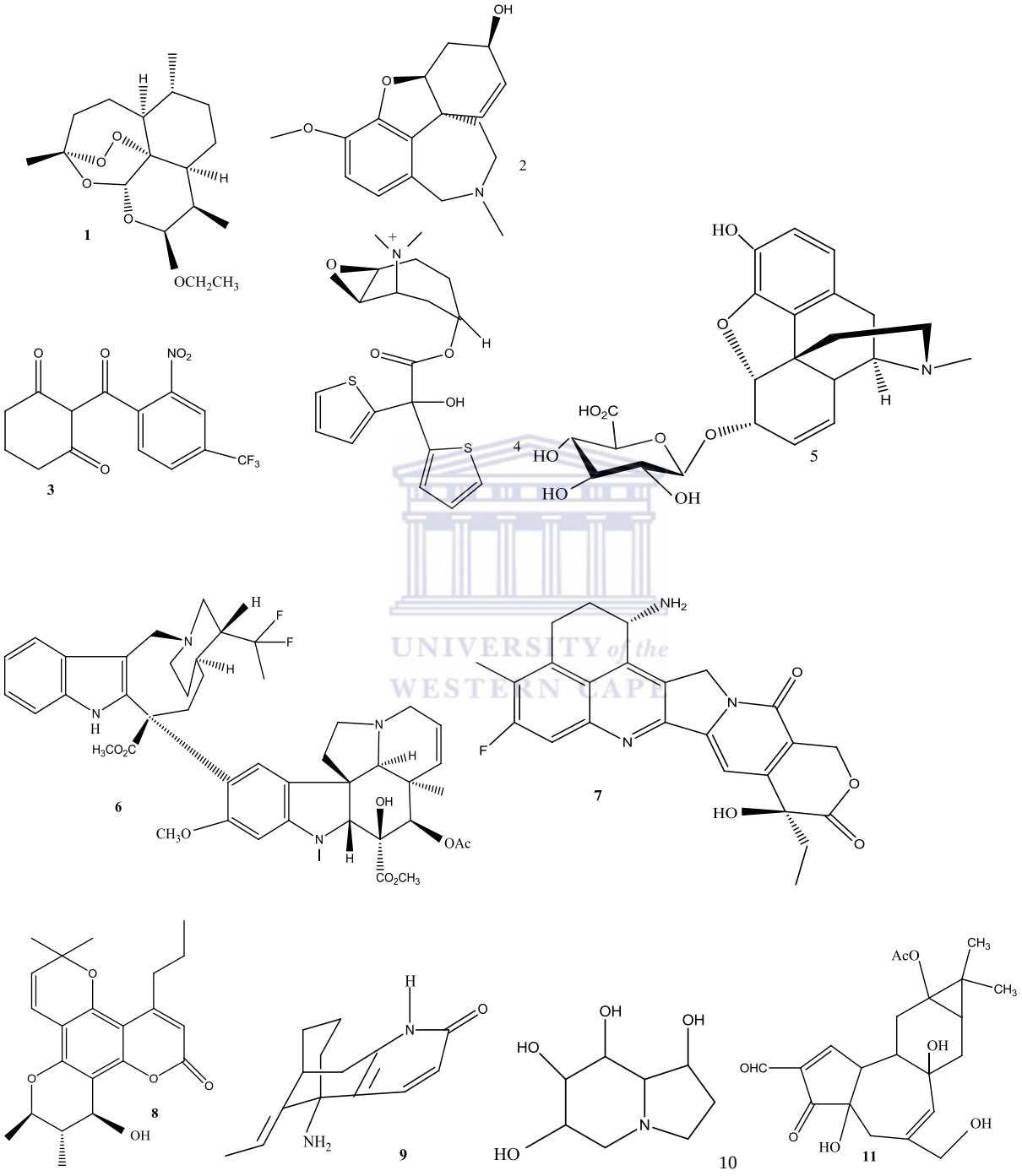

Figure1. 1. Examples of medicinal plant drugs newly introduced to the market/those in late phase clinical trials.

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1.3. Plants as a source of anti-cancer agents

Worldwide, over ten million new cancer cases (all parts of the body and sites excluding non-melanoma skin), with over six million deaths were estimated in the year 2000. There has been about 22% increases in cancer incidence and mortality since 1990 (Parkin et al., 2001). Although these figures are disconcerting, it is noteworthy that some progress has been made in cancer diagnosis and treatment as is evident through the high incidence of breast, prostate, testicular and uterine cancer and their relatively lower mortality (Parkin 2001; Jemal et al., 2005; Jatoi and Miller, 2003).

Drug discovery from medicinal plants have played an important role in treatment of cancer and most new clinical applications of plant secondary metabolites and their derivatives over the last half century have been applied towards combating cancer (Newman et al., 2003; Butler, 2004). Of all the anticancer drugs produced between 1940 and 2002, 40% were natural products or natural product - derived while 8% are considered to be natural product mimics (Newman et al., 2003). Anticancer drugs in clinical use include vinblastine, vincristine, camptothecin derivatives, topotecan and irinotecan, etoposide, derived from epipodophyllotoxin, and paclitaxel (taxol®). There are some promising new agents in clinical development based on selective activity against cancer-related molecular targets, including flavopiridol and combretastin A4 phosphate, while some agents which failed in earlier clinical studies are in more recent times stimulating renewed interest (Cragg and Newman, 2005). Other anticancer drugs for future developments include: roscovitine, betulinic acid, pervilleine A and silvestrol (Balunas and Kinghorn, 2005).

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1.4. Plant-derived anti-cancer agents in clinical use

Vinca alkaloids vinblastine (**12**) and vincristine (**13**) were the first agents to advance into clinical use for the treatment of cancer (Cragg and Newman, 2005). They were isolated from *Catharanthus roseus* (L.) G. Don (Apocynaceae) (formerly *Vinca rosea* L.) and have been used clinically for over 40 years (van Der Heijden et al., 2004). Vinblastine and vincristine have been used in combination with other cancer drugs to treat breast, lung, leukemia, advanced testicular cancer and Kaposi's sarcoma. Some of these plant derived anti-cancer agents are shown in (Fig.**1.2A** & **B**). The discovery of paclitaxel (Taxol®, **14** from the bark of the Pacific Yew, *Taxus brevifolia* Nutt. (Taxaceae) is further evidence of the success achieved in natural product drug discovery. Various parts of *Taxus brevifolia* and other *Taxus* species (e.g., *Taxus Canadensis* Marshall, *Taxus baccata* L.) have been used by several native American tribes for the treatment of some non-cancerous cases (Cragg and Newman, 2005) while *Taxus baccata* was reported to be used in the Indian Ayurvedic medicine for the treatment of cancer (Hartwell, 1982). The structure of paclitaxel was elucidated in 1971 and was clinically introduced to the US market in the early 1990s. Paclitaxel is active against ovarian cancer, advanced breast cancer and small and non-small cell lung cancer (Wani et al., 1971; Oberlies and Kroll, 2004).

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Another important anti-cancer drug is Camptothecin (**15**), isolated from *Camptotheca acuminata* Decne (Nyssaceae) which was later not used due to its severe toxicity to the bladder. However, extensive research has led to the development of more effective derivatives of Topotecan and Irinotecan which were used for the treatment of ovarian and colorectal cancer respectively (Creemers et al., 1996). The taxanes and the camptothecin accounted for approximately one-third of the global anticancer market (over 2.75 billion dollars) in 2002 (Oberlies and Kroll, 2004).

Podophyllotoxin (**16**) isolated from the resin of *Podophyllum peltatum* L. (Berberidaceae).was found to be too toxic in mice and this led to its derivatization to produce Etoposide (**17**) and Teniposide (**18**) which are semi-synthetic derivative of epipodophyllotoxin, an isomer of Podophyllotoxin. They are used in the treatment of lymphomas and bronchial and testicular cancers (Cragg and Newman, 2005).

Other anticancer drugs in clinical use include Elliptinium (**19**), a derivative of ellipticine, isolated from a Fijian medicinal plant *Bleekeria vitensis* A.C. Sm., and is being sold in France for the treatment of breast cancer (Cragg and Newman, 2005). Homoharringtonine (**20**) was isolated from the Chinese tree *Cephalotaxus harringtonia* var. *drupacea* (Sieb and Zucc.) (Cephalotaxaceae) (Itokawa et al., 2005). Racemic mixtures of harringtonine and homoharringtonine have been used in the treatment of acute myeloid leukemia and chronic myeloid leukemia in China (Cragg and Newman, 2005), while homoharringtonine was used for similar treatment in United States and Europe (Quintasa Cardama et al., 2007).

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Indirubin(**21**) a bis-indole alkaloid which has been reported to show a broad spectrum of antitumor activity (Eisenbrand et al., 2004) and is one of the components of Chinese traditional medicine called “Qingdai” used mainly for the treatment of hematemesis, eczema and other diseases (Zhongyaoshijia, 2009). Other components of “Qingdai” include Indigo and other plants comprising leaves and / or stems of *Baphicacanthus cusia* (Nees) Bremek (Acanthaceae), *Indigofera tinctoria* L. (Fabaceae), *Indigofera suffruticosa* Mill. (Fabaceae), *Isatis tinctoria* L. (Brassicaceae) and *Polygonum tinctorium* Ait. (Polygonaceae) (Deng, 1986).

Mesoindigo (**22**) is a derivative of indirubin with improved water solubility and reduced side effects. It has been reported to inhibit tumor growth in HT-29 colon cancer xenografts, cyclin-dependent kinases, promote apoptosis, and arrest cells in the G0/G1 phase of the cell cycle (Zuo et al., 2008). It is currently undergoing Phase III clinical trials in China for the treatment of chronic myelogenous leukemia (CML) (Cooperative Study Group of Phase III Clinical Trial on Meisoindigo, 1997). Perillyl alcohol, a limonene-type monoterpenoid, found in the essential oils of some aromatic herbs, such as lavender (*Lavandula intermedia*, Lamiaceae), peppermint (*Mentha piperita* L., Lamiaceae), spearmint (*Mentha spicata* L., Lamiaceae) and celery seeds (*Apium graveolens* L. Apiaceae) (Belanger, 1998) has been reported to induce apoptosis and arrest cell cycle in the G1 phase (Peffley et al., 2007; Yeruva et al., 2007). A Phase II clinical trial was sponsored by the U.S. National Cancer Institute (NCI) to study its effectiveness in treating patients with metastatic breast cancer (Bailey et al., 2008).

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Phenoxodiol (**23**), derived from genistein, a natural isoflavone constituent of soybean [Glycine max (L.) Merr. Fabaceae] has been promoted as a new promising drug for its broad anticancer spectrum and minimal toxicity Butler (2008) and is currently under advanced clinical trials for development as a ‘‘chemosensitizer’’ when combined with platinum drugs for the treatment of ovarian cancer. It has additionally been identified as a monotherapy for cervical and prostate cancers (Silasi et al., 2009). Protopanaxadiol (**24)** a dammarane-type triterpene aglycone traded under the trade name PandimexTM, is derived by hydrolysis of certain saponins from ginseng [Panax ginseng C.A. Mey.; Araliaceae] and has been reported to induce apoptosis in cancer cells and cell cycle arrest through various signaling pathways including caspases. It also displays cytotoxic activity against multidrug-resistant tumors (Li et al., 2006; Liu et al., 2007).

Protopanaxadiol (**24)** is now in Phase I clinical trial launched by PanaGin in the US for the treatment of lung cancer and other solid tumors (Saklani and Kutty, 2008; PanaGin Pharmaceuticals Inc., 2009). Additionally, it has also been approved in China for the treatment of advanced cancers of the breast, colon-rectum, lung and pancreas (PanaGin Pharmaceuticals Inc., 2009).

Among other active agents are Triptolide **(25)**, a diterpenoid triepoxide isolated from *Tripterygium wilfordii* Hook.f. (Celastraceae) and its sodium salt (14-succinyl triptolide sodium salt), a water-soluble prodrug of PG490, which is metabolized into triptolide *in vivo* after intravenous administration, has entered Phase I clinical trials for patients with solid tumors (Kiviharju et al., 2002; Fidler et al., 2003; Saklani and Kutty, 2008). Salvicine (**26**), a semi-synthetic analog of Saprorthoquinone diterpenoid quinone isolated from *Salvia prionitis* Hance (Lamiaceae), was found to exhibit significant growth inhibitory activity against a wide spectrum of human cancer cells *in vitro*, and showed effects in *in vivo* xenograft models bearing S-180 sarcoma, Lewis lung, A-549 lung, and LAX- 83 lung adenocarcinoma cells (Zhang et al., 1999). Salvicine (**26**) also demonstrated a potent cytotoxic effect on multidrug- resistant (MDR) tumor cells (Miao et al., 2003). It has entered Phase II clinical trials for the treatment of solid tumors in mainland China (Cai et al., 2008).

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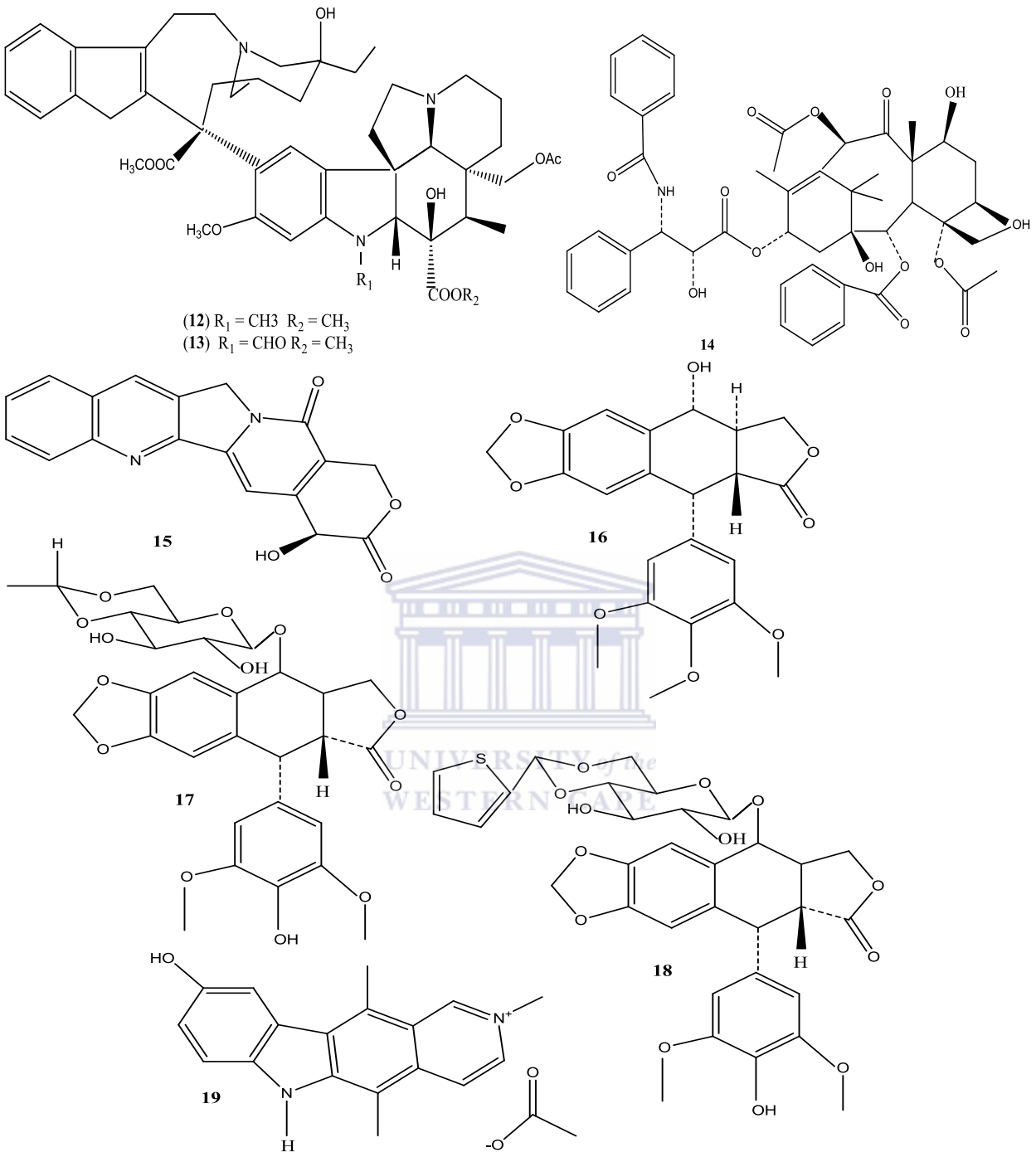

Figure 1.2A. Examples of anticancer agents in clinical use.

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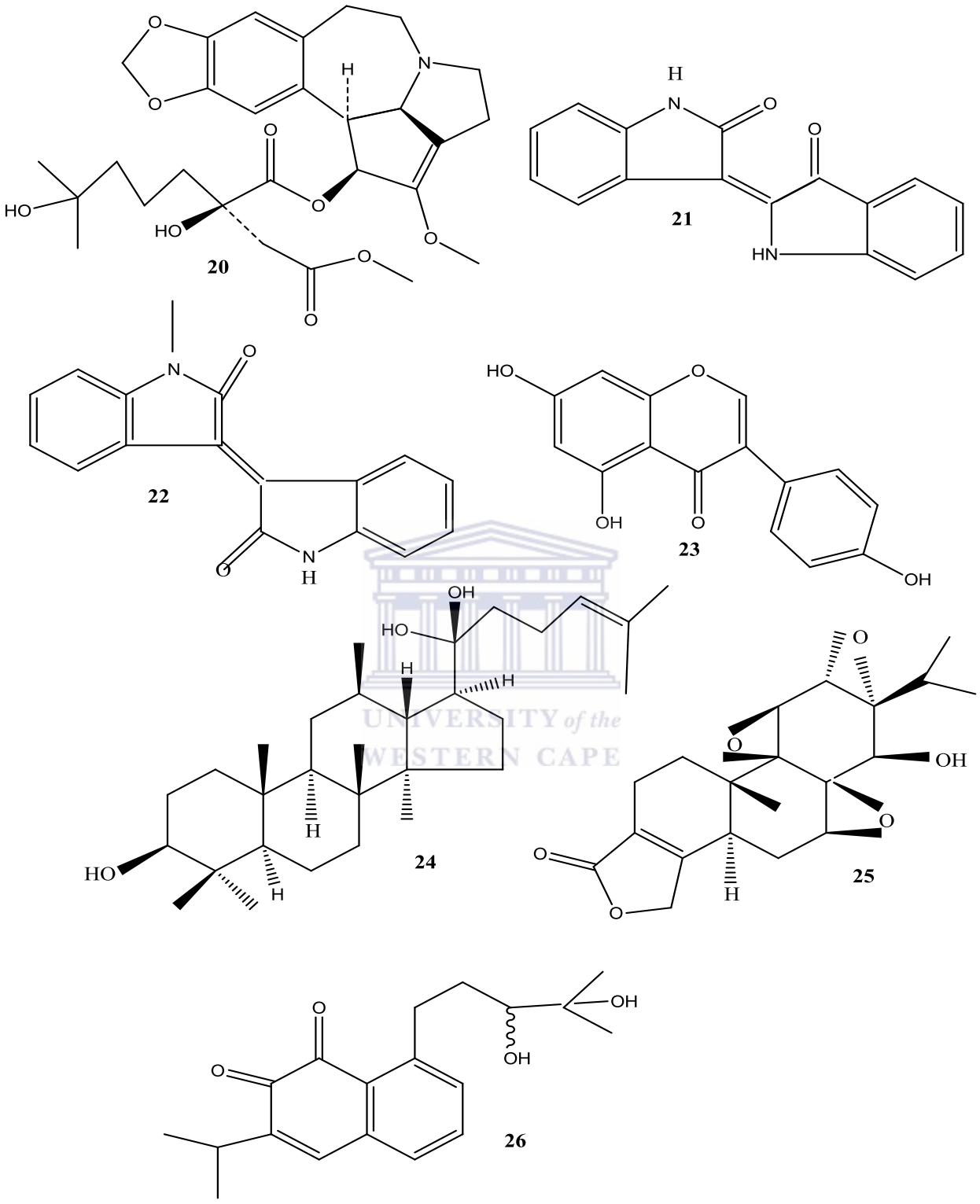

Figure 1.2B. Examples of anticancer agents in clinical use.

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1.5 Rationale for the study

Pleiocarpa pycnantha is generally known as a worm expeller but it is surprising to discover that there is no scientific data to support this claim nor any other type of biological activity reported for this plant either as a whole or any of its isolated compounds. An investigation of the anti-cancer potential of the plant extract has thus been chosen because of the increased cancer threat in Africa and the rising international interest in cancer chemotherapy, especially from medicinal plants that have not been investigated before.

Some anticancer agents have been reported from the Apocynaceae family, for example, Vinflunine is a modification of Vinblastine isolated from *Catharanthus roseus* (L.) G. Don (Apocynaceae) for use as an anticancer agent with improved efficacy (Bonfil et al., 2002; Okouneva et al., 2003). *Alstonia macrophylla*, *Alstonia glaucescens*, and *Alstonia scholaris* have been reported to be cytotoxic (Keawpradub et al., 1997). Pleiocarpamine from *Alstonia* specie (Apocynaceae), which is also a constituent of *P. pleiocarpa* was reported to demonstrate anticancer potential (Keawpradub et al., 1997). Therapeutic effects of anticancer herbs is identified by their inhibition of the cancer activating enzymes, stimulating a DNA repair mechanism, thus promoting production of protective enzymes, including antioxidant action and by enhancing activity of the immune cells. Certain biological response modifiers derived from herbs are known to inhibit growth of cancer by modulating the activity of specific hormones and enzymes. Some herbs reduce toxic side effects of chemotherapy and radiotherapy (Sabtu, 2007).

Prompted by the general anticancer potential of Apocynaceae and in the light of our ongoing research activity to identify anticancer agents from medicinal plants from Africa, we explored the anticancer potential of *P.pleiocarpa*, its extracts and isolated compounds.

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P.pycnantha is an indigenous plant from Nigeria and from some statistics already discussed indicated that many Africans are dying of cancer. Our preliminary investigation of cytotoxicity of total extracts from the leaves and some fractions demonstrated that the leaf extract and one of the fractions selectively killed the cancer cells investigated without touching the normal cells. This finding underpins the necessity to design selective anti-cancer drugs with minimal toxic side effects from the plant. On the one hand this will help cancer patients to have access to anticancer drugs from a relatively cheap origin i.e. natural sources to be able to fight the disease in Africa and Worldwide .This research will also assist to bridge the scientific gap that has long been created by the lack of useful informations about the plant and thus it is anticipated to add new knowledge to the scientific world.

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1.6. Aim/Objectives

The purpose of this study is to investigate the anticancer potential of the ethanolic extract of *Pleiocarpa pycnantha* leaves, isolate and purify the bioactive constituents.

1.6.1. Specific Objectives

- To collect, authenticate and extract the leaves of *Pleiocarpa pycnantha* into ethanol by cold maceration for 72 h.
- To phytochemically screen the ethanolic extracts of *P. pycnantha* leaves to ascertain their secondary metabolites.
- To investigate the anticancer potential of the crude extracts extract using HeLa, HT-29, MCF-7 and KMST-6 cell lines.
- To do a bioassay-guided fractionation and isolation of crude extract using the WST-1 assay on the cancer and normal cell lines.

- To isolate in pure form all bioactive compounds using a combination of chromatographic techniques.
- To determine the structures of bioactive compounds using physical methods, especially spectroscopy: including, IR, UV, NMR and MS.
- To investigate the mechanism of action of the bioactive compounds to establish a lead compound.

CHAPTER 2

2.0 LITERATURE REVIEW

This chapter gives a vivid review of the literature on Pleiocarpa family known as Apocynaceae and the genera. It also presents a general overview, their ethnomedicinal uses, phytochemistry and pharmacology.

The chapter as well summarises the subject "apoptosis" which is one of the main focus of the thesis, various apoptosis biomarkers like ROS, caspases, cell cycle and Topoisomerase I were also discussed. These biomarkers were furthermore linked with the focal topic – cancer.

2.1 Apocynaceae family

The family Apocynaceae consists of about 250 genera and 2000 species of tropical trees, shrubs and vines (Wiart, 2006). They are woody climbers, and herbs distributed primarily in tropical and subtropical areas of the world. Many species may appear as tall trees found in tropical rainforests, while some grow in tropical dry (xeric) environments while yet others are perennial herbs from temperate zones. Many of these plants have milky, often poisonous juice; smooth-margined leaves; and flowers in clusters (rarely solitary). The fruit may be berry like or fleshy but usually is a dry pod (follicle) that splits open at maturity, releasing numerous winged or tufted seeds. Members of the family are native to European, Asian, African, Australian and the American tropics and subtropics, with some temperate members (Endress and Bruyns, 2000). The most numerous genera are *Asclepias* L. (230), *Tabernaemontana* L. (230), *Cynanchum* L. (200), *Ceropegia* L. (150), *Hoya R. Braun* (150), *Matelea Aubl.* (130) and *Rauwolfia* L. (110). One of the most remarkable characteristics of Apocynaceae is that they are rich in various types of biologically active alkaloids. For instance, vincristine, vinblastine, pleiocarpine,

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strophanthilin, thevetin, peruvoiside, neriifolin, neriocorin, neriin, oleanderin, reserpine, serpentine, catharanthine are known to be present. Incidentally, alkaloids are one of the most fascinating classes of natural products, thus providing many drugs for human use (Philipson et al., 1993; Kayser et al., 2003).

2.2 Economic uses of some Apocynaceae plants

2.2.1 As drugs

Members of this family are sources of useful drugs, such as cardiac glycosides, which affect heart function. These have traditionally been used in folk medicines but are also now important in treating mental illness and cancer. They include the *Acokanthera*, *Apocynum*, *Cerbera*, *Nerium*, *Thevetia* and *Strophantus*. *Rauwolfia serpentina*, or Indian Snakeroot, yields the alkaloids Reserpine and Rescinnamine, which are useful in the treatment of high blood pressure and even some forms of psychosis (Wikipedia, accessed online on 2013-03-12). *Catharanthus roseus* is the source of the so-called vinca alkaloids, Vincristine and Vinblastine, used in cancer chemotherapy (Cragg and Newman, 2005). *Holarrhena antidysenterca* is used to treat dysentery, *Thevetia peruviana* is used to treat rheumatism and dropsy while *Alstonia scholaris* is also used to treat cancer. *Pleiocarpa pycnantha* is used as anthelmintic and to treat stomach problems (Burkill, 1985). In this research, we have demonstrated its usefulness as a potential anticancer plant. A comprehensive list of Apocynaceae plants and their uses is documented in Table **2.1**.

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2.2.2 As rubber plants

The genera *Carpodinus*, *Landolphia*, *Hancornia*, *Funtumia* and *Mascarenhasia* were used as a commercial source of inferior rubber. Valuable India rubber is extracted from these species (Burkill, 1985).

2.2.3. As ornamentals

Many members of this family are also cultivated as ornamentals, such as Vinca (periwinkle), *Thevetia peruviana*, Nerium (oleander), *Amsonia* (bluestar), *Carissa* (Natal plum, an edible fruit), *Allamanda* (golden trumpet), *Plumeria* (frangipani), *Thevetia* (lucky nut), *Mandevilla* (Savannah flower), *Tabernaemontana* (Crape jasmine), *Rauwolfia* and *Adenium* (desert-rose) (Joselin et al., 2012)

2.2.4 As dye plants and wood

Wrightia tinctoria leaves are the source of a blue dye Indigo. *Carissa edulis* is another source of dye. *Alstonia scholaris* is an evergreen tree with a dense crown, which is often planted in gardens and as avenue tree. The treated wood is used in packing and the bark has medicinal value. *Funtumia africana* is used as wood while *Strophanthus mirabilis* is used as a fodder plant.

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2.2.5. As fish poison

The juice of *Acokanthera* species such as *A. venenata* and the milky juice of the Namibian *Pachypodium* have been used as venom for arrow tips by the Bushmen (Rapanarivo and Leeuwenberg, 1999).

2.2.6. As food plants

Many genus of the plants are used as food, for example the genus *Apocynum* is used as a source of fiber by Native Americans and *Carissa edulis* also possesses edible roots. The edible flower of *Fernaldia pandurata* (common name: *loroco*) is a popular part of El Salvadorian and Guatemalan cooking while the aromatic juice of *Saba comorensis* (syn. *Landolphia comorensis*

(Bojer ex A. DC.) K. Schum.), also known as the Bungo or Mbungo fruit is popular and highly appreciated in Pemba Island and other parts of coastal Tanzania (Orwa et al., 2012).

2. 3. Mode of administration

According to published ethno-botanical data, Apocynaceae plant parts used for medicinal preparations are leaves, bark, roots, root barks stem, latex, seeds, aerial parts, fruits, flowers and the whole plant. In some cases the whole plant is used together with the roots. The most frequently used plant parts are the roots, leaves, stem bark, and root bark (Omino and Kokwaro, 1993). Generally, in herbal preparations; single plants may be used alone or in association with other plants or with other material of animal or mineral origin. Remedies are mainly prepared in the form of powder, concoction and decoction. The methods of administration of herbal medicines are internal, particularly by oral absorption and external poultice/topical application or bathing (Karou et al., 2011).

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2.4 Traditional uses of Apocynaceae family

The study of Apocynaceae species used in traditional medicine reveals that 25 species in 16 genera are of ethnobotanical interest. Nineteen species are medicinal, sixteen of which fall under the subfamily Plumerioideae which usually contains indole alkaloids (Omino and Kokwaro, 1993). The most common category of diseases treated is skin and ectoparasitic diseases followed by abdominal diseases, diseases of the head, female conditions and venereal diseases. The root is the most commonly used part of the plant and it is possible that the alkaloids play an important role in the medicinal value of the plants (Omino and Kokwaro, 1993). Apocynaceae species are also used to treat gastrointestinal ailments, fever, malaria, pain and diabetes Wiart (2006) while some are used in the treatment of cancer (Atta-ur-Rahman et al., 1986; Wang et al., 2012).

2.5 Chemistry of Apocynaceae

Plants are invaluable sources of pharmaceutical products and are recognized for their ability to produce a wealth of secondary metabolites. Mankind has used many species for centuries to treat a variety of diseases (Sadqui et al., 2006). Various natural products occur in Apocynaceae plants; an extensive phytochemical investigation has been conducted demonstrating the natural occurrence of alkaloids, terpenoids, flavonoids/phenolic acids, glycosides and iridoids .

The occurrence of indole alkaloids seemed to be a rule in this family. They occur as tetracyclic, pentacyclic or polycyclic rings. The occurrence of alkaloids in some apocynaceae family has been documented (Liu et al., 2012; Raffauf and Flagler, 1960). Some biologically active alkaloids found in Apocynaceae include: pleiocarpamine, alstoumerine, alstonerine, alstophylline, macralstonine, villalstonine, vinblastine, vincristine, pleiocarmine, strophanthilin, thevetin, peruvoiside, neriifolin, neriocorin, neriin, oleanderin, reserpine, serpentine and catharanthine.

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2.5.1 Occurrence of Alkaloids

Alstonia species are rich in important alkaloids like alstonidine, alstonine, alstovenine, chlorogenic acid, chlorogenine, ditain, ditaine, ditamine, echicaoutchin, echicerin, echiretin, echitamine, echitein, echitenin, echitin, porphyrine, porphyrosine, reserpine, venenatine, villalstonine, pleiocarpamine, *O*-methylmacralstonine, macralstonine, *O*-acetylmacralstonine, villalstonine, macrocarpamine, corialstonine, corialstonidine, nareline, scholaricine, methyl burnamine, and vallesamine (Arulmozhi et al., 2007; Rastogi et al., 1990; Cai et al., 2007).

The chemical investigation of an alkaloidal extract of *Alstonia macrophylla* bark led to the isolation and identification of two new nitrogenous derivatives, alstoniaphyllines A and B, a new indole alkaloid, alstoniaphylline C and eight known alkaloids (Cheenpracha et al., 2013).

Nine bisindole alkaloids, four from the macroline-sarpagine group, and the remaining five from the macroline-pleiocarpamine group, were isolated from the stem-bark of *Alstonia angustifolia,* (Tan et al., 2013). Nineteen monoterpenoid indole alkaloids including seven new ones, melodinines A−G were isolated from *Melodinus henryi* (Feng et al., 2010).

A new bisindole alkaloid, 19, 20-dihydroervahanine A was isolated from the stems of *Ervatamia coronaria* grown in Brazil, together with the five known alkaloids: coronaridine, heyneanine, voacristine, voacamine, descarbomethoxyvoacamine (Henriques et al., 1996). Four bisindole alkaloids, viz., 190(S)-hydroxyconodurine, conodurinine, 190(S)-hydroxyconoduramine and 190(S)-hydroxyervahanine A, in addition to conodurine and ervahanine A, were obtained from the leaf and stem-bark extracts of *Tabernaemontana corymbosa* (Kam and Sim, 2003).

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The stem bark of *Muntafara sessilifolia* has been reported to contain vobasinyl-iboga bisindole and 2-acylmonomeric indole alkaloids. Examples include 30-oxotabernaelegantine B, 30- oxotabernaelegantine A, 30(R/S)-hydroxytabernaelegantine A, oxotabernaelegantine and 30(S) hydroxytabernaelegantine C (Girardot et al., 2012). Bisindole alkaloids from the macroline–sarpagine group and macroline–pleiocarpamine group were isolated from the stem-bark extracts of *Alstonia angustifolia* which includes: perhentisine A-C, lumutinine E and villalstonidine A-E. Additional bisindole alkaloids isolated from the bark extract includes; bipleiophylline, perhentinine, perhentidines A and C, anhydromacralstonine, villalstonine, villalstonine-N-oxide, and macrocarpamine (Tan et al., 2013). Indole alkaloids condylocarpine, coronaridine, 14,15-dehydro-16-epi-vincamine, heyneanine, 10-hydroxycoronaridine,

3-oxotabersonine, 3-oxovoacangine, stemmadenine, stemmadenine-N-oxide, tabersonine and tabersonine-N- oxide. Tetrahydroalstonine, voacangine, and voacristine have been isolated from the seeds of *Tabernaemontana cymosa* (Achenbac et al., 1997).

Catharanthus roseus is a source of pharmaceutically important alkaloids with more than 130 being described to date. It is the sole source of vincristine and vinblastine used against a variety of cancers. Three new terpenoid indole alkaloids, vidolicine, normacusine B N-oxide, and lochnerine N-oxide, together with seven known ones were isolated from the whole plants of *Catharanthus roseus* (Wang et al., 2011). Globospiramine, a new spirobisindole alkaloid possessing an Aspidospermae, Aspidosperma skeleton, together with deoxyvobtusine, deoxyvobtusine lactone and vobtusine lactone were isolated from *Voacanga globosa* (Macabeo et al., 2011). Three indole alkaloids (Aspidocarpine, 11-methoxytubotaiwine and picraline) were isolated from the aqueous extract from the bark of *Aspidosperma cuspa* (Kunth) Blake, (Pérez et al., 2012).

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Zocoler et al., (2005) identified the indole alkaloids: ibogamine, coronaridine, ibogaine pseudoindoxyl, voacangine hydroxyindolenine, voacangine pseudoindoxyl, tabernanthine, catharanthine, voacangine, 19-oxovoacangine, 10-hydroxycoronaridine, affinisine, 16-*epi*-affinine, voachalotine, ibogaline, and conopharyngine from *Tabernaemontana fuchsiaefolia.* Four new aspidosperm aspidosperma-type bisindole alkaloids; 3-oxovoafrine B, voacandimine A –C were isolated from the seeds and root bark of *Voacanga Africana* (Kitajima et al., 2013).

2.5.2 Occurrence of Flavonoid/flavanone/phenolic acids

Five phenolic acids: vanillic, gentisic, syringic, 4-hydroxybenzoic and salicylic acid have been reported to be isolated from E*rvatamia coronary* (Henriques et al., 1996). Bioactive davidigenin, a dihydrochalcone was also reported from *Mascarenhasia arborescens* (Desire et al., 2010).

Paris and Duret (1974) documented the occurrence of flavonoids in the leaves of fifteen Apocynaceae collected from different geographical sources; kaempferol and quercetol were uniformly present irrespective of their geographical origin and isorhamnetol was found to be occasionally present in the plants. Other flavonoid glycosides present are isoquercitroside, rutoside, nicotifloroside and robinoside. The flavonoid content of Apocynaceae leaves were said to be low with some exceptions, depending on collection time.

2.5.3 Occurrence of Terpenoids

Iridoids are monoterpernes derived from isoprene units, which are considered the universal building blocks of all terpenoids (Dewick, 2002). They have shown a broad range of biological activities like antibacterial, antifungal, anti-inflammatory, antitumoral, hepatoprotective, cardioprotective, antioxidative, and antiprotozoal activity. They have also been reported to inhibit the growth of the hepatitis C virus. Apocynaceae has been described as the most important plant order that produces Iridoids with ca 5000 species distributed worldwide and they are concentrated in a few genera like *Plumeria, Himatanthus, Allamanda* and *Cerbera* (Amaral et al., 2013).

The isolation of four new iridoids in Apocynaceae has been reported to give; plumeridoids A-C and epiplumeridoid C which were isolated from the stem bark of *Plumeria rubra* Linn. together with fulvoplumerin, dihydroplumericin, plumieride, isoplumericin, plumericin, allamcin, and

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allamandin. Triterpenes, viz., ajunolic acid, ursolic acid, oleanolic acid, *β*-amyrin acetate, betulinic acid, lupeol and its acetate, 2,3-dihydroxypropyl octacosanoate, glucoside of *β*-sitosterol, and a mixture of common sterols (stigmasterol and *β*-sitosterol) have also been reported (Kuigoua et al., 2010).

Loganic acid, loganin, boonein, isoboonein were isolated from the bark of *Rauwolfia grandiflora* (Bianco et al.,1994). The isolation of lupeol, a pentacyclic triterpenoid was reported by (Macabeo et al., 2011). The triterpenoids lupeol linoleate, lupeol palmitate and α-amyrin linoleate are present in the stem bark of Alstonia species (Satyavati et al., 1987; Cai et al., 2007). Triterpenes: α-amyrin, β-amyrin, lupeol, betulinic acid, ursolic acid, oleanoic acid and the acetyl derivate of the cyclitol methoxy-myo-inositol were isolated from *Mucoa duckei* (Markgraf) Zarucchi (Koolen et al., 2012).

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2.5.4 Occurrence of Lignans and Lignan glucosides

(+)-Lyoniresinol, (+)-5,5'-Dimethoxy-9-O- (β-D-glucopyranosyl)lariciresinol,Glucopyranosyl (+)-3α-O-(β-D-glucopyranosyl)lariciresinol, (-)-3α-O-(β-D-glucopyranosyl)lariciresinol, (-)-2α-O-(β-D-glucopyranosyl)lariciresinol, (-)-3α-O-(β-D-glucopyranosyl) -5'-methoxyisolariciresinol and (+) -8,8'-Dimethoxy- 1-O-(β -D-glucopyranosyl) secoisolariciresinol were isolated from the seeds of *Tabernaemontana cymosa* (Achenbac et al., 1997).

2.5.5 Occurrence of Glycosides

Fanie et al., (2007) reported the isolation of two pregnane glycosides from the dried stems of *Hoodia gordonii* and *Hoodia pilifera*. Cardiac glycosides such as oleander, oleandrin, cardenolide N-1, cardenolide N-4,3 β –O-(β -D-sarmentosyl)- 16b-acetoxy-14-hydroxy-5 β,14 β -card-20-(22)-enolide and16 β -acetoxy,14-dihydroxy-5 β,14 β -card-20-(22)-enolide has been

isolated from *Nerium oleander* (Zhao et al., 2007). Hoodigoside L was reported as the major appetite-suppressant principle isolated from *Hoodia gordonii* (van Heerden, 2008).

2.5.6 Other compounds from Apocynaceae family

Sweroside and 3-O-(β-D-Glucopyranosyl) 5-O-methylgallic acids were isolated from the seeds of *Tabernaemontana cymosa* (Inouye et al., 1966; König et al., 1973). The isolation of coumarins and coumarino lignan as well as chlorinated coumarinolignan from the genus *Mondia* was reported by (Patnam et al., 2005). (−)-Loliolide which showed affinity to SERT in a binding assay has recently been reported (Neergaard et al., 2010).

2.6 Chemical structure of some isolated compounds from selected medicinal plants from Apocynaceae species

Some isolated compounds from selected Apocynaceae species from recent published articles are presented below in Figures **2.1-2.6**.

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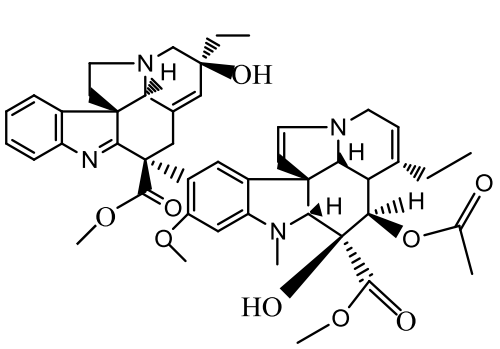
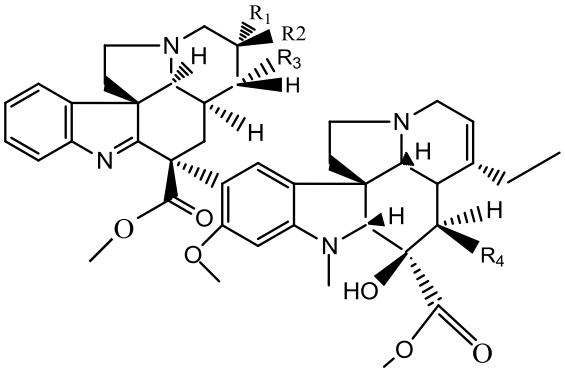
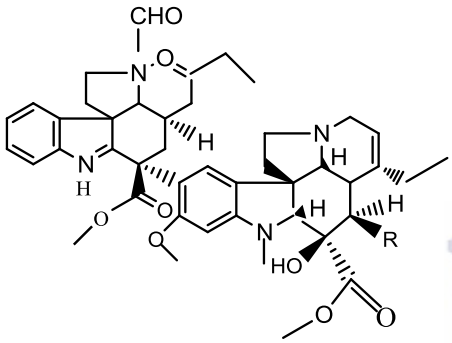


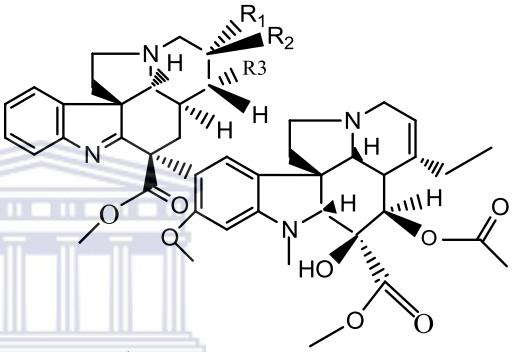
14',15'-Didehydrocyclovinblastine



17-Deacetoxycyclovinblastine $R_1=CH_2CH_3,R_2=OH,R3=R_4=H$
Cycloleurosine $R1=R_3=$ -O-,$R_2=CH_2CH_3,R_4=OCOCH_3$



17-Deacetoxyvinamidine R=H
Yinamidine $R=OCOCH_3$



Leurosine $R1=R_3=$ -O-,$R_2=CH_2CH_3$
Leurosidine $R1=OH,R_2=CH_2CH_3,R_3=H$

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Figure 2.1. Isolated compounds from *Catharanthus rose*us (Alkaloids).

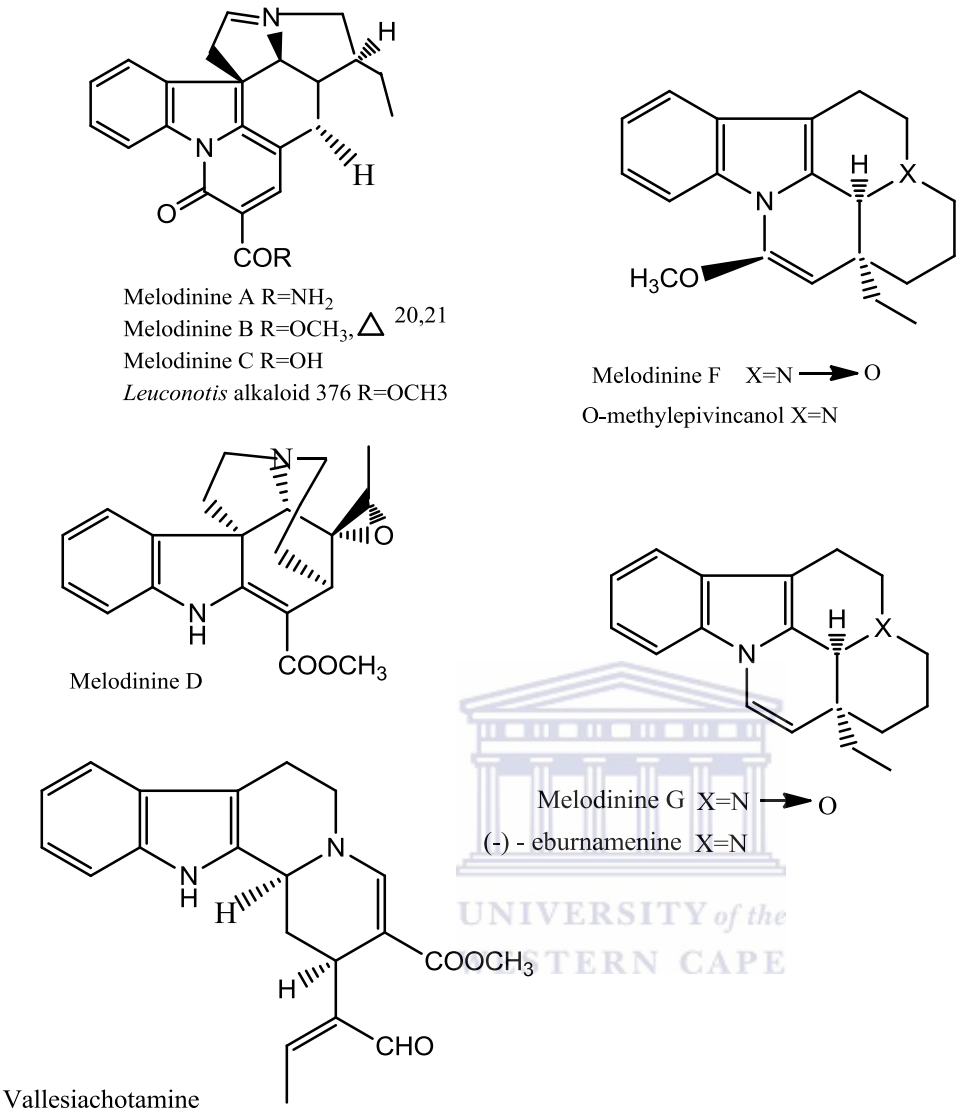

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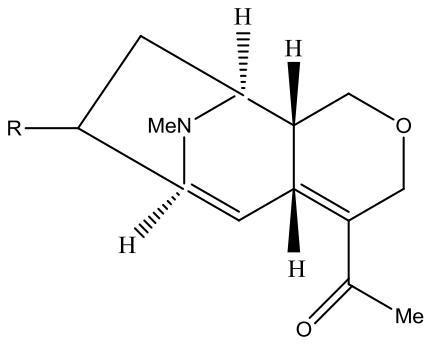
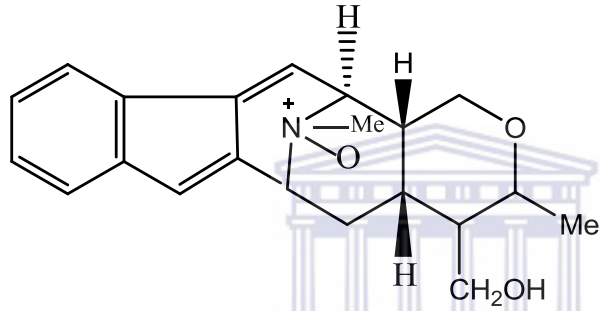
Figure 2.2. Isolated compounds from *Melondinus henryi* (Alkaloids).



Angustimaline R= α-OH

Alstoniaphylline A R= =O



Alstoniaphylline C

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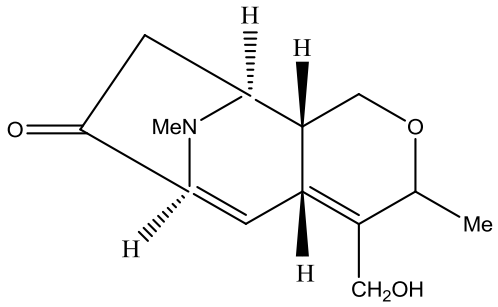
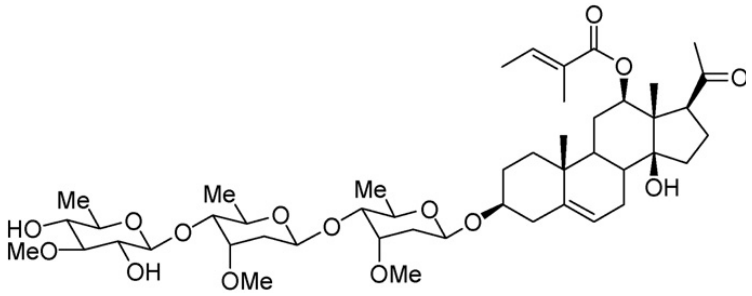
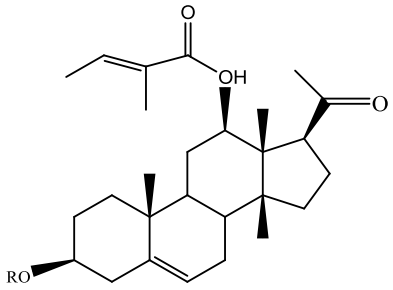


Alstoniaphylline B

Figure 2.3. Isolated compounds from *Alstonia macrophylla* (Alkaloids).



Glycoside 3β [-β-D-thevetopyranosyl-(1→4)-β-D-cymaropyranosyl-(1→4)-β-D-cymaropyranosyloxy]-12-β-tigloyloxy-14 -β -hydroxypregn-5-en-20-One (also known as P57A53)



Hoodigogenin A	R=H
Gordonoside A	R=H
Hoodigoside A	the-cym
Hoodigoside B	the-the-cym
P57A53	the-cym-cym
Hoodigoside C	the-cym-cym-cum
Hoodigoside D	the-the-cym-cym

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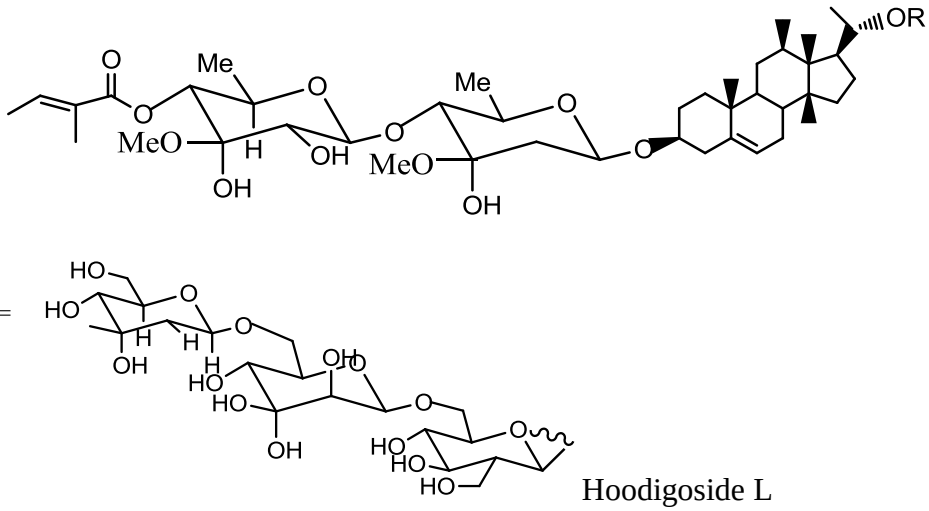
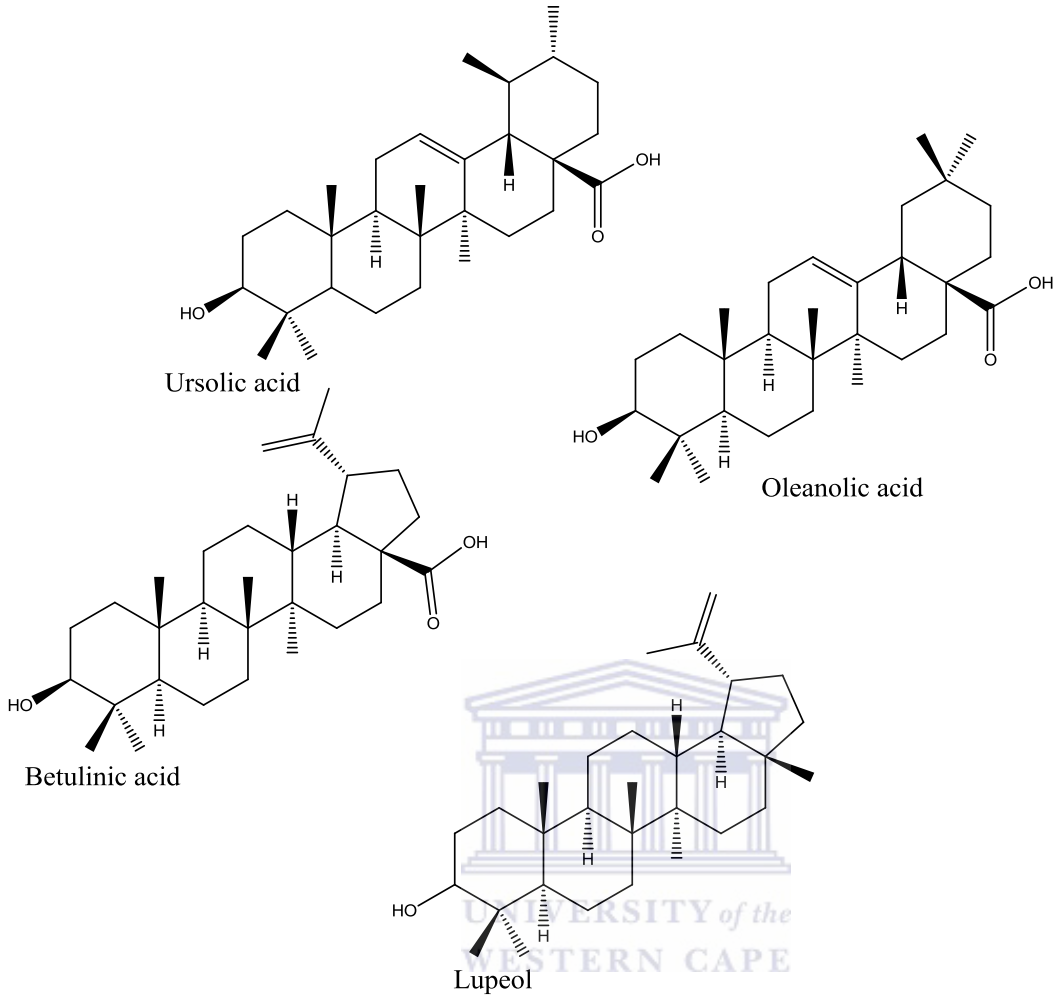


Hoodigoside L

Figure 2.4. **Glycosides derived from 12-hydroxypregnane glycoside isolated from *Hoodia gordonii.***



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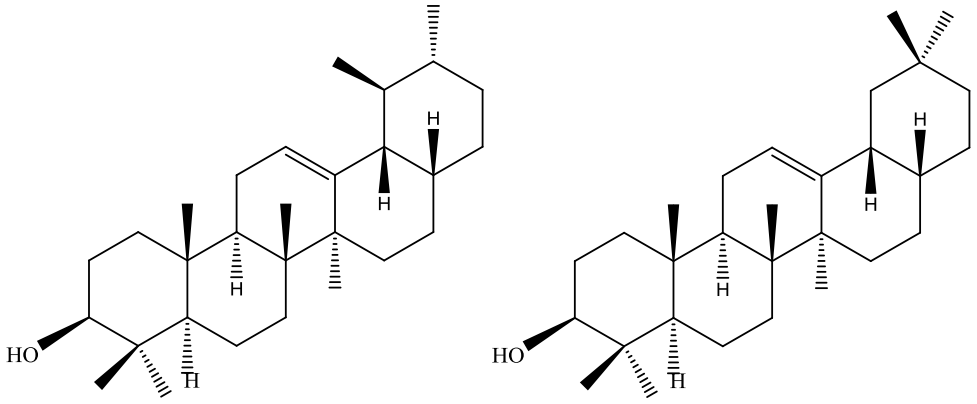
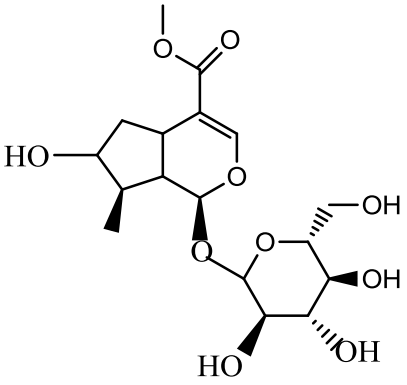
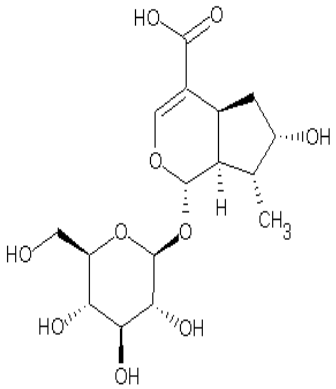


α-Amyrin β-Amyrin

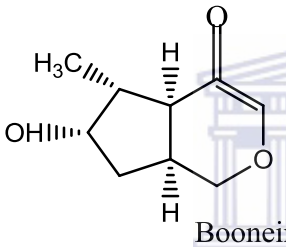
Figure 2.5. Triterpenes from *Mucoa duckei.*



Loganin



Loganic acid



Boonein

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Figure 2.6. **Terpenes from *Rauwolfia grandiflora.***

2.7 Biological activities of representative members of Apocynaceae family

Apocynaceae species have been screened for various biological activities both *in vitro* and *in vivo* using animal models. The main biological activities were antiplasmodial, anticancer, antibacterial, anti-inflammatory and antidiabetic activities.

2.7.1 Anthelmintic activity

A number of plants from Apocynaceae have shown anthelmintic activity against different organisms viz; *Thevetia peruviana* (Pers.) K. Schum, *Mondia whitei* (Hook. f.), *Acokanthera oppositifolia, Acokanthera oblongifolia*, *Asclepias fruticosa* L. and *Secamone filiformis* (Aremu et al., 2012).

2.7.2 Anti-inflammatory activity

The aqueous and alcoholic extracts of *Ervatamia coronaria*, when administered p.o. or i.p. to rats 1 h before a subplantar injection of carrageenin had a significant anti-inflammatory effect. The alcoholic extract also had an analgesic effect and increased the pentobarbital induced sleeping time, (Henriques et al., 1996). In vitro anti-inflammatory activity of *Mondia whitei* leave extracts indicated that methanol and water extracts had moderate (above 65%) COX-1 inhibition while the extract from the roots showed over 80% inhibitions (Matu and Van Staden, 2003).

Statistical analysis revealed that *Trachelospermum jasminoides* WET (0.5, 1.0 and 2.0 g/kg) significantly inhibited the development of carr-induced paw edema after 3 h of treatment when compared with control (Sheu et al., 2009). The DCM fraction of ethanolic extract of *Alstonia scholaris* has demonstrated anti-inflammatory activity (Arulmozhi et al., 2012).

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2.7.3 Antiplasmodial activity

The isolated compounds from *Muntafara sessilifolia* were evaluated in vitro for antiplasmodial activity against the chloroquine-resistant strain FcB1 of *Plasmodium falciparum*, and 30-oxo-tabernaelegantine A exhibited antiplasmodial activity (4.4 µM IC_{50}) associated with a non-significant cytotoxicity selectivity index of 48 (Girardot et al., 2012). Nicalaterine, bisnicalaterine B and bisnicalaterine C were isolated from the bark of *H. zeylanica*. Nicalaterine (IC_{50}, 0.05 µM) displayed a higher antimalarial activity against *P. falciparum* than bisnicalaterine B (IC_{50} 0.11 µM) while bisnicalaterine C showed a weak cytotoxic activity (IC_{50} value of 1.13 µM) (Nugroho et al., 2011).

The aqueous extract of a partitioned ethanolic leaf extract of *Alafia barteri* (Hook F. Icon) displayed antiplasmodial activity with an IC_{50} value of 1.5 ±0.7 µg/mL while the root aqueous extract gave 3.0 ±0.4 µg/mL comparable to the reference antimalarial drug Quinine with an IC_{50} value of 0.95 ±0.25µg/mL against *Plasmodium falciparum* strain (Lasisi et al., 2012).

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Alstonisine, isolated from *Alstonia macrophylla* exhibited antiplasmodial activity against *Plasmodium falciparum* with an IC_{50} of 7.6 µM (Cheenpracha, 2012). Four vobasinyl-iboga bisindole and one 2-acyl monomeric indole alkaloids were isolated from the stem bark of *Muntafara sessilifolia* along with eleven known compounds. Tabernaelegantine B and D displayed the highest cytotoxicity with IC_{50} values of 0.47 and 1.89 µM on MRC-5 cells and 0.42 and 2.7 µM on L-6 cells, respectively (Girardot et al., 2012). Four indole alkaloids, geissolosimine , geissospermine B , geissoschizoline and geissospermine D, were isolated from the stem bark of *Geissospermum vellosii*, with geissolosimine showing the highest cytotoxicity against the chloroquine- sensitive strain of *P. falciparum* (IC_{50} 0.96 µM)

(Mbeunkui et al., 2012).

Indole alkaloids: geissospermine D, leucoridine A N-Oxide and anhydropereirine isolated from the leaves of *L. griffithii* and evaluated for antimalarial activities against *P. falciparum* 3D7 showed potent activity at IC_{50} values of 0.09, 0.007 and 0.06 µM, respectively

(Nugroho et al., 2012).

2.7.4 Anticancer/Antineoplastic activity

Vincristine and vinblastine used against a variety of cancers such as acute leukemia, Hodgkin's disease, rhabdomyosarcomas, neuroblastoma and other lymphomas are from *C.roseus* (Mukherjee et al., 2001; Van der Heijden et al., 2004; Aslam et al., 2009). Eight indole alkaloids, 14',15'-didehydrocyclovinblastine,17-deacetoxycyclovinblastine,17-deacetoxyvinamidine, vinamidine, leurosine, catharine, cycloleurosine and leurosidine were also isolated from the whole plants of *C. roseus.* All the isolated alkaloids showed cytotoxic effects against the human breast cancer cell line MDA-MB-231, while Leurosine exhibited the most potent inhibitory activity (IC_{50} value of 0.73 µM) (Wang et al., 2012).

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Lochnericine an indole alkaloid was isolated from the whole plants of *C. roseus* and it showed cytotoxic activities against HL-60, SMMC-7721 and A549 with IC_{50} values of 11.0, 22.8 and 29.5 µM, respectively (Wang et al., 2011). Eight indole alkaloids were isolated from the whole plants of *C. roseus* among which leurosine had the most cytotoxic effects against MDA-MB-231 with an IC_{50} value of 0.73 µM (Wang et al., 2012).

The ethanolic extract, subsequent hexane fractions and fraction F-4 from *W. tomentosa* inhibited the proliferation of human breast cancer cell lines, MCF-7 and MDA-MB-231 *in vivo*.

The fraction F-4 obtained from the hexane fraction inhibited proliferation of MCF-7 and

MDA-MB-231cells in a concentration and time dependent manner with an IC_{50} of 50 µg/ml and 30 µg/ml for 24 h, 28 µg/ml and 22 µg/ml for 48 h and 25 µg/ml and 20 µg/ml for 72 h respectively. The fraction F-4 induced G1cellcycle arrest, reactive oxygen species (ROS) generation, loss of mitochondrial membrane potential and subsequent apoptosis.

Apoptosisis indicated in terms of increased Bax/Bcl-2 ratio, enhanced Annexin-V positivity, caspase 8 activation and DNA fragmentation. The active molecules isolated from fraction F-4 viz., oleanolic acid and ursolic acid inhibited cell proliferation of MCF-7 and MDA-MB-231cells at IC_{50} value of 7.5 µM and 7.0 µM respectively, whereas there is no significant cell inhibiting activity in the non-cancer originated cells, HEK-293. In both MCF-7 and MDA-MB-231, oleanolic acid and ursolic acid induced cell cycle arrest and apoptosis as indicated by a significant increase in Annexin-V positive apoptotic cell counts (Chakravarti et al., 2012).

Tumor resistance to platinum-based drugs has been an obstacle to the treatment of ovarian cancer, in order to enhance the effectiveness of platinum-based drugs, the anticancer effects of *Rauwolfia vomitoria* extract (Rau), both alone and in combination with carboplatin (Cp),

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a platinum based drug was studied. *Rauwolfia vomitoria* extract was found to decrease cell growth in tested ovarian cancer cell lines in a dose dependent manner. Apoptosis was the predominant form of Rau-induced cell death and it occurred in a time- and dose-dependent manner.

Synergy of Rau with Cp was detected with combination index values of <1 and dose reduction index values for Cp ranging from 1.7- to 7- fold. Tumor growth in mice was significantly suppressed by 36% or 66% with Rau treatment alone at a low (20 mg/kg) or a high dose

(50 mg/kg) respectively, an effect comparable to that of Cp alone. It was concluded that the combination of Rau with Cp remarkably enhanced the effect of Cp and reduced tumor burden by between 87% to 90% and ascites volume from 89% to 97% (Yu et al., 2013).

Bisleuconothine A, a bisindole alkaloid was isolated from the bark of *Leuconotis griffithii*.

It showed cell growth inhibitory activity against four human cancer cell lines HL60, HCT116, MCF-7 and A549 with IC_{50} values of 11.0, 5.7, 9.2 and 7.0 µM, respectively.

It also induced G1 phase arrest in HCT116 in a dose-dependent manner (68.3%, 0µM; 72.0%, 5 µM; 75.1%, 10 µM; 79.5%, 20 µM) after treatment for 24 h (Hirasawa et al., 2010).

The *in vitro* cytotoxic activity of the *Himatanthus drasticus* latex proteins (HdLP) was determined on cultured HL-60, MDA-MB-435, SF-295 and HCT-8 cell lines but did not give any significant *in vitro* cytotoxic effect at experimental exposure levels.

The *in vivo* antitumor activity was assessed in two experimental models, Sarcoma 180 and Walker 256 carcino-sarcoma. It was found that intraperitoneally administered HdLP was active against *in vivo* experimental tumors but inactive by oral administration. Intraperitoneal HdLP reduced tumor weight to 1.60 ± 0.18 and 1.66 ± 0.18 g on sarcoma cell lines at doses of 10 and 20 mg/kg/day, respectively. These reductions gave inhibition rates of 36.46% and 34.22% when compared with the control (2.52 ± 0.14 g). On Walker 256 carcino sarcoma, the average tumor weight of the control was 8.36 ± 0.55 g and intraperitoneal HdLP reduced tumor weight to 3.52 ± 0.94 and 3.74 ± 0.95 g at doses of 10 and 20 mg/kg/day, respectively with % inhibition rates of 57.91 and 55.23. It was concluded that HdLP had some interesting anticancer activity that could be associated with its immune stimulating properties (Mousinho et al., 2011).

The ethanolic extract of leaves of *Himatanthus obovatus* showed strong cytotoxic activity in cancer cell lines (Mesquita et al., 2009). 14-Dihydroxy-5b, 14b-card-20-(22)-enolide has shown

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in vitro cytotoxicity against T-cell, HL-60, HeLa, MCF-7, LNCap, DU145, PC3, VA-13) and HepG2 (Raghavendra et al., 2007; Zhao et al., 2007). The purified alkaloid fraction of *E. coronaria* was cytotoxic to the HT-29, A-549 and MCF-7 cell lines with IC_{50} values of 32.5, 47.5 and 72.5 µg/mL respectively. It also induced DNA fragmentation in the HT-29 cell line at a concentration of 65 µg/mL (Hullatti et al., 2013).

2.7.5 Antituberculosis activity

Globospiramine showed potent antituberculosis activity against M*ycobacterium* tuberculosis H_{37}Rv as evidenced in microplate Alamar blue assay (MIC = 4 µg/ml) and low-oxygen recovery assay (LORA MIC 5.2 µg/ml) (Macabeo et al., 2011).

2.7.6 Anticholinesterase activity

The bisindole alkaloids isolated from *Voacanga globosa*, exhibited promising anticholinesterase activity with butyrylcholinesterase, the most potent being deoxyvobtusine (IC_{50} = 6.2 µM) (Macabeo et al., 2011). Pleiocarpine had also demonstrated promising anticholinestrase activity (Naaz et al., 2013).

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2.7.7 Neurobiological activity

The ability of reserpine from *Rauwolfia serpentina* to induce depression and deplete brain amines became one of the pillars of the monoamine theory of affective disorders. The alkaloid reserpine was introduced into Western psychiatry as an antipsychotic drug in the 1950s, despite its effectiveness as an antipsychotic agent, reports of secondary depression side effects has lessened its popularity (Healy and Savage 1998; Walter and Rey 1999).

2.7.8 Aphrodisiac activity

Mondia whitei has been reported to possess aphrodisiac activity. The combined extract used as formulation: *Mondia whitei + Ekerbegia capensis,* showed a dose-dependent direct effect in increasing sexual arousability, better sexual stimulation and increase in copulatory efficiency in rats (Gundidza et al., 2009).

2.7.9 Antibacterial /Antifungal activity

Alkaloids isolated from *Tabernaemontana divaricata* (L.) R. Br were investigated for antibacterial and antifungal activities where it was found that 5-oxocoronaridine showed strong antibacterial activity against *Klebsiella pneumonia* at 50 µg/ml while tabernaelegantine D displayed maximum antifungal activity against *Penicillium chrysogenum* at 60 µg/ml (Singh et al, 2011).

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2.7.10 Anti-appetite activity

One of the two pregnane glycosides from the dried stems of *H. gordonii* and *H. pilifera* was tested for its appetite suppressant properties in rats by oral gavage at 6.25–50 mg/kg and the results showed that all doses resulted in a decrease of food consumption over an eight day period and a body mass decrease when compared to the control sample receiving only the vehicle (van Heerden et al., 2007).

2.7.11 Anticonvulsant activity

Carissa edulis exhibited dose-dependent inhibition of the convulsion induced by MEST with 20 mg/kg providing 90% protection while phenytoin (20 mg/kg) produced 100% protection.

The median lethal dose (LD_{50}) of *Carissa edulis* was 282.8 mg/kg and over 5000 mg/kg following intraperitoneal and oral administration, respectively. It also produced 40% and 20% protection against convulsion at 5 and 20 mg/kg, respectively, compared with 100% protection with benzodiazepine.The mean onset and percentage protection against convulsion in *Carissa edulis* extract-treated mice were reduced by flumazenil and naloxone. The result was said to support the ethnomedicinal claims of the use of the plant in the management of epilepsy (Ya'u et al., 2008). *C. procera* latex proteins were evaluated with respect to anticonvulsant and sedative activity in mouse models of pentylene tetrazol (PTZ)-, pilocarpine- and strychnine-induced convulsions or turning behavior and pentobarbital induced sleep. In the strychnine- and pilocarpine-induced seizure models, *C. procera* latex proteins caused no significant alterations in latencies to convulsions and death when compared with controls. In the PTZ induced seizure model, administration of *C. procera* latex proteins in high doses (50 or 100 mg/kg) and diazepam caused significant increases in latencies to convulsions and death (Lima et al., 2012).

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2.7.12 Antihyperglycaemic activity

Evaluation of antihyperglycaemic activity of the seed extracts of *Hunteria umbellate* (HU) by Oral administration with 1 mg/kg of glibenclamide, 50 mg/kg, 100 mg/kg, and 200 mg/kg of HU extract significantly and dose dependently ($p < 0.05$, <0.01 and <0.001) attenuated development of hyperglycaemia, decreased levels of plasma HbA1c, free insulin, and serum triglyceride and cholesterol, in the Groups III, IV, V and VI rats, respectively, when compared to fructose-induced hyperglycaemic (Group II) rats. Similar effects were also recorded in the dexamethasone-induced hyperglycaemic rats. The results from the study suggested that the hypoglycaemic and antihyperlipidaemic effects of HU were mediated via enhanced peripheral

glucose uptake and improvements in hyperinsulinaemia. The findings supported the folkloric use of HU to manage diabetes mellitus (Adeneye and Adeyemi, 2009).

The aqueous extract of *T. iboga* potentially stimulated glucose-induced insulin secretion in dose- and glucose-dependent manners (5.6–16.7 mM). *T. iboga* may represent a source of anti-hyperglycaemic natural products that could serve as type II diabetes mellitus therapeutics (Souza et al., 2011). Investigation of the anti-diabetic activity of unripe *Carissa carandas* Linn. fruit extract indicated that the methanol extract and its ethyl acetate soluble fraction significantly lowered the elevated blood glucose levels by 48% ($p < 0.001$) and 64.5% ($p < 0.001$) respectively at a dose level of 400 mg/kg per oral administration after 24 h as compared to diabetic control (Itankar et al., 2011).

2.7.13 Antidiarrhoeal activity

A. congensis extracts showed significant and dose-dependent antidiarrhoeal activity in treated wistar rats at oral doses of 100 and 200 mg/kg body weight. At the highest dose of 200 mg/kg body weight, the 80% hot methanol extract produced 79.87 ± 2.1% and 78.67± 0.5%, 75.0 7± 2.1% and 71.47± 2.1% inhibition of defecation and diarrhea respectively against castor oil- induced diarrhea. 75.07± 1.2% and 73.37± 1.2% inhibition were recorded against magnesium sulphate-induced diarrhea (Nsaka Lumpu et al., 2012).

2.7.14 Antiprotozoal activity

The aqueous and 80% MeOH extracts from the leaves stem bark, root bark and series of sub fractions from *Alstonia congensis* has been reported to show significant antiprotozoal activity against the KI strain of *Plasmodium falciparum* with IC_{50} values ranging from 2 to 5 µg/ml. The total alkaloid extract was active against *Leishmania infantum* with $IC_{50} < 10$ µg/ml

(Lumpu et al., 2013).

2.7.15 Analgesic activity

The DCM (30 mg/kg^{-1}) fraction of ethanolic extract of *A. scholaris* demonstrated an analgesic effect on mice with % inhibition of 45.06 ± 4.62 as compared with the control

(Arulmozhi et al., 2012).

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Table 2.1. Ethno medicinal usage of some Apocynaceae species

Scientific names	Ethno medicinal uses	Region	References
Adenium oleifolium	Root concoction is used as a tonic	Namibia	Aiyambo, 2010
Alstonia angustiloba	Stems, leaves and latex for gynecological and skin sores	Indonesia	Mulyoutami et al., 2009
	Leaves applied externally for headache especially for gastric disturbances, and as a remedy for fever. Ointment made from this plant can be applied to snake and scorpion bites	Malaysia	Lin, 2003
Alstonia boonei	Used for the treatment of malarial disease, as astringent, antifebrile, antihelmintic, antispasmodic among other indications	Nigeria	Ajibesin et al., 2008, Iwu and Klayman, 1992
Alstonia congensis	Aqueous decoction from leaves used to treat diarrhea	Congo	Lumpu et al., 2012
Alstonia oblongifolia Benth.& Hook.f.,	Roots applied topically to relieve itching		McGraw et al. 2000
Ceropegia purpurascens	The tubers are baked in the ashes before they are eaten	Namibia	Aiyambo, 2010
Ceropegia stenoloba	Tubers are eaten raw or roasted in hot ash.	Namibia	Aiyambo, 2010
Calotropis gigantea (L.)	Used to treat skin and liver diseases, leprosy dysentery, worms, ulcers, tumours and ear aches.		Rajakaruna et al., 2002
	Latex used for wound healing		Nalwaya et al., 2009
Carissa edulis	Headache, chest pain, gonorrhea, syphilis, rheumatism, rabies and as well as a diuretic		Nedi et al., 2004
	Also for fever, cell anaemia and hernia rooots are used for venereal diseases, epilepsy, malaria, heartburn, arthritis, sorcery and cancer	Kenya	Ibrahim, 1997 Jeruto et al., 2008
Catharanthus roseus	Used to treat diabetes and high blood pressure	Shebien El-Kom Menoufia	El-Seedi et al., 2013
	As a purgative, to treat indigestion and dyspepsia		Noble, 1990
	flowers milky sap applied topically to treat burns	South Africa	Govindasamy and Srinivasan, 2012
	hypertension, cancer, scalps and sore	China	TCMD, 2006
Carissa congest (Carissa carandas)	Roots were employed as a bitter stomachic, vermifuge and as an ingredient in the	India	Itankar et al., 2011
Dyera costulata Hook	Leaves and barks used for fever, Inflammation, pain		Subhadhirasakul et al., 2003
Ervatamia coronaria (Jacq) Stapf	Various parts used as anticancer, also for wounds remedy for itches		Atta-ur-Rahman et al., 1986 Talapatra et al., 1975

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Table 2.1. Continued Ethno medicinal usage of some Apocynaceae species

Scientific names	Ethno medicinal uses	Region	References
Himatanthus lancifolius	Treatment of dysmenorrhea and in the amelioration of menopausal symptoms.	Brazil	Rattmann et al., 2005
	The dried stem bark of this specie is commonly used as a febrifuge, as an emenagogue and as an abortive		
Holarrhnea floribunda			
	Stem bark is used for malaria, dysentery, fever, female sterility, skin infections veneral diseases and snake bites		
	Convulsion in children	Nigeria	Orwa et al., 2012
Hoodia gordonii	As food plant	South Africa	van Heerden, 2008
Hunteria umbellata	Use of the hot infusion of the plant seed		Adeneye and Adeyemi, 2009
	Leaves, roots and barks for fever, pain, abdominal colic and discomforts, diabetes mellitus and obesity and as an immune booster		
	plant leaves and pulp to induce labour		Falodun et al., 2006
Kopsia fruticosa (Ker.) A. DC	To treat sore and syphilis, has cholinergic effects		Nalwaya et al., 2009
Landolphia Buchananii	Leaves infusion used for wounds, gonorrhoea, molluscides	Kenya	Jeruto et al., 2008
Mascarenhasia arborescens A. DC.	To treat intestinal disorders, intestinal spasms and diarrhea	Madagascar	Desire et al., 2010
Mondia whitei	A mild laxative, to ease abdominal pains, alleviate nausea as well as for the treatment of fever, sexual dysfunction	Africa	Aremu et al., 2010
	A root decoction used to induce labour	Uganda	Ssegawa and Kasenene, 2007
	As antimalarial, anthelmintics	Nigeria Benin	Hermans et al., 2004, Idu et al., 2010 Odugbemi et al., 2004,
	To cure male infertility	Cameroon	Focho et al., 2009
	To treat ringworms, skin diseases, heart diseases and asthma	Kenya	McGeoch, 2004.
	For stress and tension in adults	South Africa	Van Wyk and Gericke, 2000
	To stimulate appetite, as an aphrodisiac, and for treatment of fits in children		Stafford et al. 2008

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Table 2.1: Continued Ethno medicinal usage of some Apocynaceae species.

Scientific names	Ethno medicinal uses	Region	References
Nerium oleander L.	Inhalation from heated decoction from roots used to treat ,headaches and colds, leaves decoction used for skin diseases, also for paralysis and serious pain	Shebien El-Kom Menoufia	El-Seedi et al., 2013
Nerium indicum Mill. (Kaner)	The roots are made into a paste with water and are applied externally to treat Chancre, ulcers and leprosy A paste of roots is applied externally on piles The paste of its leaves with oil of its root bark is used to treat ringworms and other skin diseases The roots is used for procuring abortion both by local application and internal administration	India	Parveen et al., 2007
Rauvolfia caffra Sond.	Applied topically to treat measles		McGaw et al. 2000
Rauwolfia serpentina	Dried leaves are used for snuff for headaches decoctions of the bark are used as a tranquillizer hysteria and insomnia.	South Africa	Stafford et al. 2008
Rauwolfia vomitoria	Root boiled in water to make decoction for mental illness	Nigeria	Ajibesin et al., 2008
Tabernaemontana cymosa	The leaves and fruits are remedies against the sting of millipede, and the flowers are reported to have cardiotonic properties while the latex is used to remove warts	Colombia	Achenbach, 1996
Tabernanthe iboga	Maceration of air-dried root bark used for diabetes Fatigue and hunger AIDS management	Gabon	Souza et al., 2011 Mash et al., 1998 Mefane et al., 1990
Urechites andrieuxii Muell.-Arg.	Used for the treatment of cutaneous Leishmaniasis	Yucatan Peninsula	Chan-Bacab et al., 2003
Voacanga globosa.	Used to stupefy eels and treat ulcers and wounds		Macabeo et al., 2009
Wrightia tomentosa	To treat cancer, snake and scorpion bites, renal complications,menstrual disorders		Chakravarti et al.,2012
Xysmalobium undulatum R.Br.	Roots powder applied topically to treat abscesses		VanWyk et al. 2000 Buwa and Van Staden, 2006

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2.8 The Genus Pleiocarpa

Pleiocarpa is a genus of flowering plants in the family of Apocynaceae, a native to tropical Africa and is found in roughly seven species. It is related to *Hunteria* and *Picralima*

Pleiocarpa species generally are evergreen shrubs or trees in which the stems and leaves are completely glabrous. A white latex is present in all parts but is absent in the spines and tendrils. The leaves are opposite or present in whorls of 3-5 and elliptic to obovate. The petiole is angled, often narrowly winged but stipules are absent. They are inflorescences cymose, axillary, rarely also terminal; pedicels short or absent. The calyx is glabrous with lobes free at the base, herbaceous, imbricate, and not revolute, and without scales on the inner surface. The corollas are hypocrateriform, externally glabrous, internally pubescent below stamens and the tube is often found with 5 splits developing at the level of the stamens. The lobes are contorted, overlapping to the left and are not ciliate. Their stamens arise at or above the middle of the corolla tube. The filaments are short and the anthers are dorsifixed, introrse, longitudinally dehiscent while their ovaries are glabrous, composed of 2-5 separate carpels with 1-4 ovules per carpel. The clavuncle is present and the stigma are reduced to a sessile area or rarely represented by a rudimentary apiculus. Their fruits are a compound berry of 2 variously shaped, fleshy to slightly fibrous mericarps with a rounded to hook-shaped apex. The seeds are variously shaped with smooth brown testa. The embryo is straight, spathulate, surrounded by a thick, rather starchy and hard endosperm, leaving a hole around the radicle base. The cotyledons are elliptic, thin and leafy, while the radicle is almost cylindrical (Louppe, 2008; GAP, 2013; Govaerts et al., 2011).

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Photograph of *P.pycnantha (Family: Apocynaceae)*

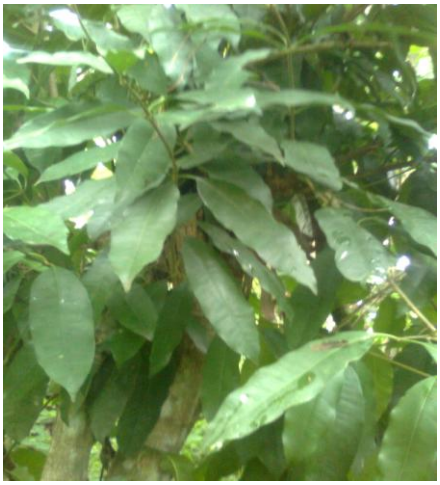

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Figure 2.7. The photograph of Pleiocarpa pycnantha leaves.

Kingdom: Plantae

Phylum: Magnoliophyta

Class: Magnoliopsida

Subclass: Asteridae

Order: Gentianales

Family: Apocynaceae

Genus: Pleiocarpa

Figure 2.8. Scientific classification of Pleiocarpa pycnantha.

Chemical constituents found in Pleiocarpa species includes alkaloids: (+)-pycnanthine, pleiomutinine,(+)-pleiocarpamine, (+)-quebrachamine, (+)-macusine B, (-)-eburnamine, tubicine, Tubifoline,(+)-pycnanthinine, kopsinine, tubotaiwine, tubifolidine, kopsinilam 19, 20-dihydroakuammicine tuboxenine and (-) 1, 2-dehydroaspidospermidine. Others are (-)-pleiocarpinine, (-)-aspidofractinine, (+)-aspidospermidine, pleiocarpinilam, huntrabrin methochloride, kopsinic acid methochloride, Na methylsarpagin methochloride, pleiocarpine, macusine B chloride, macusine B rhodanide, pleiomutine, pleiomutinone, pleiomutinol, eburnamylpleiocarpinine, 10,11-dioxopleiocarpine,1-carbomethoxy-β-carboline, isotuboflavine and norisotuboflavine. Also, triterpenes lupeol, α- and β-amyrin and their corresponding acetates (Thomas et al., 1965, 1966; Achenbach and Biemann, 1965).

2.8.1 Phytochemistry of selected Pleiocarpa species

2.8.1.1 Pleiocarpa tubicina

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Some alkaloids have been reported from the leaves of *Pleiocarpa tubicina* STAPF; (+)-quebrachamine ((+)-I), (−)-1, 2-dehydroaspidospermidine ((−)-II) and (−)-aspidofractinine (IV) have been isolated in small quantities. The interesting conversion of (−)-quebrachamine ((−)-I) into (+)-1, 2-dehydroaspidospemidine ((+)-II) has been achieved by controlled per-manganate oxidation (Bycroft et al., 1964). Others are quaternary indole alkaloids from the root bark: huntrabrine methochloride, kopsinic acid methochloride, $N_{(a)}$-methyl-sarpagine methochloride and macusine B chloride (Khan et al., 1967). Tuboxenin isolation was reported by Kump et al., (1964) and tubotaiwine (Schripsema et al., 1987).

2.8.1.2 Pleiocarpa mutica

Three new β-carboline derivatives were isolated from the stem bark of Pleiocarpa mutica Benth. which are 1-carbomethoxy-β-carboline, isotuboflavine and norisotuboflavine. Other known compounds reported are; pleiocarpine, 1,2-dehydro- aspidospermidine, quebrachamine, eburnamine, pleiocarpamine (Schmid and Kump, 1961; Achenbach and Biemann, 1965).

The roots have been reported to contain five known alkaloids, pleiocarpine, kopsinine, pleiocarpamine, eburnamine and pleiomutinine (Addae-Kyereme et al., 2001).

The stem bark also contained eburnamyl pleiocarpinine (Thomas et al., 1965), flavocarpine (Buchi, 1962) and pleiocarpoline (pleiocarpine N_b,-oxide), pleiocarpolinine (pleiocarpinine N_b,-oxide) and kopsinoline (kopsinine N_b,-oxide) (Thomas et al., 1966).

2.8.1.3 Pleiocarpa talbotii

Several alkaloids have been isolated from the bark and root bark of *P.talbotii* which includes; talbotine, talpinine, talcarpine, and 16-epi-affinine (Naranjo et al., 1972).

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2.8.1.4 Pleiocarpa pycnantha

Some indole alkaloids: pycnanthine, pleiocarpamine, quebrachamine, macusine and

(-) ebunarmine have been isolated from *Pleiocarpa pycnantha* roots and bark (Gorman and Schmid, 1967; Gorman et al., 1969).

2.9 Pharmacological activity of some *Pleiocarpa* species

Alkaloids from the roots of *Pleiocarpa tubicina* Stapf and the leaves, roots and seeds from *Pleiocarpa mutica* Benth have been shown to be hypotensically active (Tsao et al., 1961; Schmid and Kump, 1961). Pleiomutinine from the roots of *P. mutica* was found to show significant

antiplasmodial activity against *P. falciparum* (IC_{50}=5 μM). Pleiocarpine was inactive against malaria parasites in vitro, but moderately active against *P. berghei* in mice. A dose of 25 mg kg^{-1} daily for 4 days reduced parasitaemia by 28.5% compared to untreated controls

(Addae-Kyereme et al., 2001). Aqueous and organic extracts were prepared from *P. mutica* Benth from Belgium Congo; all the aqueous extracts lowered the blood pressure when administered to rats while the ethyl acetate extracts produced the most significant hypotensive activity. The mechanism of action was traced to epinephrine blockade but was not altered by atropine. These extracts also showed some nematocidal activity on *Panagrellus redivivus* (L.) (Tsao et al., 1961).

Pleiocarpine, kopsinine, pleiocarpamine from *P. mutica* were analyzed for their anti-cholinergic action through docking with acetylcholinesterase (AChE) as target.of all the alkaloids tested Pleiocarpine showed promising anti-cholinergic potential, while its amino derivative showed about six-fold higher anti-cholinergic potential than pleiocarpine. Pleiocarpine and its amino derivative were found to be better inhibitors of AChE, as compared to commonly used drugs Tacrine (brand name : Cognex) and Rivastigmine (brand name: Exelon), indicating that the molecules could serve as potential therapeutics in future (Naaz et al., 2013). Kopsinine also displayed some hepaprotective activity against CCl_4 – toxicity in mice and also shortened the barbital-induced sleeping time in mice. Eburnamine had a similar biological activity as strychnine and has been found to be a useful cerebrovascular agent (Schmelzer et al., 2008).

A comprehensive project was conducted as part of the Associated Project #3 of the International Cooperative Biodiversity Group Program under the direction of the Walter Reed Army Institute for Research which focuses on the study of West and Central Africa plants.

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In this study, a total of 41 plant extracts and eight compounds were tested in vitro on strains of African trypanosomes (*Trypanosoma brucei* group) and of pathogenic trichomonads (*Trichomonas vaginalis* and *Tri-trichomonas f*oetus). *P. pycnantha* was one of the plants that showed IC_{50} value of $\leq$ 20 µg/ml. The leaf extract did not show any positive results for the antimicrobial study (Iwu, 2001; Bacchi, 2002).

2.10 Ethnobotanical information on Pleiocarpa genus

The ground bark of *Pleiocarpa mutica* is rubbed against the bodies of inhabitants in Sierra Leone to prevent fever. A drink decoction of the grated bark is used to treat stomach ache in Côte d'Ivoire while it is used for oedema of legs by the Ebrié people. The root bark decoction can also be used to treat kidney diseases and malaria. In Ghana, the decoction from roots is taken as a febrifuge, antimalarial and also for jaundice and convulsions. The ground bark in palm wine is used as laxative (Schmelzer et al., 2008). *Pleiocarpa pycnantha* roots are added to palm-wine in Ghana to give it potency. Ground roots mixed with seeds of *Aframomum melegueta* K.Schum and palm wine is taken as a laxative. In Benin, leaf maceration with lemon juice is given to patients suffering from jaundice, oedema, reduced urine excretion and infection by roundworms (Burkill, 1985). The Yoruba speaking part of West Africa combine a blend of leaves of *Pleiocarpa pycnantha* (Apocynaceae), leaves of *Spondias mombin* (Anacardiaceae) and a fruit of *Aframomum melegueta* (Zingiberaceae) which is then administered to gain and retain good memory (Fatumbi, 1995). The Ekiti people of Nigeria administer the leaf decoction with lime juice for people suffering from roundworm (Personal Information). The cold infusion from the bark of *Pleiocarpa talbotii* can be used to treat stomach pain (Schmelzer et al., 2008).

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2.11 Pleiocarpa pycnantha(K.Schum.) Stapf.

Protologue Oliv., Fl.trop.Afr.4 (1):99(1902)

Family Apocynaceae

Synonyms: Pleiocarpaflavescens Stapf (1902)

Pleiocarpa micrantha Stapf (1902)

Origin and geographical distribution: It is widespread from Senegal east to Kenya and South to Angola and Mozambigue.It is widely distributed throughout tropical Africa.

Uses: The wood is used for local construction, plane blocks, comb, and pestles and in carving in which case it is used to make pipe stem in Uganda. Its ground roots with seeds of *Aframomum melegueta* K.Schum are taken as laxatives. The leaf maceration with lemon juice is given to patients suffering from jaundice, oedema, reduced urine excretion and worm infestion in Benin (Burkill, 1985).

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Properties: The wood is yellow to brown, hard and durable.

Isolated compounds: Some indole alkaloids have been isolated from P. pycnantha namely: pycnanthine, pleiocarpamine, macusine B and quebrachamine.

Botanical description: An evergreen shrub or tree 1·5–30 m. tall; bark grey, smooth or slightly scaly; slash whitish, cream or pale brown with darker yellow striations, brownish yellow beneath, yielding milky latex and the branches are subsarmentose. Young twigs are glabrous, angled and sometimes may have a verticillate branching-pattern.

The leaves thinly coriaceous, opposite or in whorls of 3–5, completely glabrous; petiole 3–20 mm. long, angled, often narrowly winged almost to the base by a continuation of the lamina; lamina (4) 6–13·5 x (1·3) 2·2–5·5 cm., elliptic to obovate or narrowly obovate, the apex

cuspidate with short rounded acumen or acute with rounded tip, the base attenuate; upper surface glossy, with all nerves raised (midrib both prominent and channeled); lower surface paler and mat, all nerves raised; lateral nerves ± parallel, 2–4 mm apart, joining near the margin in a neatly looped vein; edge of leaf forming little pleats when pressed flat.The flowers are white, fragrant, in dense axillary fascicles on new and old wood; pedicels up to 1 mm long, glabrous. Calyx 1·5–2 mm long; sepals ovate, free, glabrous,corolla tube 5–8 mm long, narrowed in the middle, glabrous externally, pubescent internally in upper half, hairs longest just below the anthers; corolla lobes 2–4 x 1·5–2·5 mm, elliptic, glabrous, not strongly overlapping. Their stamens are inserted at about 4-6 mm above the base of the corolla tube, the filaments are minute,and the anthers is usually about 0·5–1 mm long. Gynoecium 4–6·5 mm long; ovary c. 1 mm long, cylindrical, glabrous, of 2 free carpels each containing 1–4 ovules; style slender, flattened; clavuncle 2-lobed; stigma reduced to a flat region at apex of clavuncle. Pleiocarpa fruit comprising 2 clavate mericarps 7–20 x 4·5–18 mm, pale green to orange when ripe, each containing 1 or 2 salmon-pink seeds (Burkill, 1985; Hyde et al., 2013). It has about five species and it is related to Hunteria and Picralima. *P. pycnantha* flowers and fruits throughout the year.

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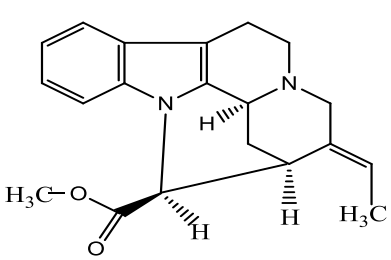
Ecology: It occurs in the understorey of rainforests as well as in gallery and montane forests up to 2300m altitude. It can also be found in disturbed forests.

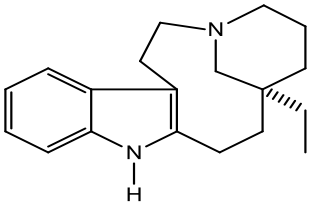
Genetic resources and breeding: It is generally not threatened by erosion because it is found in various forest types and it is widely distributed.

Future prospects: It is unlikely to become an economically important timber tree due to its relatively small size, but will retain its usage in the construction of local houses and production of implements because of its hardness and durability.

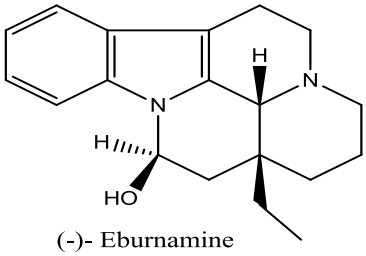
Some isolated compounds from Pleiocarpa genus are documented in Fig.**2.9**.



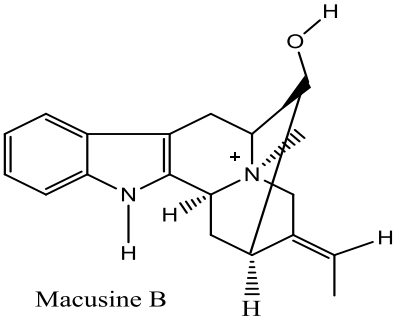
Pleiocarpamine



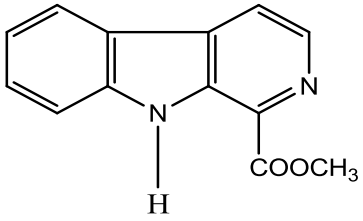
(+) - Quebrachamine



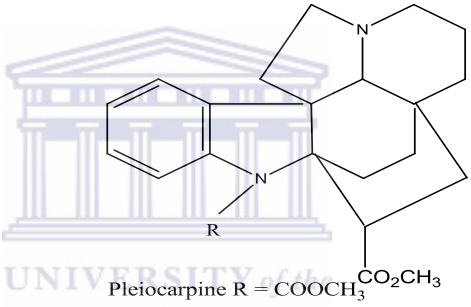
(-)- Eburnamine



Macusine B



1-Carbomethoxy-β-Carboline



Pleiocarpine R = $COOCH_3$
Pleiocarpinine R= CH3
Kopsinine R=H

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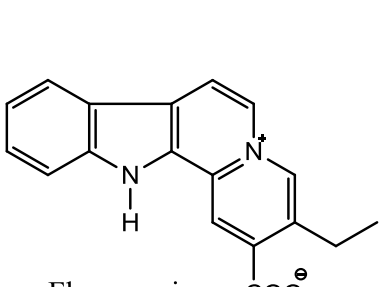
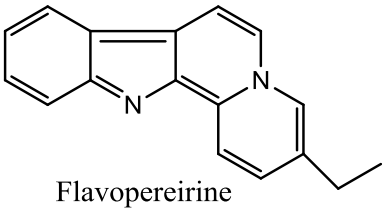
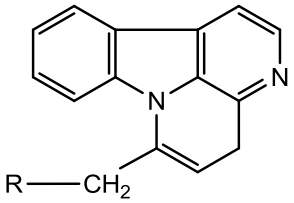
Figure 2.9. Some isolated compounds from Pleiocarpa genus.



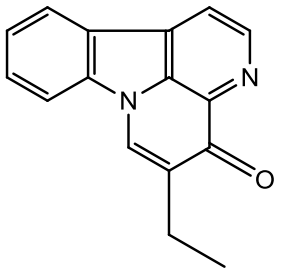
Flavocarpine



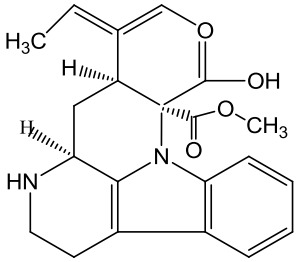
Flavopereirine



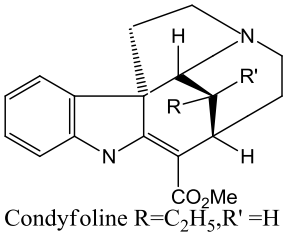
norisotuboflavine R= H
Isotuboflavine R =CH_3



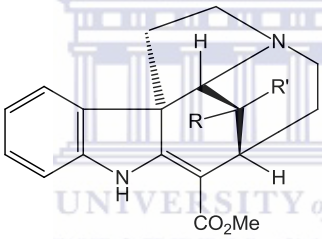
Tuboflavine



Talbotine



Condyfoline R=C_2H_5,R' =H
20 -epi-condyfoline R'=C_2H_5,R =H



Tubotaiwane(20S) R=C_2H_5,R' =H

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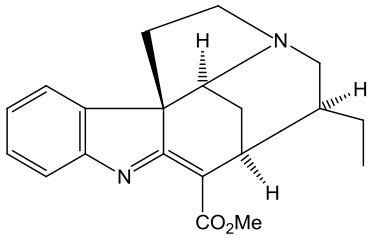
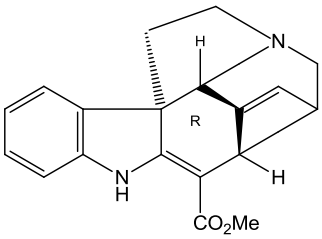
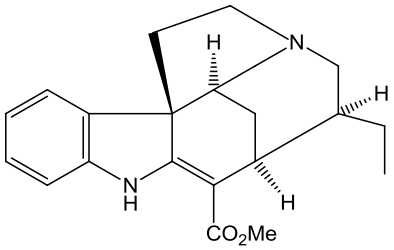


Tubifoline



Condylocarpine



19,20-dihydroakuammicine

Figure 2.9. Some isolated compounds from Pleiocarpa genus continued.

2.12 The development of Apoptosis

Cell death plays a considerable role during physiological processes of multicellular organisms, particularly during embryogenesis and metamorphosis (Lockshin and Zakeri, 2001). The term programmed cell death was introduced in 1964 in which it was proposed that cell death during the development stage is not accidental but occurs in a well regulated fashion. The term apoptosis had been used to describe the morphological processes leading to controlled cellular self-destruction and was first introduced in a publication by Kerr, Wyllie and Currie (Kerr, 1972).

It is of Greek origin, with the meaning "falling off or dropping off", in analogy to leaves falling off trees or petals dropping off flowers. This emphasizes that the death of living matter is an integral and necessary part of the life cycle of organisms. Apoptosis is different from other forms of cell death called necrosis in which uncontrolled cell death leads to lysis of cells, inflammatory responses and, potentially, to serious health problems. Apoptosis seemed to be the most common type of programmed cell death. Other non-apoptotic deaths are also of great biological significance (Liest, 2001).

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2.13 The importance of Apoptosis

The development and maintenance of healthy multicellular biological systems depends on a sophisticated interplay between the cells forming the organism z and that the way in which most cells die is conserved from worm to mammal. Optimum body maintenance means that about

10 billion of our cells will die on a normal day just to counter the number of new cells that arise through mitosis. During development, many cells are produced in excess, which eventually undergo programmed cell death and thereby contribute to sculpturing many organs and tissues

(Meier, 2000). Apoptosis is of great biological importance, and it is involved in differentiation, development, proliferation, homoeostasis, regulation and function of the immune system. Thus, failures in apoptosis may result in autoimmune diseases, neoplasia and spreading of viral infections. Conversely, excessive apoptosis has been associated with other sorts of diseases, such as AIDS, neurodegenerative disorders and ischemic diseases (Gewies, 2003).

2.14 Morphological features of apoptosis

Apoptotic cells may be recognized by stereotypical morphological changes namely; cell shrinkage, deformation and lost contact with the neighboring cells. Its chromatin condenses and marginates at the nuclear membrane, the plasma membrane blebbs or budds, and finally the cell is fragmented into compact membrane-enclosed structures, called 'apoptotic bodies' which contain cytosol, the condensed chromatin, and organdies (Fig.**2.10**). The apoptotic bodies are engulfed by macrophages and thus are removed from the tissue without causing an inflammatory response. These morphological changes are as a result of characteristic molecular and biochemical events occurring in an apoptotic cell, most notably the activation of proteolytic enzymes, which eventually mediate the cleavage of DNA into oligonucleosomal fragments as well as the cleavage of a multitude of specific protein substrates which determines the integrity and shape of the cytoplasm or organelles (Saraste, 2000; Gewies, 2003).

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Apoptosis is therefore in direct contrast to the necrotic mode of cell-death. In necrosis, the cells suffer a major insult that results in a loss of membrane integrity, swelling and the consequent disruption of cells. During necrosis, the cellular contents are released in an uncontrolled manner into the cell's environment which results in damage of the surrounding cells and a strong inflammatory response in the corresponding tissue (Leist, 2001).

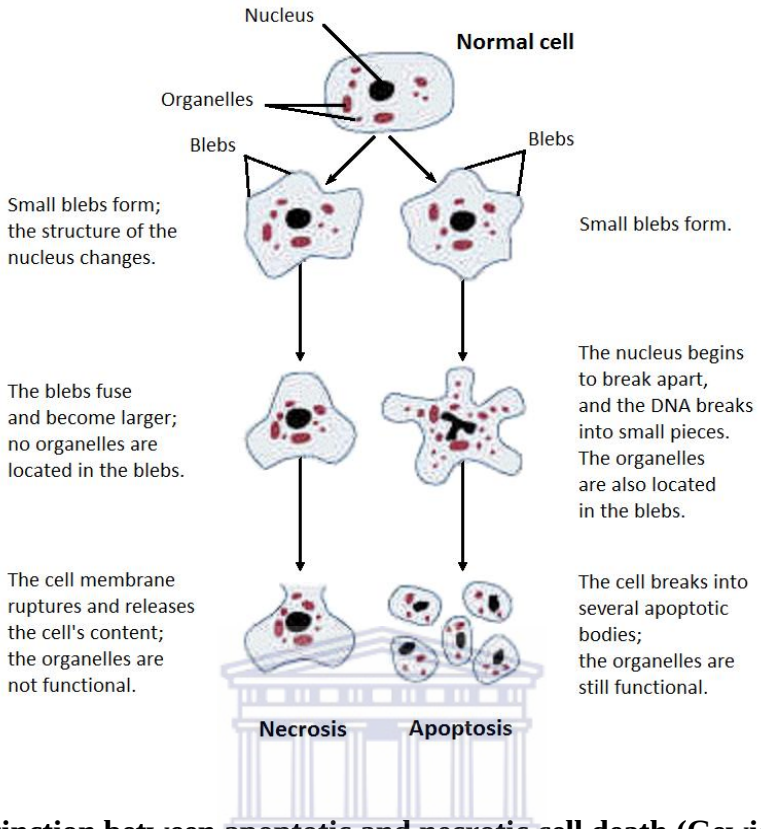

Figure 2.10. **Dinstinction between apoptotic and necrotic cell death (Gewies, 2003).**

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2.14 Stimuli for apoptosis

Several factors can trigger apoptosis from both external and internal stimulus, for example; ligation of cell surface receptors, DNA damage as a cause of defects in DNA repair mechanisms, treatment with cytotoxic drugs, irradiation, lack of survival signals, contradictory cell cycle signaling developmental death signals.

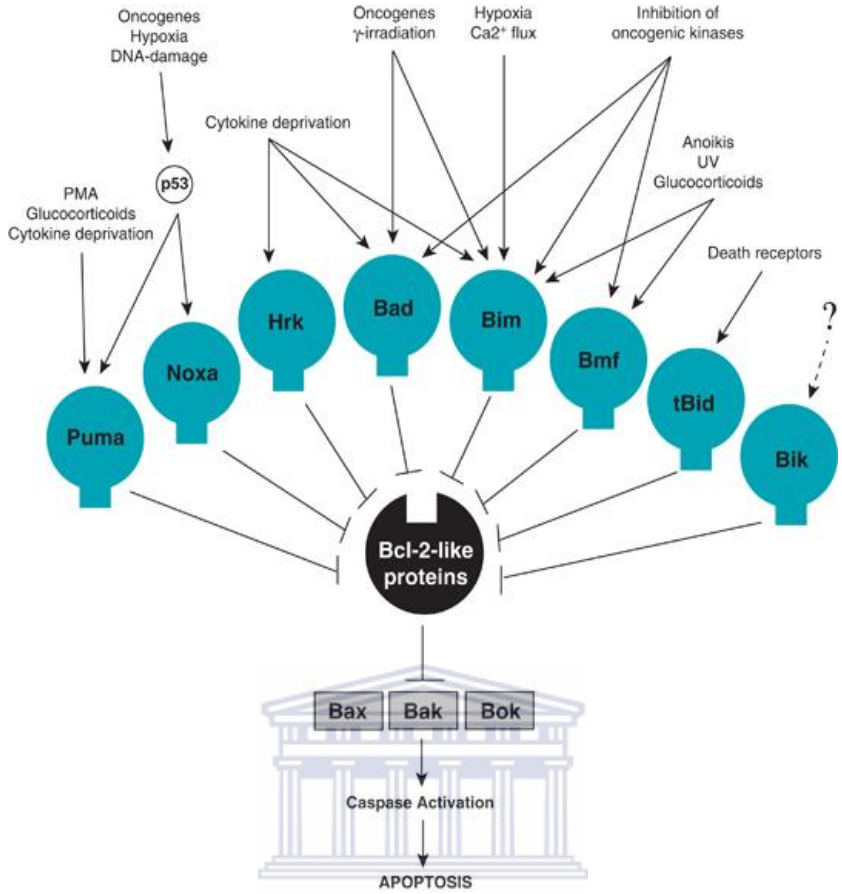

Figure 2.11. Apoptosis stimuli factors (Kelly and Strasser, 2011).

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There are four main groups of stimuli for apoptosis; Group I stimuli includes ionizing radiation and alkylating anticancer agents, causing DNA damage as shown in Fig.**2.10**. Group II stimuli induce apoptosis through receptor mechanisms either by receptor activation mediated by glucocorticoids (acting on the thymus) see Fig.**2.11**, tumour necrosis factor-a (TNT-a), or by withdrawal of growth factors (nerve growth factor and interleukin (IL)-3. Group III stimuli comprise biochemical agents that enhance the downstream components of the apoptotic pathway which includes phosphatases and kinase inhibitors like calphostin C, staurosporine.

The Group IV agents cause direct cell membrane damage and includes heat, UV light and oxidizing agents like superoxide anion and hydrogen peroxide (Wyllie, 1980; Santana, 1996).

Excessive production of reactive oxygen species (ROS) may generate free radicals that can damage the lipid membranes, proteins and nucleic acids. Many of these stimuli cause necrosis in larger doses. Following an appropriate stimulus, the first stage or 'decision' phase of apoptosis is the genetic control point of cell death which is followed by the second stage or 'execution' phase, responsible for the morphological changes of apoptosis. Two main pathways trigger the cell to enter the first stage or apoptosis viz. signaling via mitochondria, and signaling via 'death-receptors' such as CD95 or Fas and both result in the destruction of the same apoptotic substrates (Wyllie, 1980; Santana, 1996; Kam and Ferch, 2000).

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2.15 Apoptotic pathways

2.15.1 The extrinsic (receptor) pathway

The extrinsic apoptosis pathway is mediated by the activation of death receptors, which are at the cell surface and transmit apoptotic signals after binding to specific ligands as indicated in Fig.**2.12**. The extrinsic apoptosis pathway involves procaspase-8 which is recruited by its death effector domains (DEDs) to the death inducing signaling complex (DISC), a membrane receptor complex formed to the ligation of a member of the tumor necrosis factor receptor (TNFR) family. Once bound to the DISC, several procaspase-8 molecules are joined in close proximity and then are capable to activate each other by autoproteolysis. Death receptors belong to the tumor necrosis factor receptor (TNFR) gene superfamily, including TNFR-1, Fas/CD95 and the TRAIL receptors DR-4 and DR-5, after which the signaling is transmitted through the

cytoplasmic part of the death receptor, which contains a conserved sequence termed the death domain (DD). Adapter molecules like FADD (Fas-activated Death Domain) or TRADD
(Toll receptor activated death domain) have their own DDs by which they are recruited to the DDs of the activated death receptor, thereby forming the DISC and the local concentration of several procaspase-8 molecules at the DISC leads to their autocatalytic activation and release of active caspase-8. Active caspase-8 then processes downstream effector caspases, which subsequently cleave specific substrates resulting in apoptosis (Denault and Salvesen, 2002; Gewies, 2003).

Cells that possess the capacity to induce this direct and mainly caspase-dependent apoptosis pathway are classified as type I cells. On the other hand, in type II cells, the signal coming from the activated receptor does not mediate a caspase signaling cascade strong enough for execution of cell death on its own. In this case, the signal needs to be amplified via mitochondria-dependent apoptotic pathways. The link between the caspase signaling cascade and the mitochondria is provided by the Bc1-2 family member Bid.

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Bid is cleaved by caspase-8 into the truncated form (t-BID), which translocates to the mitochondria where it acts in concert with the pro-apoptotic Bcl-2 family members Bax and Bak to induce the release of cytochrome c and other mitochondrial pro-apoptotic factors into the cytosol (Denault and Salvesen, 2002; Gewies, 2003).

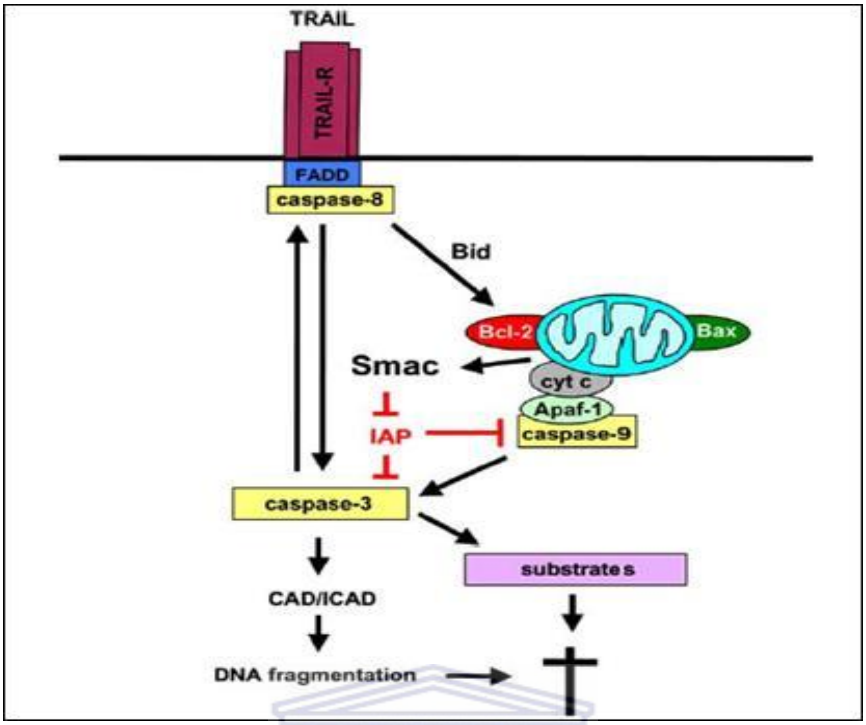

Figure 2.12. **Receptor mediated apoptosis (Fulda, 2009).**

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2.15.2 The intrinsic (mitochondrial) pathway

The mitochondrial pathway is activated by a variety of extracellular and intracellular stresses, including oxidative stress and treatment with cytotoxic drugs. The apoptotic signal leads tothe release of cytochrome c from the mitochondrial intermembrane space into the cytosol, where it binds to the Apoptotic Protease Activating Factor-1 (Apaf-1), and a mammalian CED-4 homologue. Intrinsic apoptosis pathways involve procaspase-9 which is activated downstream of mitochondrial pro-apoptotic events at the so called apoptosome, a cytosolic death signaling protein complex that is formed upon release of cytochrome C from the mitochondria, see Fig.**2.13**. In cancer, the intrinsic pathway is activated by DNA damage resulting from cytotoxic chemotherapy as well as irradiation, and also mediates apoptosis from other stimuli including

hypoxia, defective cell cycle events, and deprivation at growth factors (Denault and Salvesen, 2002).

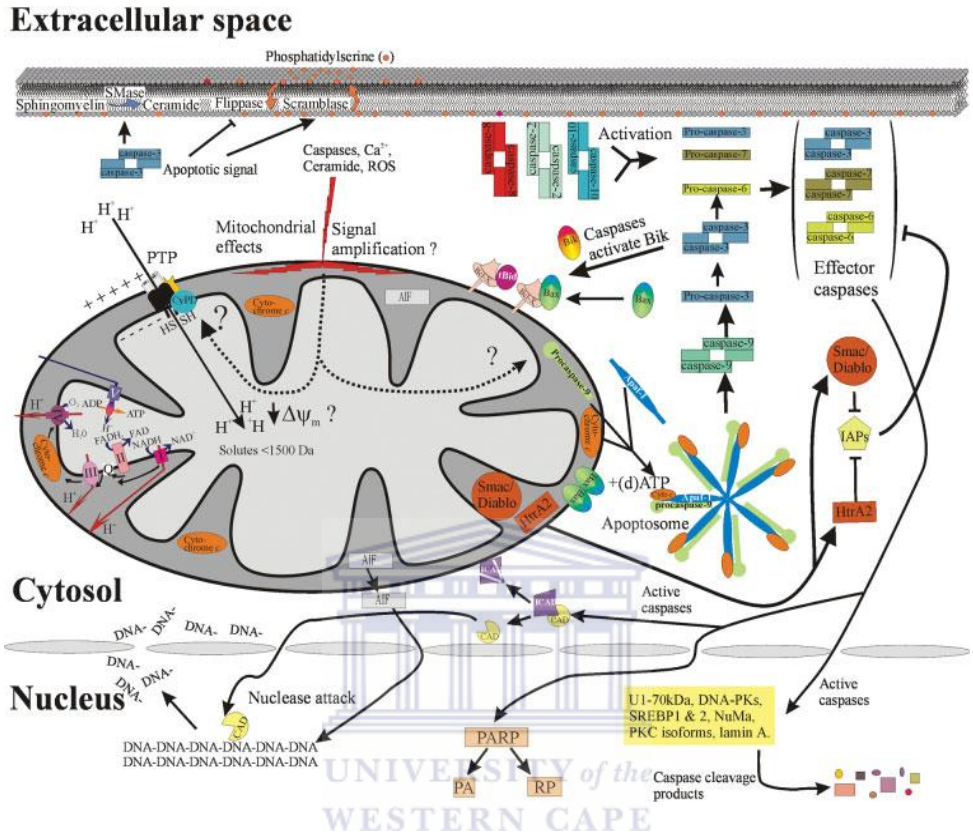

Figure 2.13. **Apoptotic intrinsic pathway (Lawen, 2003).**

Following an apoptotic stimulus, Bax (the pro-apoptotic protein), undergoes homodimerization and oligodimerization through interaction with the BH3-regions and associates with the mitochondrial membrane with insertion into the mitochondrial membrane (Denault and Salvesen, 2002). Permeabilization results in loss of membrane potential, and consequently release of apoptogenic factors including cytochrome C, ATP and SMAC/DIABLO (second mitochondria-derived activator of caspase/direct IAP binding protein with low p1). ATP binds to the nucleotide-binding domain of apoptotic protease-activating factor 1 (APAFI) resulting in the formation of large oligomers (heptamers). Cytochrome c binds to APAF1 and through a CARD

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in APAFI, binds to a complementary CARD on precaspase-9 leading to activation of caspases 9 and of the downstream ”executioner” caspases 3, 6, and 7. These activated caspases auto-induce activation of themselves as well as downstream caspases and the protocolytic cascade, that ultimately cleaves substrates essential for cell viability finally resulting in the characteristic biochemical sad morphological changes of apoptosis (Denault and Salvesen, 2002).

2.16 Reactive Oxygen Species (ROS)

Reactive oxygen species (ROS) are chemically reactive molecules containing oxygen which includes oxygen ions and peroxides. It forms as a natural byproduct of the normal metabolism of oxygen and plays an important role in cell signaling and homeostasis. However, during environmental stress like UV or heat exposure, ROS levels increase (Devasagayam et al., 2004). This may consequently result in significant damage to cell structures with the resultant effect known as oxidative stress. ROS can be generated exogenously or endogenously.

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Exogenous sources include ionizing radiation, pollutants, tobacco, smoke, drugs or xenobiotics while endogenous sources are intracellularly generated through multiple mechanisms and depending on the cell and tissue types, may result in being the major sources of the "professional" producers of ROS NADPH oxidase (NOX) complexes (7 distinct isoforms) in cell membranes, mitochondria, peroxisomes and endoplasmic reticulum (Muller, 2000; Han et al., 2001).

Reactive oxygen species are implicated in cellular activity towards a variety of inflammatory responses including cardiovascular disease. They may also be involved in hearing impairment via cochlear damage induced by elevated sound levels. ROS are also implicated in mediation of apoptosis and ischemic injury. Other harmful effects of reactive oxygen species on the cell are

DNA damage, lipid peroxidation, amino acid oxidation and inactivation of specific enzymes by co-factors in the oxidation process (Brooker, 2011).

2.17 ROS and Cancer

ROS are constantly generated and eliminated in biological systems and are required to drive regulatory pathways (Dickinson and Chang, 2011). Under normal physiological conditions, cells control ROS levels by balancing the generation of ROS with their elimination by scavenging systems. Under oxidative stress conditions, excessive ROS can damage cellular proteins, lipids and DNA, leading to fatal lesions in cells that contribute to carcinogenesis (Kang et al., 2013). Cancer cells exhibit greater ROS stress than normal cells do, partly due to oncogenic stimulation, increased metabolic activity and mitochondrial malfunction. At low levels, ROS facilitates cancer cell survival since cell-cycle progression driven by growth factors and receptor tyrosine kinases (RTK) require ROS for activation and chronic inflammation, a major mediator of cancer, is regulated by ROS while at high level can suppress tumor growth through the sustained activation of cell-cycle inhibitor and induction of cell death as well as senescence by damaging macromolecules. In fact, most of the chemotherapeutic and radiotherapeutic agents kill cancer cells by augmenting ROS stress (Irani et al., 1997; Ramsey and Sharpless, 2006; Takahashi et al., 2006; Renschler, 2004; Toler et al., 2006). The ability of cancer cells to distinguish between ROS as a survival or apoptotic signal is controlled by the dosage, duration, type and site of ROS production. Modest levels of ROS are required for cancer cells to survive, whereas excessive levels kill them (Gupta et al., 2012).

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Metabolic adaptation in tumors balances the cells' need for energy with an equally important need for macromolecular building blocks and tighter control of redox balance. As a result,

production of NADPH is greatly enhanced, which functions as a cofactor to provide reducing power in many enzymatic reactions for macromolecular biosynthesis and at the same time rescuing the cells from excessive ROS produced during rapid proliferation. Cells counterbalance the detrimental effects of ROS by producing antioxidant molecules, such as reduced glutathione (GSH) and thioredoxin (TRX), which rely on the reducing power of NADPH to maintain their activities (Cairns et al., 2011). Most of the risk factors associated with cancer interact with cells through the generation of ROS, which then activate various transcription factors such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), activator protein-1 (AP-1), hypoxia-inducible factor-1α and signal transducer and activator of transcription 3 (STAT3), leading to expression of proteins that control inflammation, cellular transformation, tumor cell survival, tumor cell proliferation, invasion, agiogenesis and metastasis. ROS can also control the expression of various tumor suppressor genes such as p53, retinoblastoma gene (Rb), phosphatase and tensin homolog (PTEN) (Gupta et al., 2012).

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2.18 ROS and cell death

Cancer cells can die in three ways: apoptosis, necrosis and autophagy. Excessive ROS can induce apoptosis through both the extrinsic and intrinsic pathways as earlier stated. In the extrinsic pathway of apoptosis, ROS are generated by Fas ligand as an upstream event for Fas activation via phosphorylation, which is necessary for subsequent recruitment of Fas-associated protein with death domain and caspase 8 as well as apoptosis induction (Gupta et al., 2012).

In the intrinsic pathway, ROS functions to facilitate cytochrome c release by activating pore-stabilizing proteins (Bcl-2 and Bcl-xL) as well as inhibiting pore-destabilizing proteins (Bcl-2-associated X protein, Bcl-2 homologous antagonist/killer) (Martindale and Holbrook, 2002).

A high level of ROS can result in both apoptosis and necrosis in cancer cells. ROS can also induce cell death through autophagy; a self-catabolic process involving sequestration of the cytoplasmic contents (exhausted organelles and protein aggregates) for degradation in lyposomes (Hampton and Orrenius, 1997; Shrivastava et al., 2011).

2.19 Caspases

Caspases (*c*ysteine-*asp*artic proteases or **c**ysteine-dependent aspartate-directed proteases) are a family of proteases that play an essential role in apoptosis, necrosis and inflammation. They are a large family of evolutionarily conserved proteases found from *Caenorhabditis elegans* to humans and the structure is shown in Fig.**2.15**. Although the first caspase was identified as a processing enzyme for interleukin-1β, genetic and biochemical data have collectively revealed that many caspases are key mediators of apoptosis, the intrinsic cell suicide program essential for the development of and tissue homeostasis. Each caspase is a cysteine aspartase and employs a nucleophilic cysteine in its active site to cleave aspartic acid peptide bonds within proteins

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(Shi, 2002; Alnemri et al., 1996; Thornberry et al., 1992, 1998).

Two major general pathways for the induction of apoptosis exists viz., the receptor or extrinsic pathway and the mitochondrial or intrinsic pathway. Both apoptotic signaling pathways converge at the level of the specific proteases—the caspases (Fig. **2.13**). Currently about 14 mammalian caspases has been identified (Fig. **2.14**). They are synthesized as pro-enzymes, which usually undergo proteolysis and activation by other caspases in a cascade (Earnshaw et al., 1999). Peptide caspase inhibitors can inhibit downstream caspase activation and subsequently apoptosis, of the caspases, about 30 can be grouped into subclasses in various ways. Functionally, one can distinguish three classes of caspases; (i) the initiator caspases that are characterized by long

prodomains (>90 amino acids) containing either DED domains (caspase-8 and caspase-10) or a caspase recruitment domain (CARD) (caspase-2 and caspase-9; CED-3); (ii) the executioner or effector caspases containing short prodomains (caspase-3, caspase-6 and caspase-7) and (iii) the remaining caspases whose main role lies in cytokine maturation rather than apoptosis.

Upon activation, the prodomains are cleaved off and the large and small subunits are separated by caspase action (all cleavages occur after Asp residues). The active site is formed by the interface of the two subunits by 1 Arg, 1 His, 1 Cys of the large subunit and 1 Arg of the small subunit.

The active caspases form heterotetramers, Grütter (2000) while the initiator caspases cleave and activate effector caspases. These then cleave cellular substrates, which leads to the apoptotic morphology. One effector caspase, caspase-3, when activated is capable of cleaving many important cellular substrates, including ICAD (inhibitor of caspase-activated DNase), ROCKI (Rho-associated coiled coil forming kinase I), poly (ADP-ribose) polymerase (PARP, a DNA repair enzyme), actin, fodrin and lamin. Active caspase-3 can cause membrane blebbing (via ROCKI cleavage that leaves the kinase constitutively active, permanently phosphorylating myosin light chain, disassembly of the cell structure and DNA fragmentation [via ICAD (inhibitor of caspase-activated DNase)] cleavage that sets CAD (caspase activated DNase) free to move into the nucleus and cleave DNA internucleosomally, which eventually leads to cell death (Lawen, 2003).

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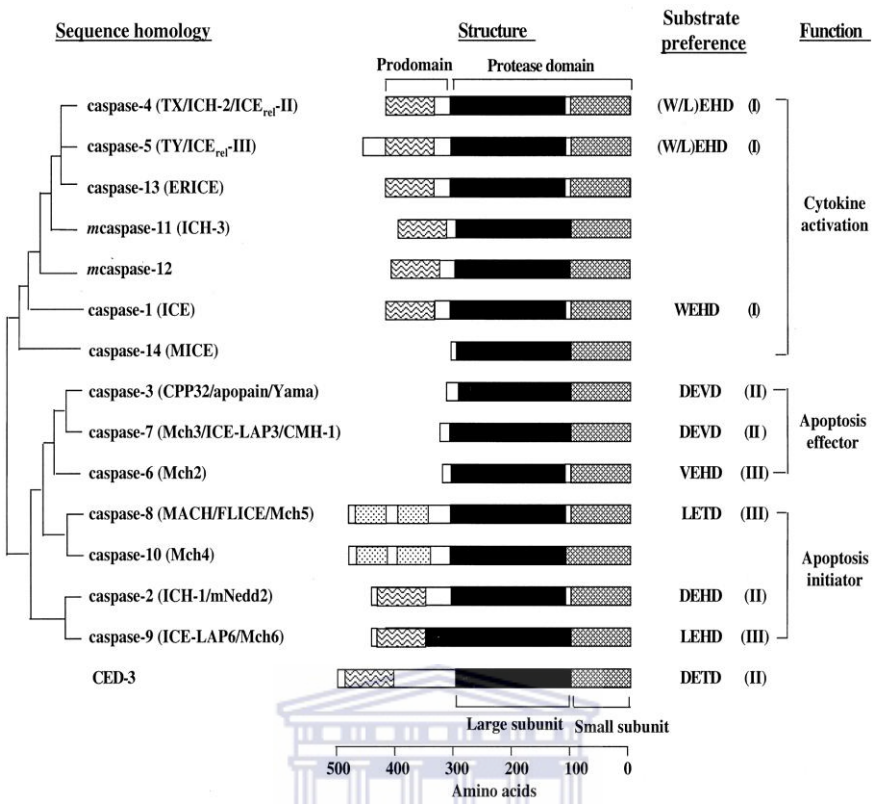

Figure 2.14. Mammalian caspase family and *C.elegans* caspase CED-3 (Chang and Yang, 2000).

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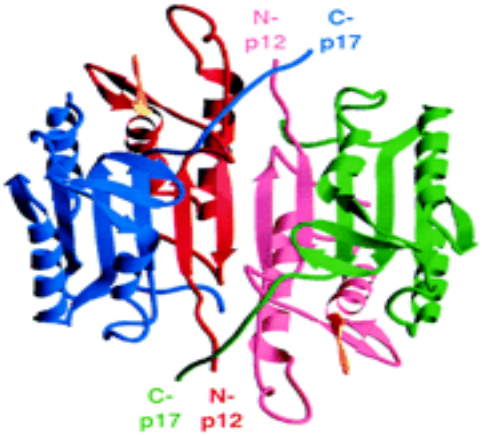

Figure 2.15. Structure of caspase -3 tetramer in complex with AC-DEVD-CHO (Chang and Yang, 2000).

CHAPTER 3

3.0 MATERIALS AND METHODS

This chapter deals with the description of the materials and methods used in this research.

The plant was collected, identified and extracted using 95% ethanol by cold maceration for 72 h. The resultant product (ethanolic extract) was subjected to phytochemical screening to ascertain the secondary metabolites after which it was fractionated on column chromatography.

The crude extract and the various fractions P1-13 was screened using WST-1 assay for cytotoxicity on HeLa, HT-29, MCF-7 and KMST-6. Based on the result obtained from the cytotoxicity profile, some fractions were selected for further purification and tested for their ability to reduce the cell viability on both cancer and normal cells. The details of the procedure for isolation, cell culture, cytotoxicity and mechanistic studies are described in this chapter.

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3.1 Materials and Instrumentation

The details of the material and specific instrumentation conditions are described in sections **3.1.1** and **3.2.1**.

3.1.1 General conditions

Melting points (uncorrected) were determined using Stuart melting point SMP10 apparatus.

UV spectra were recorded in methanol on a Shimadzu UV-2501, and Shimadzu UV2UO PC spectrophotometer. IR spectra were acquired on a Varian 1000 FT-IR (Scimitar Series).

^{1}H-NMR, ^{13}C-NMR and 2D-NMR spectra (COSY, HSQC, HMBC, NOESY and DEPT) spectra were recorded on Bruker Avance 200, 400 and 600 MHz spectrometers using TMS as reference.

Mass spectrometry studies were carried out on Varian MAC 112 mass spectrometer. Column

chromatography was carried out using 60 Å silica gel (230-400 mesh), as the stationary phase and HPLC was also used to purify the polar fractions.

The size-exclusion column- chromatography was performed using Sephadex LH-20 (Sigma) pre-swollen in the specified solvent before loading onto the column. Unless otherwise specified, a glass column (2.5 x 50 cm) was used and 5 ml volumes were collected.

The preparative TLC was performed using Merck Silica gel 60 PF_{254} on glass plates (20 cm x 20 cm) and with a thickness of 0.5 mm. Also the analytical TLC was conducted on normal-phase Merck Silica gel 60 PF_{254} on precoated aluminium plates. Separated compounds on TLC and PTLC plates were visualized under UV light at (254 and 366 nm), and spraying of the plates where required was carried out using 2% vanillin in H_2SO_4 reagent followed by heating at $110^{o}C$ for 2– 4 mins.

All solvents used in this research were of Analytical, NMR and HPLC grade, those that required distillation were distilled prior to use.

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Materials for the biological analysis includes; DMSO, APO-DIRECT ^{TM} kit, RNase, Propidium Iodide, crystal violet, Phosphate buffer saline (PBS), Dubelco's modified eagles medium (DMEM), Foecal calf serum (FCS), Caspase 9 and 3/7 , trypsin, Tissue culture flasks, 12-well and 96-well plates. Others are Tetrazolium salt (4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) (WST-1) reagent, Topoisomerase I enzyme, 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA) and NucBlue^{TM} Live Cell Stain Hoechst 33342.

3.2 Plant collection, authentication and preparation

3.2.1 Plant collection and Identification

Fresh leaves of *Pleiocarpa pycnantha* was collected in January 2011 at Ikere Ekiti, Ekiti State, South West Nigeria and authenticated by the herbarium curator, Mr. Femi Omotayo of the Department of Plant Sciences, Ekiti State University (EKSU), Ado-Ekiti where a voucher specimen e-Herbarium UHAA 45 was deposited.

3.2.2 Plant preparation

The leaves were washed with distilled water and dried at room temperature in a ventilated room for three weeks based on the nature of the plant materials. They were then milled separately to a fine powder using an electric grinder and 1.0 kg of the powder was stored in a well labeled air-tight container prior to analysis.

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3.3 Plant extraction

1.0 kg of the dried and powdered plant material was extracted for three days with 6L of 95% ethanol. The resulting extract was filtered and the filtrate concentrated with a rotary evaporator at 50^0C to afford 81g of crude *Pleiocarpa pycnantha* extract.

3.4. Phytochemical Screening

1.0 mg samples of the plant extract was dissolved in methanol or dichloromethane for the purpose of being screened for various secondary metabolites using standard procedures

(Wagner and Bladt, 2001; Trease and Evans, 1996) which were identified based on the colour produced after spraying with respective identifying reagents.

3.6 Chromatographic Techniques

3.6.1 Thin Layer Chromatography (TLC)

The analytical TLC was performed on silica gel precoated aluminium plates (GF_{254}) 0.25mm thick while the preparative TLC was performed using Merck Silica gel 60 PF_{254} on glass plates (20 cm x 20 cm) and with a thickness of 0.5 mm.

3.6.1.1 Solvent systems for TLC

The solvent systems used for the analytical TLC are:

Dichloromethane: Methanol (95:5).

Dichloromethane: Methanol (90:10).

Ethyl acetate (100%).

Ethyl acetate: formic acid: glacial acetic acid: water (100:11:11:26).

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3.6.2 Visualization/chromogenic reagents

Analytical TLC plates were treated with the following chromogenic reagents;

(i). 1%w/v Aluminium chloride in ethanol.

This was prepared by dissolving 1g of $AlCl_3$ in 100ml of ethanol and sprayed on analytical plates. Observation was in daylight and under UV.

(ii). Vanillin –Sulphuric acid reagent.

15g of Vanillin was added to 250mL of ethanol and 1.0mL of sulphuric acid was slowly added to make Vanillin reagent.

(iii). Ammonia vapor.

3.7 Column chromatography

A portion of the crude ethanolic extract of *P.pycnantha* (62.22g) was adsorbed on silica gel (Merck, 0.040- 0.063 mm particle size) and fractionated by AGC using the following solvent mixtures: Hexane-EtOAc (5L each, 90%-50%), then 3L (30%), 2L (20%), 2L (100% EtOAc) and 2L (20%) EtOAc-MeOH. Fractions collected (1L each) were analyzed by TLC using DCM–MeOH (99%, 97%, 95% and 90%), then 70% and 99% (Hex-EtOAc) as the mobile phase. Fractions showing the same TLC characteristics were bulked together and concentrated in vacuo. Thirteen fractions coded P1 – P13 were obtained and the results are summarized in Table **3.1**. The general scheme of the fractionation of the crude extract is given in Fig. **3.1**.

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Table 3.1. Fractions grouped from the column.

Tubes	Weight	Fractions
1	0.16g	P1
2	9.78g	P2
3	0.74g	P3
4-6	9.79g	P4
7-9	4.94g	P5
10	0.91g	P6
11-12	2.93g	P7
13-16	3.28g	P8
17-19	3.62g	P9
20-22	5.91g	P10
23-27	5.96g	P11
28-31	1.70g	P12
32	1.62g	P13

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3.7.1 Column chromatography of P12 fraction

Fraction P12 (1.60g) was rechromatographed on a silica gel column using the solvent system: Hex-EtOAc [(5L, 50%), (6L, 60%)] and (0.3L, 100%) $EtOA_{C,}$ 100mL fractions were collected. The solvent systems used to evaluate the fractions were, 95% DCM-MeOH, 50 and

70% Hex-EtOAc. Fractions were bulked together according to their TLC characteristics and

were coded A-H.

The table below summarizes the result and the scheme of the fractionation is presented in Fig. **3.2**.

Table 3.2. Fractions obtained from P12.

Tubes	Weight(mg)	Code
1-2	10.0	A
3-4	230.0	B
5	120.0	C
6-10	240.0	D
11-16	140.0	E
17-21	10.0	F
22-27	40.0	G
28-56	360.0	H

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$P_{12}C$ was washed with 10% EtOAc –Hex and then with 100% DCM to afford a white amorphous powder identified as **C1** (33mg).

The chromatography of $P_{12}D$ (220mg) on a silica gel column using the solvent system 70% EtOAc –Hex (1.5L) for the main column, 95% DCM-MeOH and 50% EtOAc -Hex for TLC afforded five fractions (DI-DV) illustrated in Table **3.3**. The volume collected was 50mL per tube.

Fraction (DIII) when washed as described above was found to be similar to **C1** obtained previously.

The sub fraction DV (20mg) could not be further purified on HPLC due to its limited quantity.

Table **3.3** summarizes the results.

Table 3.3. Showing the sub- fractions collected from $P_{12}D$.

Tubes	Weight(mg)	Code
1-2	5.0	DI
3-4	20.0	DII
5-6	70.0	DIII
7-8	40.0	DIV
9-14	20.0	DV

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Chromatography of $P_{12}E$ (140mg) on a sephadex column using 100% ethanol as the solvent system was then effected with 5mL fractions being collected from the column. The fractions were monitored with 97, 95 and 90% DCM-MeOH on TLC to afford three sub-fractions (EI-EIII). The results are presented in Table **3.4**.

Table 3.4. Showing the sub-fractions from $P_{12}E$ column.

Tubes	Weight(mg)	Code
1-38	10.0	EI
39-46	80.0	EII
47-66	20.0	EIII

Fraction EIII was combined with $P_{12}F$ fractions for further purification.

3.7.2 Column chromatography of (P11) fraction

Fraction P11 (5.60g) was rechromatographed on a silica gel column using the solvent system: Hex-EtOAc [(1L, 80%), (2L, 70%), (2L, 60%), (2L, 50%) and (1L, 40%)]. Fractions of 250 mL were collected, 95% DCM-MeOH, 50 and 70% Hex-EtOAc were used as solvent systems to monitor fractions from the column. The fractions were bulked together according to their TLC characteristics and were coded I-N, Table **3.5** summarizes the result and the scheme of the fractionation is given in Fig. **3.3**.

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Table 3.5. Sub-fractions from P11 column.

Tubes	Weight(g)	Codes
1-4	0.03	I
5-13	2.03	J
14-15	0.22	K
16-17	0.48	L
18-20	0.91	M
21-31	2.20	N

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The fraction $P_{11}M$ (0.91g) precipitated in the tube and the supernatant liquid was carefully decanted, concentrated in vacuo to afford two sub fractions; $P_{11}M$ (0.74g) supernatant and $P_{11}M$ (0.17g) residue.

The sub fraction $P_{11}M$ (0.74g) supernatant was further chromatographed on Sephadex column using 100% ethanol as the solvent. Fractions of 5mL each was collected from the column. Based on the TLC profile of the fractions, four fractions (MI-MIV) were obtained and the result is hereby presented in Table **3.6**.

Table 3.6. Sub-fractions from P11M.

Tubes	Weight(mg)	Codes
1-7	5.0	MI
8-24	30.0	MII
25-39	540.0	MIII
40-62	110.0	MIV

$P_{11}L$ (0.48g) and $P_{11}M$ (0.17g) residues were found to be similar on TLC and therefore they were rechromatographed together on silica gel column using EtOAc -Hex (25%, 1L and 30%, 5L). From the TLC analysis of fractions, four fractions coded LI-LIV were obtained.

Table **3.7** summarizes the result.

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Table 3.7. Sub-fractions from the combined extracts $P_{11}L$ and M.

Tubes	Weight(mg)	Codes
1-8	15.0	LI
9-18	150.0	LII
19-24	20.0	LIII
25-50	30.0	LIV

Fractions MIII (supernatant) 0.54mg, P11N (2.20g) and P12 E(0.02g) were pooled together and renamed as $P_{11}O$ based on their TLC profile and rechromatographed on a silica gel column using the solvent system EtOAc-Hex(25%, 18L), EtOAC(100%, 0.8L) and EtOAc -MeOH(50%,

0.8L). Fractions of 100mL were collected in tubes numbered 1-147 while 250mL volumes were collected in tubes numbered 148-168. Mobile phases of 90% DCM-MeOH, 95% DCM-MeOH and 50% EtOAc-Hex (double run) were used to monitor fractions on aluminium precoated plates, based on the TLC profile, fractions were pooled together to afford twenty one fractions. The result is summarized in Table **3.8**.

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Table 3.8. Sub-fractions obtained from combined extracts from $P_{11}O$.

Tubes	Weight(mg)	Codes
1-6	1.1	I
7-13	1.0	II
14-16	2.0	III
17-32	110.0	IV
33-45	75.0	V
46-52	30.0	VI
53-78	0.5	VII
79-91	2.0	VIII
92-97	2.0	IX
98-102	10.0	X
103-113	5.0	XI
114-120	4.0	XII
121-129	5.0	XIII
130-136	20.0	XIV
137-146	30.0	XV
147-153	20.0	XVI
154-168	80.0	XVII
169-170	10.0	XVIII
171	130.0	XIX
172-173	90.0	XX
174-179	40.0	XXI
180-182	50.0	XXII

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Fractions VIII-XXII were washed with DCM-Hex (50%) to afford precipitates while the sub-fractions XIX (130mg) and XX (90mg) were further chromatographed repeatedly on a sephadex L-H 20 column using DCM-MeOH (95%) as solvent and then on HPLC using 80%

(MeOH-H_2O) with UV detector to afford compounds **C2** (5.5mg) and **C3** (7.3mg).

The scheme of the fractionation of $P_{11}O$ sub fraction is given in Fig. **3.4**.

3.7.3 Column chromatography of P9 fraction

P9 (3.20g) was placed on silical gel column using the solvent systems EtOAc-Hex (20%, 14L) and100% EtOAc (1L). Fractions of 0.1L were collected in tubes 1-5 while fractions of 0.2L were collected in tubes 6-74. These were combined based on their TLC profiles and coded Q1-13. Table **3.9** summarizes the results, while Fig. **3.5** gives the fractionation scheme.

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Table 3.9. Sub-fractions from P9.

Tubes	Weight(mg)	Codes
1-6	30.0	Q1
7-12	10.0	Q2
13-17	120.0	Q3
18-21	310.0	Q4
22-27	590.0	Q5
28-31	300.0	Q6
32-37	470.0	Q7
38-44	270.0	Q8
45-50	100.0	Q9
51-55	80.0	Q10
56-61	90.0	Q11
62-68	70.0	Q12
69-74	380.0	Q13

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Fractions Q3-Q8 was washed with 100% DCM to yield some precipitates which were not purified further. Q4 (310mg) was washed in a mixture of 10% Hexane/EtOAc to give compound **C1 (100mg)** which has already been isolated before.

3.7.4 Column chromatography of P7 fraction

Fraction P7 (2.53g) was chromatographed on a Silical gel column using the solvent systems EtOAc-Hex [(10%, 8L), (20%, 4L)] and 100% EtOAc (1L). 100mL was collected in tubes 1-30 while 200mL was collected in tubes 31-64. Fractions were combined based on their TLC profiles and coded R1-R11. Table **3.10** summarizes the results.

Table 3.10. Sub-fractions from P7.

Tubes	Weight(mg)	Codes
1-6	20.0	R1
7-12	30.0	R2
13-20	60.0	R3
21-25	60.0	R4
26-28	130.0	R5
29-34	120.0	R6
35-40	220.0	R7
41-47	220.0	R8
48-51	170.0	R9
52-60	710.0	R10
61-64	180.0	R11

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Fraction P7 (2.53g) was chromatographed on a silical gel column to afford sub-fractions R1-11 R8 and R10 were washed with 10% EtOAc-Hex to afford a white precipitate which was fairly

clean. R8 was further separated on Prep TLC to give compounds **C1** (15mg) (already isolated) and **C4** (6.1mg) as an inseparable mixture of two triterpenes, the structure of which was not elucidated in this study. The fractionation of P7 is further presented in Fig. **3.6**.

Based on their ^{1}H-NMR spectra, R6 and R7 (340mg) was combined and renamed as R_7A and further purified on a Sephadex L-H 20 column using 100% ethanol as the solvent with fractions of 5mL being collected. TLC was used to pool the various fractions together to afford a total of four fractions (R_7AI-IV). Table **3.11** summarizes the results.

Table 3.11. Sub-fractions from R_7A.

Tubes	Weight(mg)	Codes
1-13	10.0	I
14-28	280.0	II
29-36	10.0	III
37-52	5.0	IV

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R_7A (280mg) could not be separated further by chromatography. Fraction **R_7A (**III) was fairly clean but too little to be further purified. The sub-fraction **R_7A** (II) were then purified on a Sephadex L-H 20 column using 100% ethanol as the solvent with fractions of 5mL being collected per tube. TLC was used to pool the fractions together to afford a total of five fractions (IIa-IIe). Table **3.12** summarizes the results.

Table 3.12 .Sub-fractions from R_7A.

Tubes	Weight(mg)	Codes
1-17	30.0	II a
18-22	170.0	II b
23-24	10.0	II c
25-34	50.0	II d
35-44	5.0	II e

Fraction R10 (710mg)was chromatographed on a Sephadex L-H 20 column using 100% ethanol as the solvent with fractions of 5mL being collected per tube . TLC was used to pool the various fractions together to afford a total of six fractions (I-IV). Table **3.13** summarizes the result.

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Table 3.13 Sub-fractions from R10.

Tubes	Weight(mg)	Codes
1-17	30.0	I
18-31	140.0	II
32-48	310.0	III
47-98	70.0	IV
99-112	30.0	V
113-160	87.0	IV

3.7.5 Column chromatography of (P4) fraction

Fractions P4 (6.00g) was chromatographed on a Silical gel column using the solvent system EtOAc-Hex [(2%, 500 mL), (4%, 2L), (6%, 2L), (8%, 2L), (10%, 2L), (15%, 2L), (20%, 2L), (30%, 1L)] and 100% EtOAc (500 mL). Fractions of 100 mL were collected per tube and these were combined based on their TLC profiles to afford fractions T1-T10, Table **3.14** summarizes the result.

Table 3.14 Sub-fractions from P4.

Tubes	Weight(g)	Codes
1-6	0.03	T1
7-11	0.03	T2
12-16	0.06	T3
17-18	0.06	T4
19-25	2.01	T5
26-30	3.02	T6
31-37	0.52	T7
38-40	0.04	T8
41-45	0.17	T9
46-72	0.05	T10

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Fractions T6 needle- like crystals were washed with 100% Hexane to afford a **novel** compound **C5 (0.97g)**. Several attempts to purify T5 and T7 on sephadex, silica column and silver nitrate

impregnated silica were not successful. The scheme of fractionation of P4 is presented in Fig. **3.7**.

3.8 Synthesis of compound C6

33 mg of **C5** was dissolved in methanol to which methanolic –KOH was added and the mixture was refluxed for 3hours then cooled and acidified. Extraction of the mixture with EtOAc afforded a residue which was chromatographed using 70% Hex – EtOAc as eluent to afford compound **C6** (20 mg)**.**

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3.9 Scheme of extraction

A general overview of the extraction protocol for the dried plant material is presented in scheme **3.9.**

3.9.1 The crude fractionation

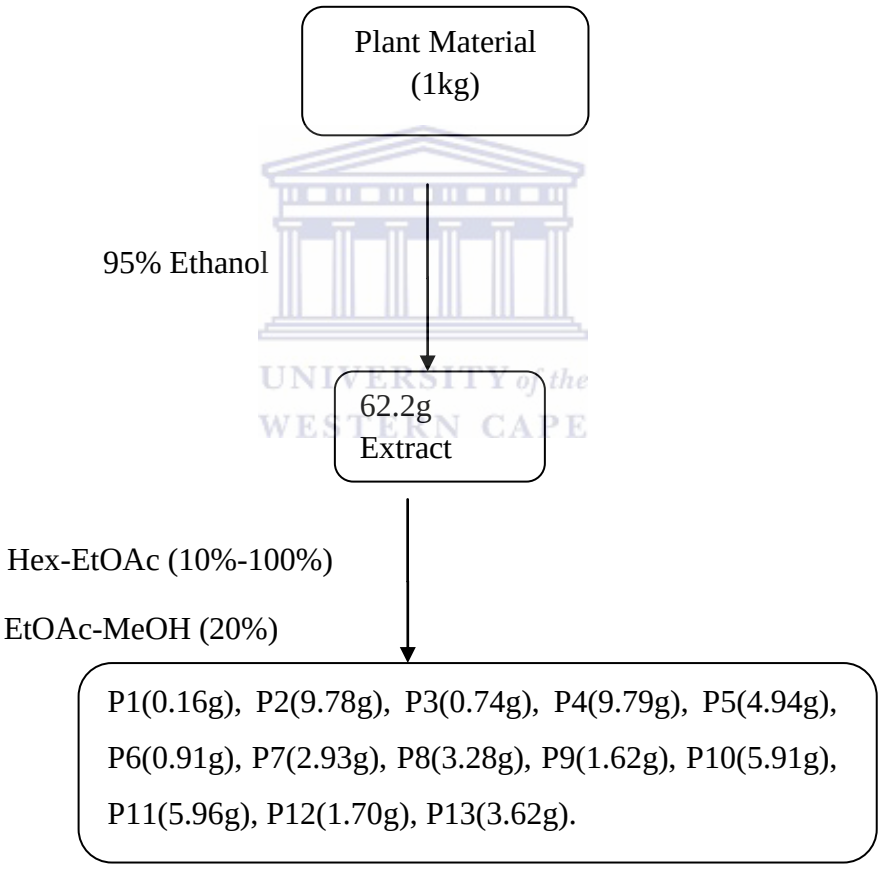

Figure 3.1. Generalized scheme of extraction for total extract.

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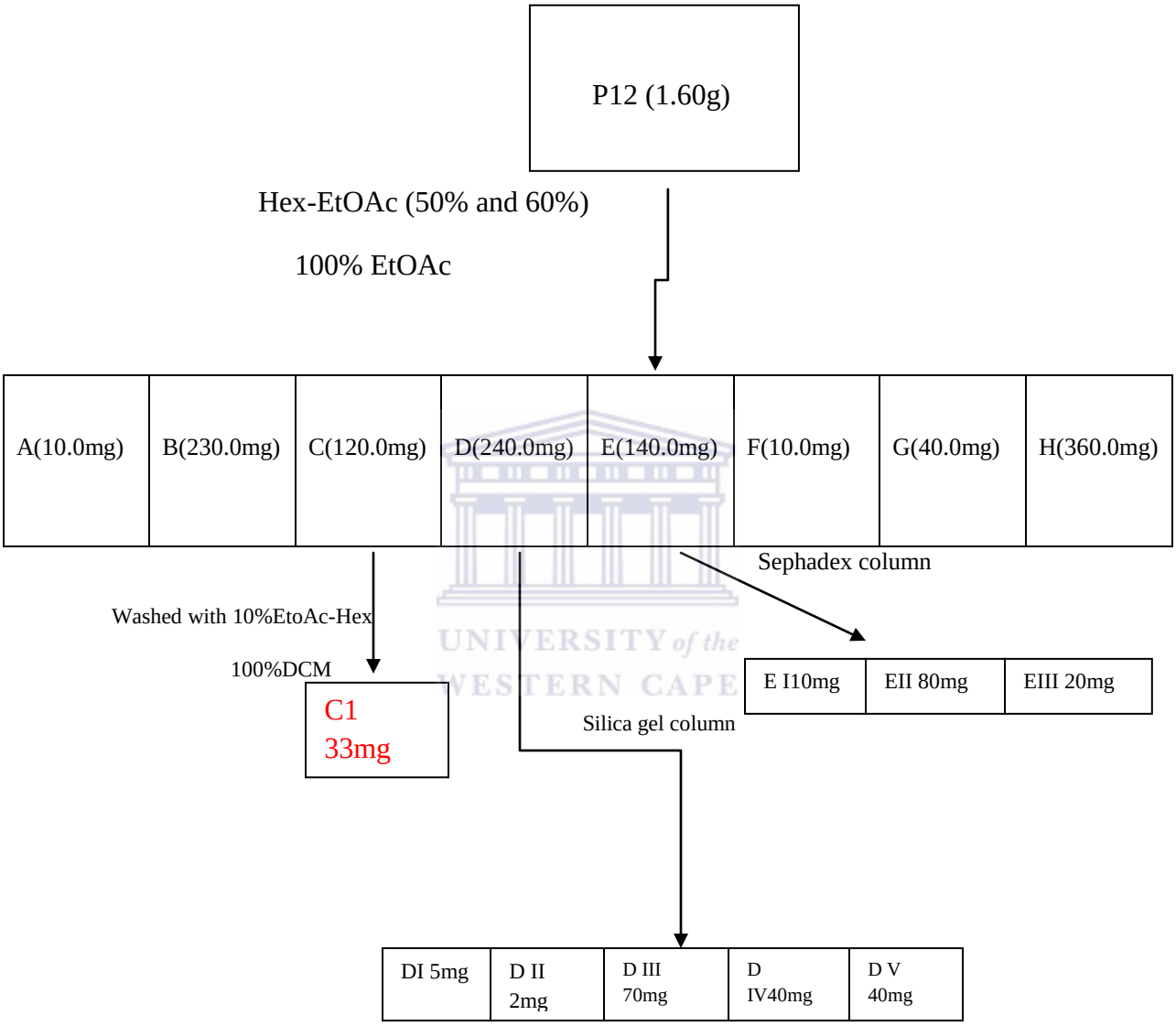

Figure 3.2. Isolation scheme for fraction P12.

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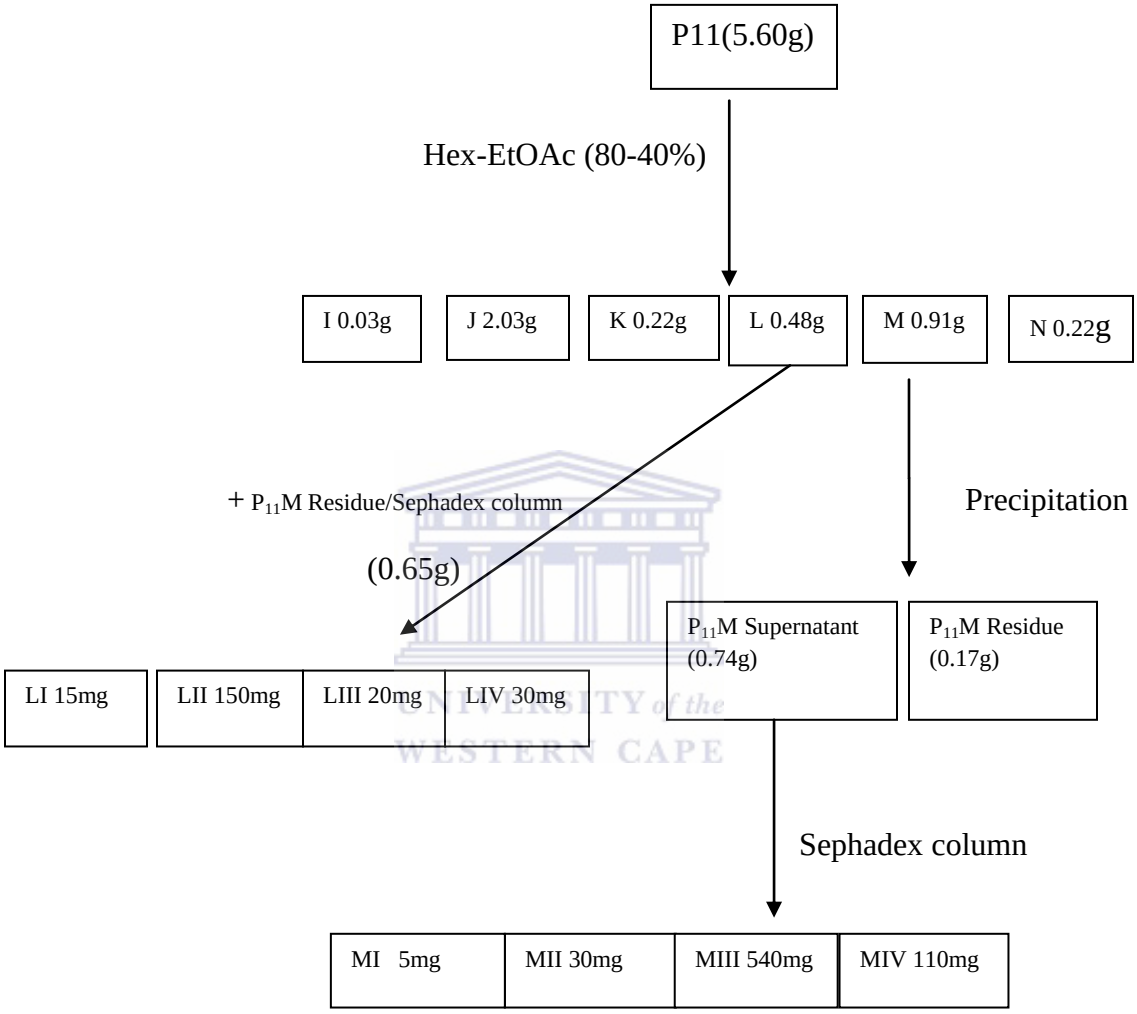

Figure 3.3. Isolation scheme for fraction P11.

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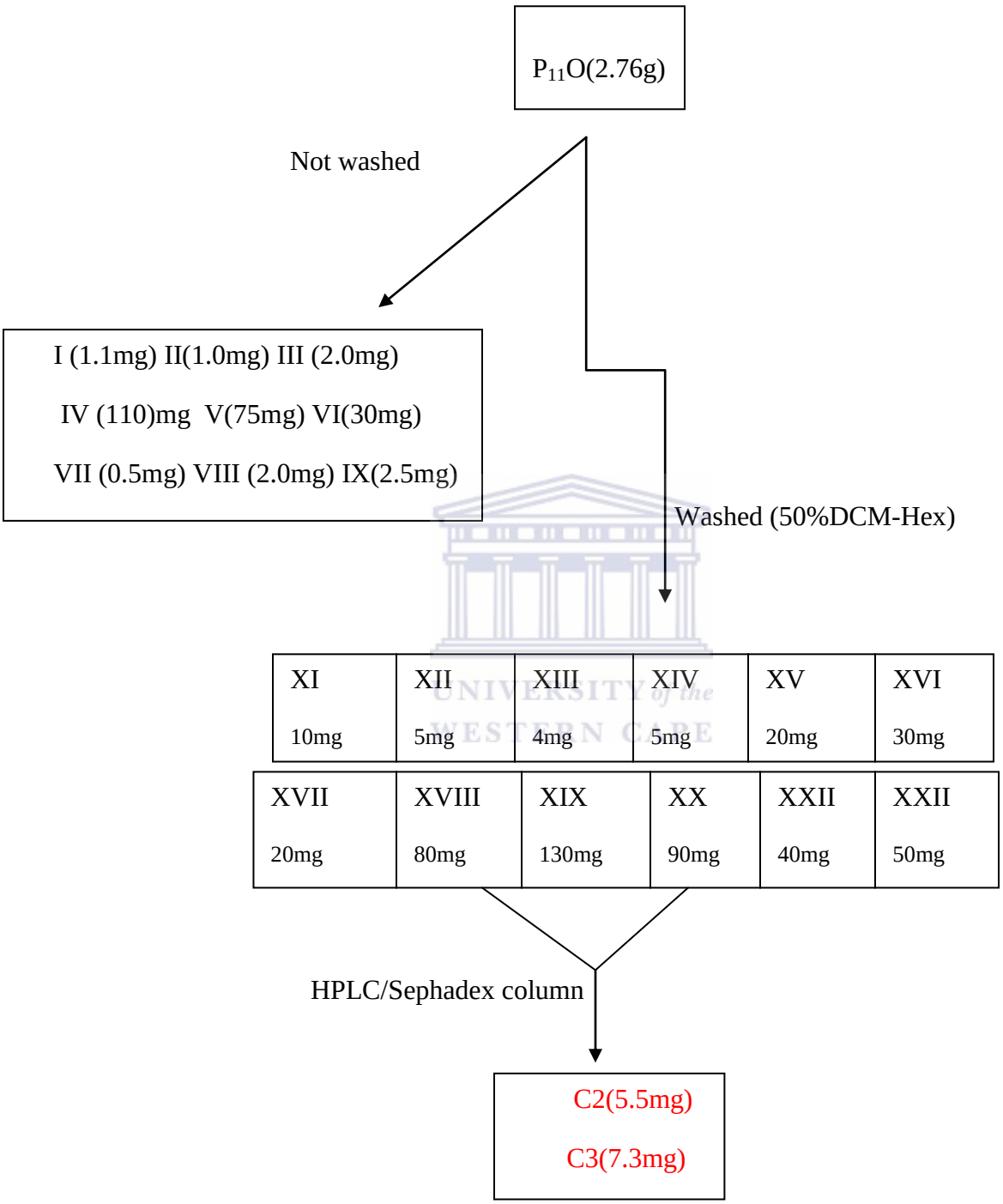

Figure 3.4. Isolation scheme for fraction $P_{11}O$.

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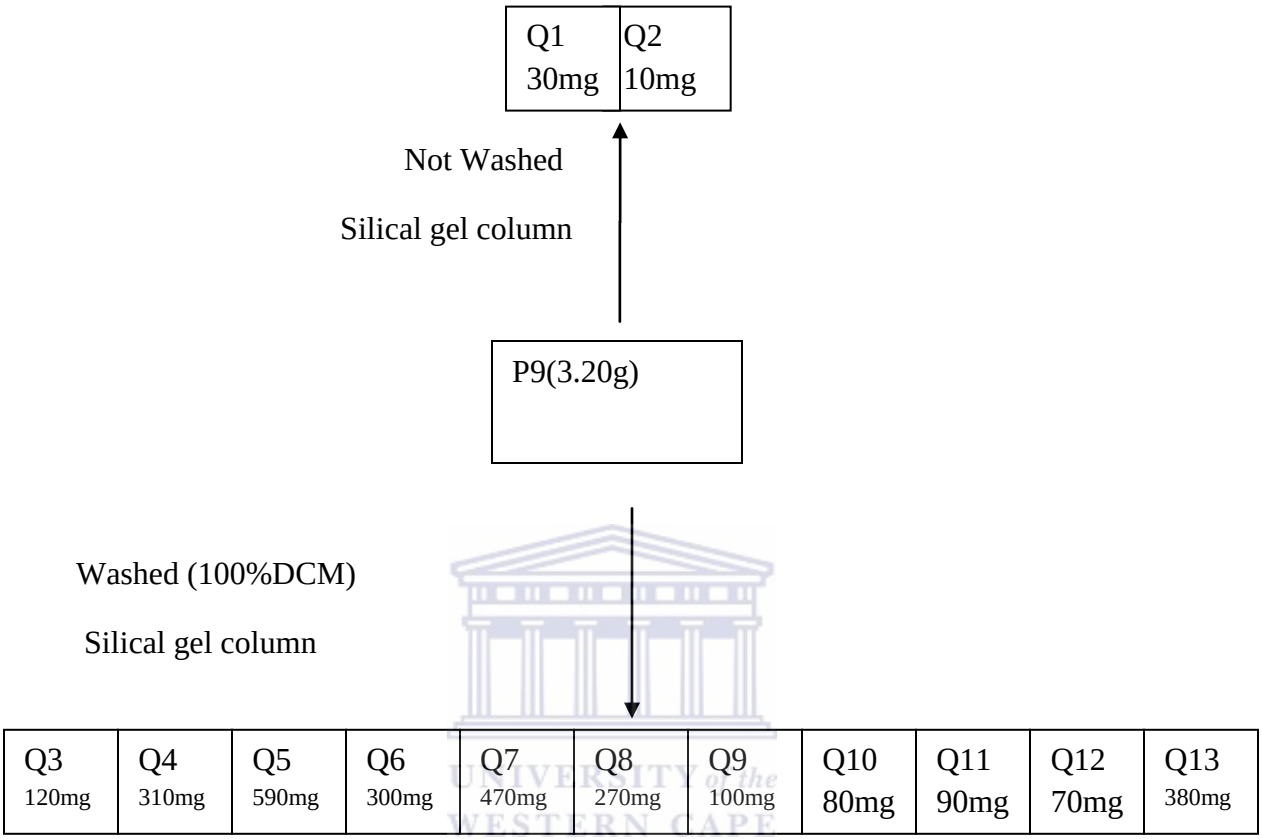

Figure 3.5. Isolation scheme for fraction P9.

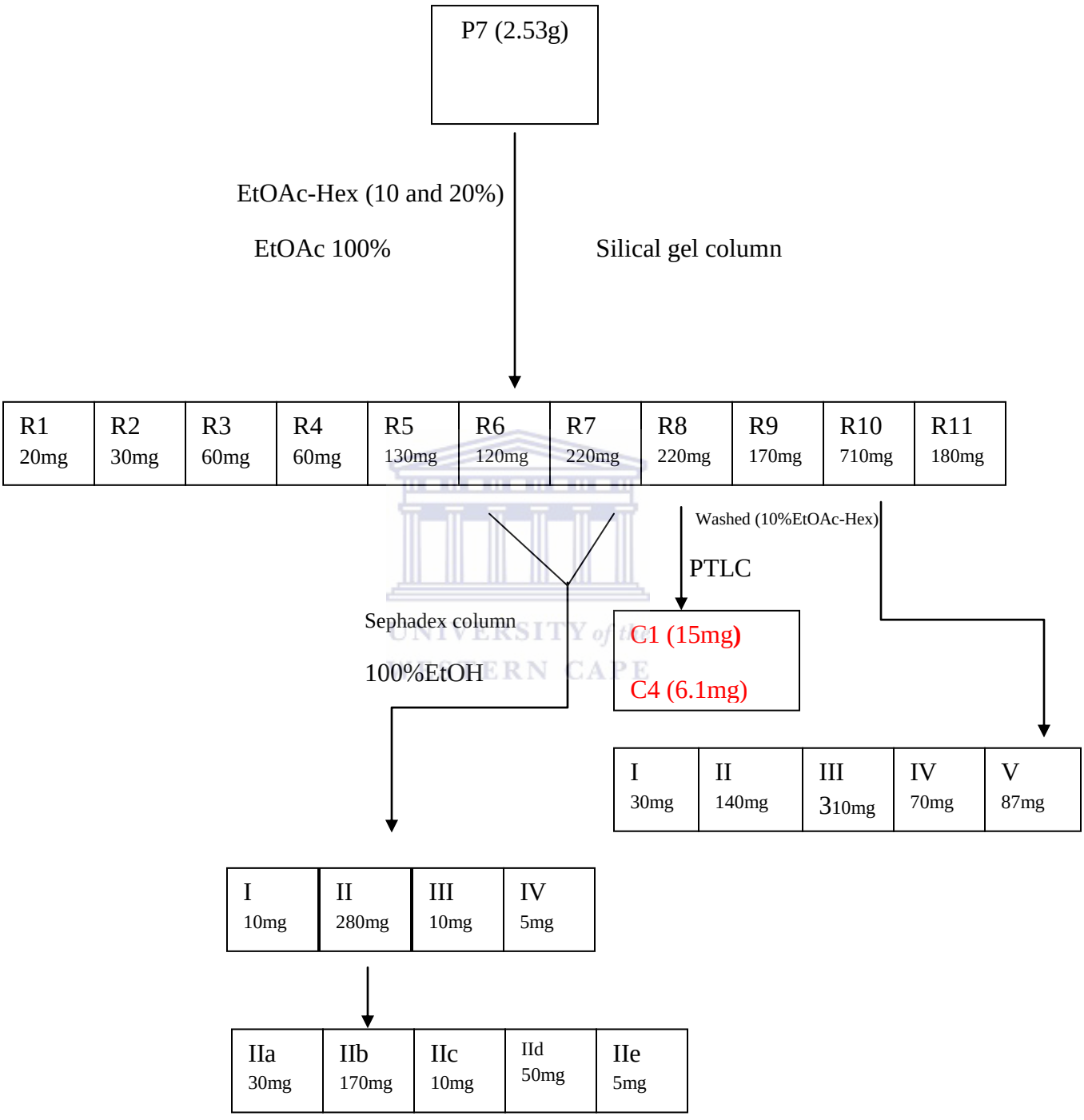

Figure 3.6. Isolation scheme for fraction P7.

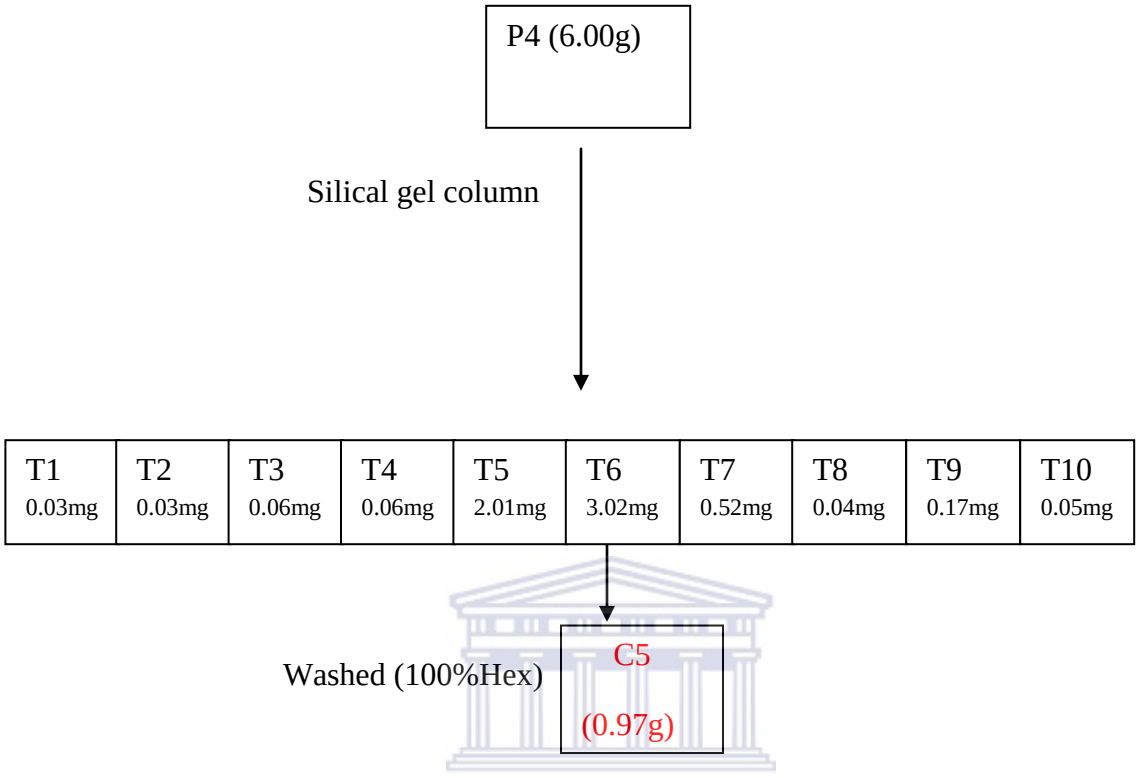

Figure 3.7. Isolation scheme for fraction P4.

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3.10 BIOLOGICAL EVALUATIONS CONDUCTED

3.10.1 In vitro culturing of mammalian cells

3.10.1.1 Seeding of cells

A vial of the respective frozen cells was taken from the -150^{o}C freezer and rapidly thawed in a water bath set at 37^{o}C by submerging the vial in water. The cells were transferred into tissue culture flasks containing the pre-warmed media DMEM and incubated at 37^{o}C in a 5% CO_2 humidified incubator.

3.10.1.2 Trypsinization and cryo-preservation of cell lines

Once the cells approached 70-90% confluency, they were washed with 1X PBS and incubated for 5-10 minutes in 5 ml of 0.125% trypsin if cells were cultured in T-25 cm^2 flasks or in 10 ml of the 0.125% trypsin if 75 cm^2 flasks were used. Trypsinization was stopped by the addition of 2-3 ml of culture medium. The detached cells were recovered by aspiration and transferred into a centrifuge tube which was centrifuged for 3 minutes at 200 xg in a bench top centrifuge.

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The supernatant was discarded and the cell pellet resuspended in a solution of 90% media and 10% DMSO, a small aliquot of cells (100-200 µl) was removed and a cell viability count performed. If the cell viability was in excess of 90%, the cells were frozen and if not, the cells were discarded and a new culture grown.

The cells were dispensed in 1ml aliquots in labeled cryo vials at a concentration of 2x10^6 cells per ml and frozen immediately at -150^{o}C. A quality control was set up by removing another aliquot of 50-100 µl from the cell solution to be frozen to confirm whether they were

contaminated or not. This would be done for 24-48 hours and if they were found to be contaminated, the vials were withdrawn and discarded.

3.10.1.3 Cell counting

Cell count was performed using a Countess^{TM} automated cell counter (Invitrogen).

10 µL of cell solution already tripsinized and in fresh media was withdrawn from stock into an eppendorf tube and 10 µL of trypan blue added. The two solutions were thoroughly mixed to homogenize them efficiently after which 10 µl of the mixture was transferred onto a cell counter slide to calculate the percentage viability of the live cells using trypan blue staining solution. Visually, the dead cells appeared dark in colour, while the live cells had bright centers.

3.10.1.4 Morphological evaluation of treated and untreated cells

Cell lines were cultured in 6well plates to 90% confluency and induced to undergo apopotosis with various concentrations of the isolated compounds ranging from 3.125-50 µg/ml with both negative and positive controls as monitoring vectors. The cells were incubated for 24 hours at 37^{o}C in a CO_2 incubator. The cells were inspected under a Nikon inverted light microscope using a 10X and 20X objective and photographs were taken using a Leica EC3 digital camera.

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3.10.2 Tetrazolium salt WST-1-(4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1, z3-benzene disulfonate) Assay

Cells were seeded at a concentration of 5×10^4 cells/well in 100 µl culture medium containing Penicillin/FBS(1g/ml) and various amounts of extract, fractions and compounds were added at different concentrations into microplates (96 wells - flat bottomed tissue culture grade).

The cells were incubated for 24 hours at 37^{o}C in a CO_2 incubator. A volume of 10 µl of cell proliferation reagent WST-1 was added and incubated for 4 h at 37°C and 5% CO_2.

Mixtures were thoroughly shaken for about 5minutes and the absorbance measured against a background control as blank using a micro- plate (ELISA) reader at 450 and 750 nm.

% Viability = OD of treated well –blank/OD of untreated control well –blank

IC_{50} values were calculated using Graphpad prism software. Triplicate experiments were conducted and the results expressed as mean±SD.

3.10.3 The APOPercentage^{TM} apoptosis assay

This assay was done as described by Masalu et al., (2010). Briefly, the cells were cultured to 90% confluence in 12 well plates at a density of 1x10^{5}cells/ml and incubated for 24 hours at 37^{o}C in a 5% CO_2 incubator. The cells were treated with various concentrations of the test fraction or isolated material at concentrations varying from 3.125-50 µg/ml while the positive control was 50µM Camptothecin. Floating cells were transferred to 15ml centrifuge tubes while adherent cells were tripsinized and added to the tubes containing floating cells.

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The cells were harvested by centrifugation and washed twice with 1X PBS and then stained with Apopercentage^{TM} dye (1:160) in complete media for 30 minutes and incubated at 37^{o}C in a 5% CO_2 incubator. The cells were washed twice with 1X PBS again and the pellet resuspended in 400 µl 1X PBS and analyzed on an Attune flow cytometer (Applied Biosystems) apparatus with a 488nm Argon laser as the light source. Apopercentage^{TM} dye fluorescence was measured using the FL3 channel and the results are expressed as percentage cell death against concentration.

3.10.4 Determination of ROS

To evaluate the intracellular ROS generation, the molecular probe 5-(and 6)-chloromethyl-2´, 7´-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA) was employed using the method described by Wei et al., (2000) with slight modifications. The cells were cultured in 12 and 6well plates and treated with 12.5 or 25 µg/ml of compounds on HeLa, MCF-7 and HT-29 cells with 50 µM of Camptothecin being used as a positive control in a 5% CO_2 humidified incubator at 37^{o}C for 24 hours. The adherent cells were transferred to 15 ml tubes and washed twice with PBS after which they were spun at 300 xg for 5 minutes. The cells were stained in 7.5 µM of the probe prepared in DMSO and reconstituted in PBS for 30minutes in a 5% CO_2 humidified incubator at 37^{o}C and analyzed on a flow cytometer (BD Calibur) instrument equipped with a 488nm argon laser and the cell fluorescence (DCF) measured using the FL-1 channel.

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3.10.5 ROS determination by fluorescence imaging

To further investigate apoptotic effects of isolated compounds, the cells were treated with 12.5 µg/ml of compounds for 4 hours and stained the nucleus with NucBlue^{TM} live cell staining dye for 20 mins and then with 1 µM CM-H_2DCFDA molecular ROS probe for another 30 mins and viewed under Axio vision fluorescence microscope.

3.10.6 Measurement of Caspase 3/7activity

Caspases 3/7 were measured using the assay kits from Promega (Promega, Corp., Madison, WI) according to the manufacturer's instructions and the method described by Thaler et al., (2013) with slight modifications. In brief, after treatment, cells were lysed and the substrates cleaved by caspases 3/7 were measured using the luminescent signal obtained with a 96 well Luminometer

(Glomax, Promega) and the assays were done in triplicate and repeated as and when the need arose.

3.10.7 Measurement of Caspase 9 activity

Caspases 3/7 were measured using the assay kits from Promega (Promega, Corp., Madison, WI) according to the manufacturer's instructions and the method described by Thaler et al., (2013) with slight modifications. Cells were seeded in 96well tissue culture plates at a concentration of $5x10^4$ml per well, after treatments cells were lysed and the substrates cleaved by the caspases 9 were measured using the luminescent signal obtained from Luminometer (Glomax, Promega). The assay was done in triplicates.

3.10.8 Topoisomerase inhibition determination

Topoisomerase I assay is based on the inhibition of the relaxation of supercoiled circular DNA by topoisomerase I (Osheroff et al., 1983).

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1 U of topoisomerase I will relax 0.5 µg of supercoiled pBR322 when incubated in assay buffer in a total reaction volume of 30µl. A master mix containing ddH_2O, 1X assay buffer [20mM Tris. HCl (pH 7.5), 200mM NaCl, 0.25 mM EDTA & 5% glycerol], 0.5 µg supercoiled DNA, 1U topoisomerase I enzyme and the test fractions was prepared. 5 µl of the stock solutions of test fractions was added to 30 µl of the total reaction volume. Preparation was done on ice before incubating the mixtures at 37^0C for 2 h, after which it was terminated with 6 µl 6x stop buffer (3% SDS, 60 mM EDTA, 50% glycerol, 0.25% bromophenol blue). The reaction products were determined by electrophoresis on 1% agarose gel in Tris-acetate-EDTA (TAE) running buffer at 6.5 V/cm for 2 h. The gels were stained with 0.5 µg/ml ethidium bromide for 30 mins. Gels were directly scanned with an image analyzer (Biometra, Germany) for visualization.

3.10.9 Cell cycle analysis

The effect of compounds on cell cycle phase distribution was analyzed using the method described by Shao et al., (2011) with slight modifications.

2×10^5 of cells were seeded in each well of a 6-well plate and treated with 5 compounds; C**2** (12.5 µg/ml), C**3** (12.5 µg/ml), **C4** (12.5 µg/ml), **C5** (12.5 µg/ml) and **C6** (6.25 µg/ml) .

Untreated cells were also included as controls. After incubation for 24 h cells were trypsinized and washed with PBS, centrifuged and resuspended repeatedly into single cells prior fixation with 70% ethanol. Fixed cells were kept at -20°C for 48 h and later washed with 1X PBS twice and the supernatant was discarded. Cell pellets were resuspended in 200 µl of 1X PBS and 500 µl of PI master mix and the cell suspension incubated for 15 minutes on ice in the dark. FACS Calibur (BDBiosciences, USA) and Cell Quest Pro software (BD Biosciences, USA) was used to determine the cell cycle distribution.

A total of 10,000 of cells were acquired each time using FACS Calibur flowcytometer. Flowcytometric data were analyzed using Modfit software and displayed in histogram cell count versus DNA content.

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CHAPTER 4

The rundown of the isolation procedure is given in this chapter. It further explained in detail the identification of the isolated compounds with the use of spectroscopic, chromatographic and X-ray crystallographic techniques in their structural elucidation. The structures of isolated compounds and similar compounds from literatures are shown in Fig. 4.2 – 5.0 while their NMR spectra are given in Tables 4.2 – 4.6.

4.1 STRUCTURAL ELUCIDATION OF ISOLATED COMPOUNDS

4.1.1 Summary of isolation procedure

The ground air-dried leaves (1 kg) were soaked in 95% ethanol for three days and the extract filtered and concentrated in vacuo to yield 81.00 g of the crude extract. The crude extract was screened separately phytochemically using GC-MS coupled with a library search within the GC-MS instrument's memory as well as other standard procedures (Wagner and Bladt, 2001).

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For the fractionation of the extract, 62.22 g of the ethanolic extract was adsorbed on silica gel (Merck, 0.040-0.063mm particle size) and chromatographed using column chromatography coupled with a gradient elution of a Hexane-EtOAc mixture as mobile phase.

Fractions showing similar TLC characteristics were bulked together and concentrated in vacuo; giving rise to thirteen fractions coded **P 1** – **P 13**. Fractions **P1 - 13** were screened for cytotoxicity using the WST-1 assay and were further purified either based on bioassay or NMR results. Fractions **P4**, **P7**, **P8**, **P9** and **P12** displayed significant cytotoxicity and were chosen for further purification while **P11** was chosen because of its proapoptotic effect and the unusual signals in the NMR spectra to evaluate the compounds present for biological activity.

P9 was further chromatographed on a silical gel column using gradient elution with Hexane-EtOAC mixture to afford **Q1-13**. In this case fraction **Q4** (310 mg) was washed in a mixture of 10% Hexane/EtOAc several times to give compound **C1 (100 mg)**.

P11 (5.60 g) was adsorbed on a silical gel column using a Hex-EtOAc mixture 80-40% as eluting solvent to afford sub fractions P_{11}**I-N**. **P_{11}M** (0.91 g) precipitated in the tube and was carefully decanted to give **P_{11}Ma** (0.74 g) supernatant and **P_{11}Mb** (0.17 g) residue. The supernatant fraction was further chromatographed on a sephadex LH-20 column using absolute EtOH as eluting solvent to give sub fractions (**P_{11}MI-MIV**). The fractions **P_{11}L** (0.48 g) and **P_{11}Mb** (0.17 g) were found to be similar by TLC and thus pooled together to be run on a separate silical gel column using EtOAc-Hex 25% and 30% as solvent system to produce sub-fractions **P_{11}LI-LIV**.

Fractions **P_{11}MIII** (supernatant) 0.54mg, **P_{11}N** (2.20g) and P_{12} **E**(0.02 g) were pooled together and renamed as **P_{11}O** based on TLC profile and separated on silica gel column using the solvent system EtOAc –Hexane (25%, 100%), and EtOAc -MeOH (50%,), giving rise to twenty two fractions coded as sub-fraction **I-XXII**. Sub-fractions **XIX** (130 mg) and **XX** (90 mg) were further chromatographed repeatedly on sephadex L-H 20 using DCM-MeOH (95%) as solvent and HPLC (MeOH-H_2O) 80% with UV detector to afford compound **C2** (5.5 mg) and **C3** (7.3 mg) which was initially pure, but later isomerized into a mixture of **C2** and **C3**.

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Fraction **P7** (2.53g) was chromatographed on silical gel to afford sub-fractions **R1-R10**.

R10 was further separated on Prep TLC to give compound **C4** which is a mixture of two triterpenes but the structure was not elucidated in this study.

Fraction **P4** (6.00g) was adsorbed on a silical gel column using a gradient elution of Hexane-EtOAc 98%-70% and 100% EtOAc as eluent solvent system to afford sub-fractions **T1-T10**, sub fraction

T6 crystallized overnight to form needle-like crystals which were carefully decanted and recrystallized in Hexane-DCM mixture to produce compound **C5** (0.97g).

Compound **C6** was synthesized by refluxing 33 mg of **C5** dissolved in methanolic - KOH for 3hours to afford **Z1** mixture. The cooled mixture was acidified and extracted into ethyl acetate and purified on a silical gel column to give **C6** (20.0mg, 60.6%).

Further detailed isolation procedures were discussed and illustrated in Chapter **3**.

It should be noted that the plant parts used in this study were air-dried to prevent the decomposition of thermo labile constituents which might occur at high temperatures.

Ethanol was chosen as solvent to mimic the preparation of herbal extracts as used in South -West Nigeria and the percentage (95%) was chosen to represent organic and aqueous extracts.

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Table 4.2. Qualitative phytochemical analysis of *Pleiocarpa pycnantha.*

Test	Results
Alkaloids	
Draggendorf	Absent
Wagner	Absent
Hager	Absent
Terpenoids	Present
Saponins	Present
Flavonoids	
$FeCl_3$	Absent
$AlCl_3$	Absent
Lead Acetate	Absent

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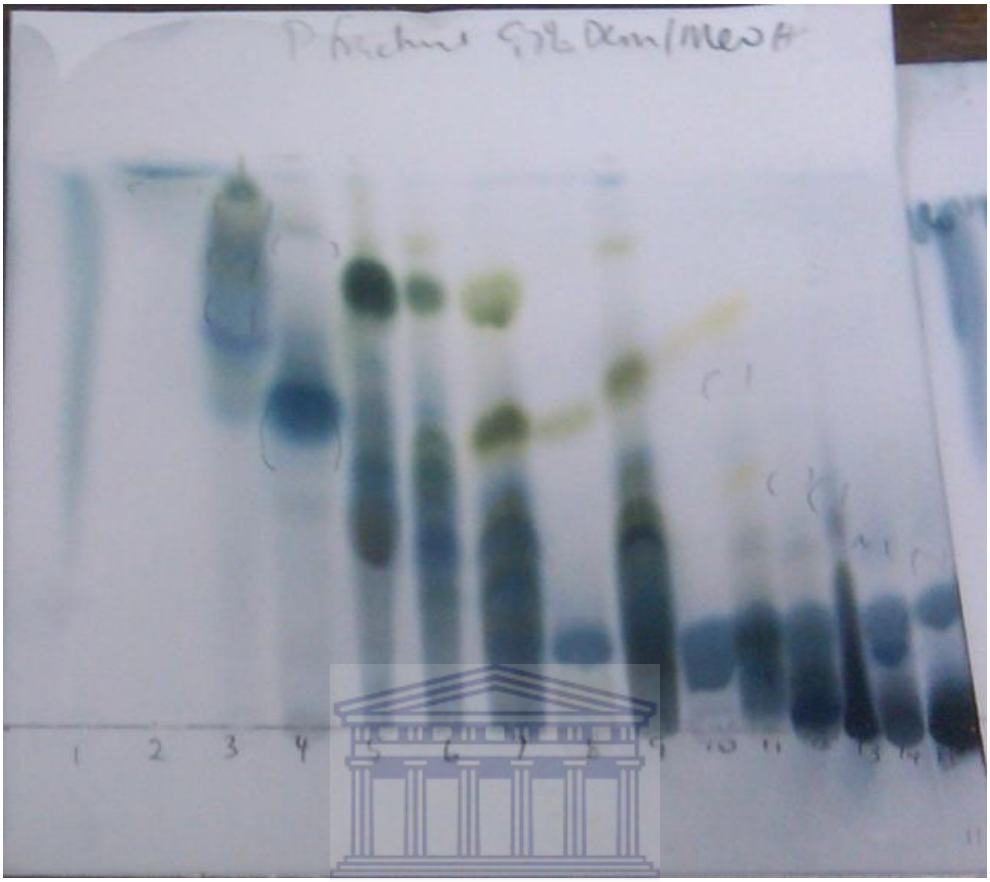

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Figure 4.1. Showing TLC characteristics of fractions P1 to P15 (now P1-P13) from *P. pycnantha* leaves when sprayed with vanillin-sulphuric acid reagent.

The preliminary phytochemical screening of the ethanolic extract obtained from *P. pycnantha* leaves as illustrated in Table **4.1** and Figure **4.1** indicated that the major component of the leaves are Terpenoids which have been reported to be useful anticancer agents (Neto, 2007).

4.2 Physico-chemical/spectroscopic parameters of isolated compounds

Any minor discrepancies in δ and *J* values are due to the different solvent system used in determining the NMR spectra.

Compound **C1** (Ursolic acid)

White amorphous powder (MeOH)

Melting point: 273-274^{o}C

HREIMS m/z (rel.int.): 456.3856 $[M+H]^{+}$ (calculated 456.7003)

Molecular formula $C_{30}H_{48}O_3$

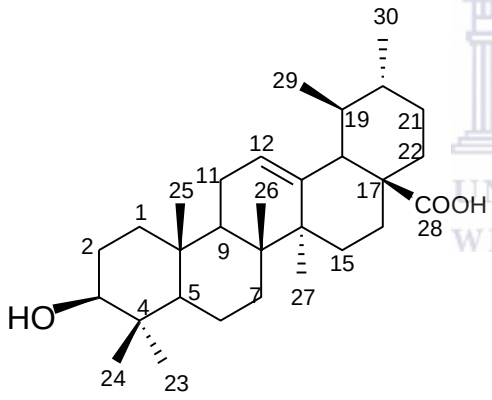

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Figure 4. 2. Molecular structure of compound C1.

The HREIMS of compound 1(**C1**) gave the ion $[M+H]^{+}$ at 456. 3856 which has the molecular formula $C_{30}H_{48}O_3$ (calculated 456.7003) and assigned for ursolic acid as deduced from an analysis of ^{1}H, ^{13}C NMR and DEPT spectra. The proton spectrum indicated the presence of seven methyl groups in the molecule with characteristic peaks typically at high field and supported by the DEPT spectrum (Table **4.2**). One acid carbonyl signal appeared at δ 181.6 in the ^{13}C NMR

spectrum. The signals at δ 139.6 and 126.9 are assigned to the two olefinic carbons C-13 and C-12 respectively based on the DEPT spectrum.

The ^{1}H NMR spectrum further showed the presence of an oxygenated methine signal at δ 3.13 (1H, dd, *J* = 6.0 and 2.0 Hz, H-3) and a trisubstituted olefinic signal at δ 5.22 (1H, br s, H-12).

On this basis, compound **C1** was identified as ursolic acid, which had similar spectra to those reported by Murphy et al., (2003) as well as Hamzah and Lajis, (1998) and is illustrated in Table **4.2**.

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Table 4.2. ^{1}H and ^{13}C NMR data of C1 and Ursolic acid in CD_3OD (δ values, *J* in parenthesis in Hz).

Position	Compound C1		Ursolic acid	
	$\delta^{13}C$	$\delta^{1}H$	$\delta^{13}C$	$\delta^{1}H$
1	39.8		39.8	
2	27.8		27.8	
3	79.7	3.13dd 1H (6.0,2.0)	79.6	3.14m 1H
4	39.9		39.9	
5	56.7		56.7	
6	19.4		19.4	
7	34.3		34.3	
8	40.7		40.7	
9	47.6		47.6	
10	38.1		38.1	
11	24.3		24.3	
12	126.9	5.23m 1H	126.8	5.21m 1H
13	139.6		139.6	
14	42.8		42.8	
15	29.2		29.2	
16	25.3		25.3	
17	48.0*		47.6	
18	54.3	2.18d 1H (11.2)	54.3	2.20d 1H (11.3)
19	40.4		40.4	
20	40.4		40.4	
21	31.7		31.7	
22	38.1	1.54m	38.1	1.55m
23	28.7	1.12s 3H	28.7	1.10 s 3H
24	16.0	0.94 s 3H	16.0	0.93 s 3H
25	16.4	0.78 s 3H	16.3	0.76 s 3H
26	17.6	0.90 s 3H	17.6	0.95 s 3H
27	24.0	0.98 s 3H	24.0	0.96 s 3H
28	181.6		181.6	
29	17.8	0.87 d 3H	17.8	0.87 d 3H
30	21.6	0.83 d 3H	21.5	0.83 d 3H

Ursolic acid ^{1}H and ^{13}C in CD_3OD (Hamzah and Lajis, 1998)

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Compound **C2** (27- p-*E*-coumaroloxy ursolic acid)

An off-white amorphous powder (MeOH)

Melting point: 284-286^{o}C

HREIMS m/z (rel.int.): 618.3914 $[M+H]^{+}$

Molecular formula $C_{39}H_{54}O_6$ (calculated 618.8423)

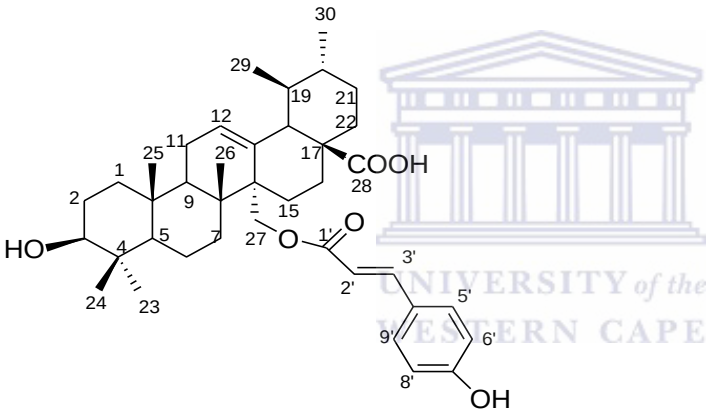

Figure 4.3. Molecular structure of compound C2.

High Resolution EIMS was used to determine the molecular formulae of both **C2** and **C3** which were found to be $C_{39}H_{54}O_6$. The two compounds were isolated in pure form initially and it was subsequently discovered that **C3** had over a short period of time isomerized into a mixture of the two geometric isomers **C2** and **C3**. Inspection of the downfield signals in the proton NMR spectra indicated the presence of a p-hydroxycoumaric acid ester moiety.

Compound **C2** displayed characteristics peaks at δ 7.51 (1H, d, *J* = 16.0 Hz, H-3'), 7.38 (2H, d, *J* = 8.0 Hz, H-5', 9'), 6.78 (2H, d, *J* = 8.0 Hz, H-6', 8') and 6.19 (1H, d, *J* = 16.0 Hz, H-2')

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(Table **4.3**). The large *J* value of 16 Hz clearly supports the *trans* nature of the side chain double bond. The agreement of the proton chemical shifts and coupling constants of **C2** with other reported triterpene hydroxycoumaroyls is further evidence supporting the presence of this moiety.

Our spectra were also compared to the earlier reported data for *Uncaria rhynchophylla* Lee et al., (2000) and *Plemeria obtusa* Siddiqui et al., (1990), see Table **4.3** for NMR data.

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Table 4.3. ^{1}H and ^{13}C NMR data of C2 ($CHCl_3$+ 1drop CD_3OD) and 27-p-*E*-coumaroyloxyursolic acid (pyridine-d_5) (δ values, *J* in parenthesis in Hz)

Position	Compound C2		27-p-*E*-coumaroloyursolic acid	
	$\delta^{13}C$	$\delta^{1}H$	$\delta^{13}C$	$\delta^{1}H$
1	37.2		37.4	
2	27.9		28.1	
3	78.8	3.38m	78.1	3.38 dd(7.8, 2.0)
4	38.2		38.5	
5	55.0		56.0	
6	18.1		18.9	
7	33.0		33.2	
8	39.9		39.8	
9	46.1		48.2	
10	37.2		38.5	
11	23.7		23.8	
12	127.2	5.43 br s	125.6	5.46 br s
13	137.3		134.6	
14	46.1		47.2	
15	28.1		28.3	
16	23.9		24.1	
17	48.6		48.2	
18	55.0	2.58 d (11.5)	53.3	2.60 d (11.6)
19	38.7		39.4	
20	38.2		39.1	
21	30.7		30.7	
22	36.7		37.4	
23	29.8	1.00 s	29.3	1.00 s
24	16.1	0.88 s	16.0	0.98 s
25	16.5	0.90 s	16.6	0.99 s
26	16.9	1.06 s	16.9	1.05 s
27	65.7	4.23 d (13.2) 4.03 d (12.6)	66.2	4.73 d (13.0) 4.54 d(13.0)
28	180.8		179.9	
29	18.8	1.18 d (8.0)	17.0	1.19 d (7.2)
30	21.2	0.83 d (8.0)	21.4	0.93 d (7.1)
1’	167.0		167.2	
2’	115.2	6.19 d (16.0)	115.6	6.67 d (15.9)
3’	142.6	7.51 d (16.0)	145.2	7.96 d (15.9)
4’	132.3		134.6	
5’	130.0	7.38 d (8.0)	130.6	7.60 d (8.5)
6’	116.7	6.78 d (8.0)	116.9	7.13 d (8.5)
7’	158.1		161.5	
8’	116.7	6.78 d (8.0)	116.9	7.13 d (8.5)
9’	130.0	7.38 d (8.0)	130.6	7.60 d (8.5)

^{1}H and ^{13}C NMR of 27-p-*E*-coumaroyloxyursolic acid (Siddiqui et al., 1990).

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Compound **C3** (27- p-*Z*-coumaroloxy ursolic acid)

An off-white amorphous powder (MeOH)

Melting point: 199-202^{o}C

HREIMS m/z (rel.int.): 618.3914 $[M+H]^{+}$

Molecular formula $C_{39}H_{54}O_6$ (calculated 618.8423)

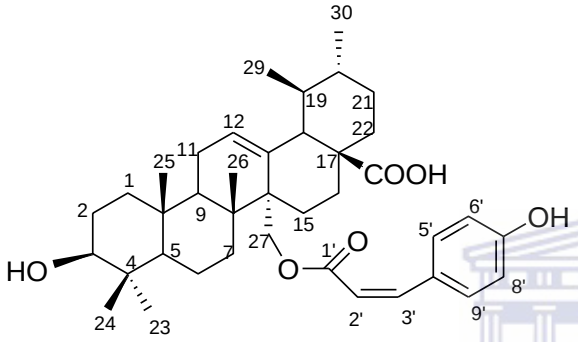

Figure 4.4. Molecular structure of compound C3.

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Compound **C3** was identified as 27- p-Z-coumaroloxy ursolic acid. It later isomerized into a mixture of **C2** and **C3** on remaining in a solution of deuterated chloroform/methanol mixture. The *cis* configuration of the C2'-C3' double bond was confirmed by comparison with published data on *cis*-hydroxycoumaroyls (Rios et al., 2001). As illustrated in Table **4.4**, the respective proton signals for **C3** appeared at δ 7.36(2H, d, *J* = 8.0 Hz, H-5', 9'), 6.82(1H, d, *J* = 12.0 Hz, H-3'), 6.79 (2H, d, *J* = 8.0 Hz, H-6,'8') and 6.15 (1H, d, *J* = 12.0 Hz, H-2') to support the *cis* geometry within the side chain of the molecule. The ^{13}C spectra for **C2** and **C3** are nearly identical with the major difference being in the signals of C2' and C3' being at 116.0/115.2 and 144.8/142.6 for *cis* and *trans* isomers respectively (Tables **4.3** and **4.4**).

Table 4.4. ^{1}H and ^{13}C NMR data of C3 ($CHCl_3$+ 1drop CD_3OD) and 27-p-*Z*-coumaroyloxyursolic acid (^{1}H, CD3COCD3), (^{13}C, CD3OD) (δ values, *J* in parenthesis in Hz)

Position	Compound C3		27-p-*Z*-coumaroloyursolic acid	
	$\delta^{13}C$	$\delta^{1}H$	$\delta^{13}C$	$\delta^{1}H$
1	37.1		37.9	
2	27.9		28.1	
3	78.6	3.13m	78.5	3.08 m
4	38.9		40.1	
5	55.2		56.2	
6	19.5		19.1	
7	33.6		34.3	
8	41.0		41.0	
9	48.9		49.2	
10	38.4		38.0	
11	23.9		24.3	
12	130.2	5.57 br s	130.9	5.52 t (3.5)
13	132.3		134.5	
14	46.1		46.6	
15	29.3		28.7	
16	26.7		25.0	
17	48.6		47.9	
18	52.4	2.40m	53.6	2.38m
19	40.9		40.9	
20	39.0		39.2	
21	30.5		30.8	
22	39.8		39.9	
23	30.3	0.94 s	30.3	0.89 s
24	17.4	0.81 s	16.3	0.82 s
25	17.6	0.92 d (7.8)	16.4	0.87 d (8.0)
26	18.2	0.96 s	18.1	0.92 s
27	65.9	4.27 d (12.0) 4.13 d (12.0)	66.2	4.41 d (12.8) 4.13 d(12.8)
28	180.5		178.6	
29	18.2	1.21s	18.8	1.25 s
30	22.6	0.75s	21.5	0.76 s
1’	167.4		166.7	
2’	116.0	6.15 d (12.0)	116.8	5.76 d (13.0)
3’	144.8	6.82 d (12.0)	144.1	6.87 d (13.0)
4’	132.3		134.4	
5’	130.0	7.36 d (8.0)	133.7	7.78 d (8.8)
6’	115.0	6.79 d (8.0)	115.8	6.84 d (8.8)
7’	159.2		159.9	
8’	115.0	6.79 d (8.0)	115.8	6.84 d (8.8)
9’	132.3	7.36 d (8.0)	133.7	7.78 d (8.8)

^{1}H and ^{13}C NMR of 27-p-*Z*-coumaroyloxyursolic acid (Rios et al., 2001).

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Compound **C5:** Pycanocarpine A (2, 3-seco-taraxen-14-ene -2, 3- lactone)

Colourless needle-like crystals (DCM)

Melting point: 246-249^{o}C

IR (KBr) γ_{max} 3054, 2914, 2856, 1738, 1474, 1077cm^{-1}

HREIMS m/z (rel.int.): 440.3556 $[M+H]^{+}$

Molecular formula $C_{30}H_{48}O_2$ (calculated 440.7009)

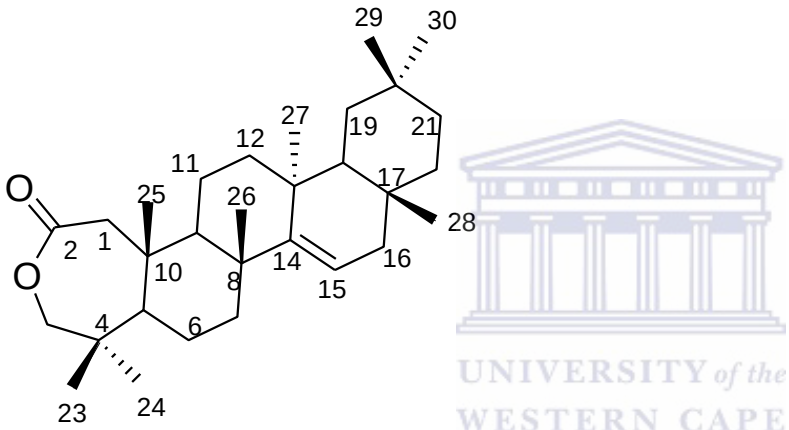

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Figure 4.5. Molecular structure of compound C5.

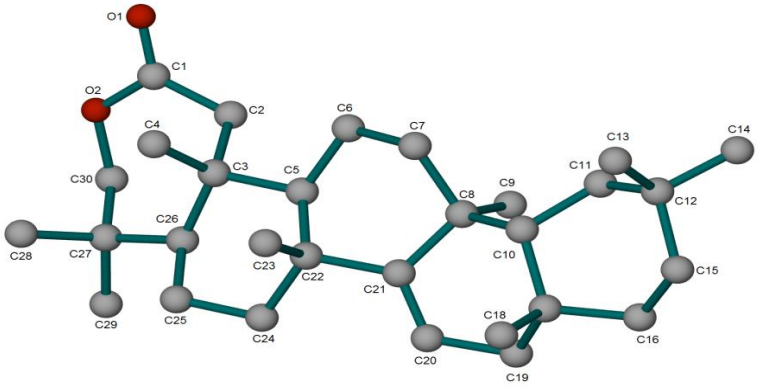

Figure 4.6. Molecular structure of compound C5 as determined by single X-ray crystallography.

Compound **C5** was obtained as a needle-like white crystals with a molecular formula $C_{30}H_{48}O_2$ deduced from the HRMS of the 440.3556 $[M+H]^+$(calculated 440.7009), single X-ray crystallography, 2D-NMR and ^{13}C DEPT spectra. The IR spectrum exhibited absorption bands at 3054, 2914, 2856 (C-H stretching), 1738(C=O stretching of a lactone) 1474 (C-O, carboxylate ion. The ^{13}C DEPT spectra showed 30 signals (Table **4.5**) which are attributed to 8 methyls, 10 methylenes, 4 methines and 8 quartenary carbons. The molecule was chemically deduced to be a triterpenoid through a purple vanillin-sulphuric acid test and supported by the appearance of 8 methyl groups in the ^1H NMR spectrum (Table **4.5**).

The signal due to the olefinic proton at δ 5.54 (m) together with ^{13}C NMR signals of C-14 (δ 157.7) and C-15 (δ 117.1) suggested a taraxerane moiety (Mahato and Kundu, 1994; Giang et al., 2013). The ring A of compound **C5** was compared to the ring A of 4- hydroxy-3,4-seco-ursan-12-en-28-oic acid 3,4 lactone which is a 3,4- secoursan -12- ene derived from ursolic acid (Tu et al., 2009) and compared very favourably (Table **4.6** and Fig. **4.7**). The C-2 carbon was assigned on the basis of the chemical shift at δ 174.8, the structure of **C5** is as illustrated in Fig.**4.5**. The NMR data were assigned unambiguously from the hydrolyzed product of compound **C5**. The structure was finally established from X-ray diffraction studies, Fig. **4.6** illustrates the perspective view of the molecule with its relative configuration.

The compound was thus assigned as 2, 3-seco-taraxer-14-en-2, 3-lactone and given the name pycanocarpine.

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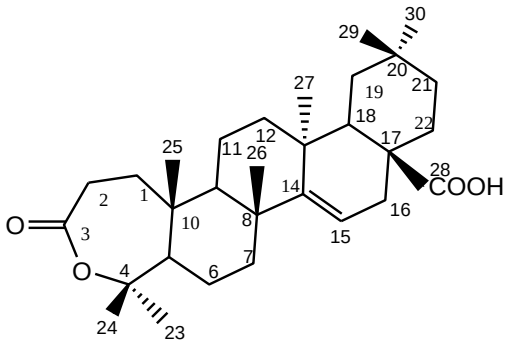
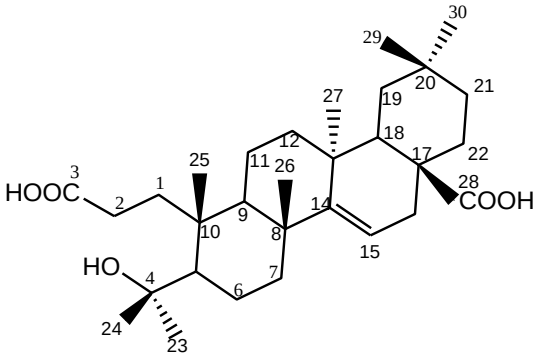


4-Hydroxy-3,4-seco-ursan-12-en-28-oic acid 3,4 lactone



3,4-seco-Ursan-12-en-3,28-dioic acid

Figure 4.7. **Molecular structure of ursolic acid seco derivatives.**

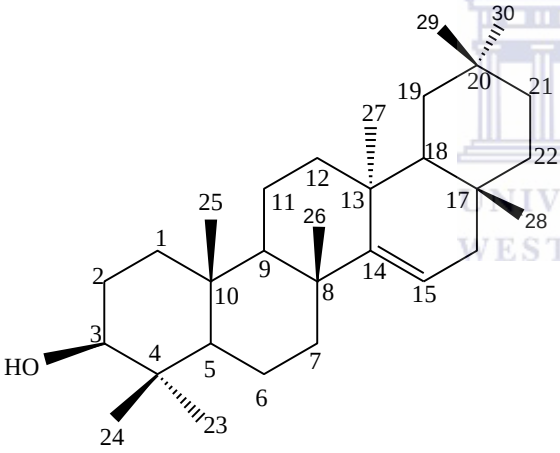


Figure 4.8. Molecular structure of 3β-taraxerol.

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Table 4.5. ^{1}H and ^{13}C NMR data of C5 ($CDCl_3$) and C6 ($CDCl_3$+ 1drop CD_3OD) (δ values, *J* in parenthesis in Hz)

Position	Compound C5		Compound C6	
	$\delta^{13}C$	$\delta^{1}H$	$\delta^{13}C$	$\delta^{1}H$
1	44.9 t	2.44 br s; 2.78 br s	43.1 t	2.36 d; 2.35 d(12.0)
2	174.8 s		172.9 s	
3	77.9 t	3.79 br s; 4.08 br s	72.1 t	3.33 d; 3.34 d(7.0)
4	38.4 s		38.6 s	
5	48.7 d	0.97*	44.9 d	1.72*
6	20.6 t	1.57*; 1.66*	21.8 t	1.60*; 1.53*
7	35.1 t	1.02*; 1.35*	35.6 t	0.95*; 1.34*
8	35.8 s		34.9 s	
9	40.9 d	1.64*	42.8 d	1.76*
10	46.7 s		43.6 s	
11	18.6 t	1.57*; 1.75 br s	19.5 t	1.18* 2H
12	36.6 t	0.98 dd; 1.34*(12.0,4.5)	37.5 t	1.49*; 1.83 td (9.0,3.5)
13	37.4 s		37.0 s	
14	157.5 s		157.3 s	
15	117.1 d	5.54 dd(8.0,3.5)	116.8 d	5.46 dd(9.0,4.5)
16	37.8 t	1.64*; 1.92 dd(9.0,2.0)	36.5 t	0.90*; 1.22
17	40.6 s		40.2 s	
18	48.7 d	0.97*	48.5 d	0.92* 2H
19	39.4 t	1.37*; 2.04 dt (9.5,3.5)	39.7 t	1.33*; 1.96 dd (3.5,8.5)
20	28.7*s		28.6 s	
21	33.1 t	1.25 dt (9.5,3.5); 1.32*	32.9 t	1.44*; 1.59*
22	33.6 t	1.60*; 1.64*	33.8 t	1.55*; 1.60*
23	19.4 q	1.02 s	26.3 s	0.93 s
24	28.7 q	0.95 s	24.4 d	1.04 s
25	17.5 q	1.13 s	18.5 q	1.02 s
26	25.8 q	1.11 s	20.6 q	0.86 s
27	29.6 q	0.92 s	29.5 q	0.83 s
28	21.1 q	0.90 s	23.9 q	1.00 s
29	33.5 q	0.95 s	33.1 q	0.87 s
30	29.6 q	0.83 s	29.7 q	0.71 s

*overlapped signals

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Compound **C6:** Pycanocarpene A (2, 3-seco-Taraxen-4-hydroxy-14-en-2-oic acid)

An off-white amorphous powder (MeOH)

Melting point: 237-241^{o}C

IR (KBr) γ_{max} 3055, 2921, 2857, 1740, 1473, 1067cm^{-1}

HREIMS m/z (rel.int.): 458.3810 $[M+1]^{+}$

Molecular formula $C_{30}H_{50}O_3$ (calculated 458.7162)

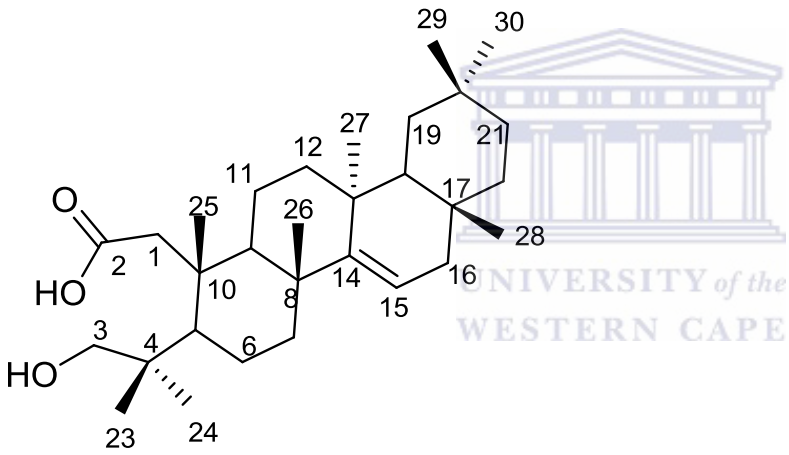

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Figure 4.9. Molecular structure of compound C6.

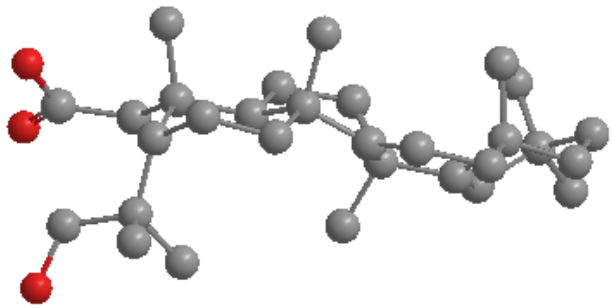

Figure 5.0. Ball and Stick minimized energy model for C6. [Generated by ChemDraw Ultra 8.0 (Cambridge)].

Compound **C6** was synthesized by refluxing 33.0mg of **C5** (33.0mg, 0.075mmol) in 5% methanolic-KOH(50 ml) for 3 hours at 110^0C after which the resultant mixture **Z1** was cooled, diluted with deionized water (30 ml), acidified with 1M HCl and extracted with EtOAC (50 ml). The residue was chromatographed on silica gel column using 70% Hex-EtOAC to yield **C6** (20.0mg, 60.6%). This alkaline hydrolysis of compound **C5** produced the unstable derivative *viz* 2,3-seco-3-hydroxy-taraxer-14-en-3-oic acid (**C6)** as an off-white amorphous powder having the molecular formula $C_{30}H_{50}O_3$ based on HREIMS of 458.3810 $[M+H]^+$ (calculated 458.7162) as well as ^{13}C NMR data. The IR spectrum exhibited a strong absorption band at 3055 cm^{-1} for an OH and 2921and 2857 cm^{-1} (C-H stretching), 1740 cm^{-1} (C=O stretching), 1473 cm^{-1} (C-O, carboxylate ion) and 1067 cm^{-1} (C-O stretching).

The^{13}C DEPT spectra showed 30 signals (Table **4.5**) attributed to 8 methyls, 10 methylenes, 4 methines and 8 quaternary carbons. The product was deduced to be a triterpenoid through a purple vanillin-sulphuric acid test and the appearance of the methyl groups δ 0.71, 0.83, 0.86, 0.87, 0.93, 1.00, 1.02 and 1.04 in the ^{1}H NMR spectra(Table **4.5**). The olefinic proton at δ 5.46 (m) together with ^{13}C NMR signals of C-14 (δ 157.3) and C-15 (δ 116.8) showed a tri-substituted double bond which when compared to similar molecules suggested a taraxer-14-ene structure (Giang et al., 2013).

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It was noticeable that the free carboxylic and hydroxyl groups could easily reform the lactone ring after a short time in organic solvents such as methanol and chloroform. The NMR data of **C6** is similar to the parent compound **C5** and is illustrated in (Table **4.5**). Minor changes were observed between **C5** and **C6** for carbons C-11, -23, -24 and -26. The stereochemistry of both compounds, especially ring A, may partially explain this.

In compound **C5** the carbonyl group is directed into the same plane similar to C-24, -26 and -11. On the other hand, in the derivative **C6**, the minimized energy conformation (Fig. **5.0**) showed different orientations and distances for the C_3-OH and C_2-OOH groups.

Seco-taraxerane triterpenoids have not been reported in the Apocynaceae family and *Pleiocarpa* genus. This is the first report of the isolation of triterpenes from *Pleiocarpa pycnantha* and the second from the genus.

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Table 4.6. ^{1}H and ^{13}C NMR data of Taraxerol $CDCl_3$, 4-Hyroxy-3, 4-seco-ursan-12-en-28-oic acid 3, 4 lactone and 3, 4-seco-Ursan-12-en-3, 28-dioic in CD_3OD (δ values, *J* in parenthesis in Hz)

Position	Taraxerol $\delta^{13}C$	$\delta^{1}H$	4-Hydroxy-3,4-seco-ursan-12-en-28-oic acid 3,4 lactone(Ring A) $\delta^{13}C$	$\delta^{1}H$	3, 4-seco-Ursan-12-en-3, 28-dioic (Ring A) $\delta^{13}C$	$\delta^{1}H$
1	38.0		*		*	
2	27.1		31.8		30.4	2.30 1H m, 2.49 1H m
3	79.1	3.24 1H dd (4.7, 11.0)	175.8		181.7	
4	39.0		74.9		76.2	
5	55.5		52.5		53.0	
6			23.9		21.6	
7	35.1	2.03 1H dt (3.1, 12.6)	29.6		32.7	
8	38.7		45.3		43.8	
9	48.7		37.0		54.4	
10	37.5		*		40.6	
11	17.5		17.0		17.8	
12	35.8		125.3	5.251H t (3.6)	127.2	5.27 1H t (3.6)
13	37.6		138.1		139.4	
14	158.1		42.1		42.1	
15	116.9	5.53 1H dd (3.2,8.2)	27.9		29.2	
16	36.6	1.92 1H dd (3.0, 14.6)	24.0		24.1	
17	37.7		47.9		49.0	
18	49.2		55.2	2.20 1H d(11.2)	52.9	2.22 1H d (11.6)
19	41.3		39.0		40.1	
20	28.8		38.8		38.1	
21	33.7		30.5		31.8	
22	33.1		36.6		35.7	
23	28.0	0.98 3H s	*	1.28 3H s	33.7	1.27 3H s
24	15.4	0. 80 3H s	19.7	1.26 3H s	28.3	1.25 3H s
25	15.4	0.93 3H s	13.6	0.80 3H s	18.1	0.97 3H s
26	29.8	1.09 3H s	16.8	1.01 3H s	17.7	1.10 3H s
27	25.9	0.91 3H s	20.8	1.07 3H s	23.4	1.14 3H s
28	29.9	0. 82 3H s	182.3		181.7	
29	33.3	0.95 3H s	23.3	0.95 3H d(6.0)	20.6	0.91 3H d (6.4)
30	21.3	0.90 3H s	21.1	0.85 3H d (6.4)	23.8	0.89 3H d (6.4)

NMR data of Taraxerol (Chávex et al., 2012), 4-Hydroxy-3, 4-seco-ursan-12-en-28-oic acid 3, 4 lactone and 3, 4-seco-Ursan-12-en-3, 28-dioic (Tu et al., 2009).

* omitted in the reference article.

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CHAPTER 5

The chapter is dedicated to the results and discussions of the biological evaluation of the isolated compounds. This chapter reports on the cytotoxicity of the extract, fractions and compounds from *P. pycnantha* leaves via the WST 1 assay on cervical carcinoma (HeLa), colorectal adenocarcinoma (HT-29) breast adenocarcinoma (MCF-7) and nontumorigenic immortalized human cell lines (KMST-6). Cytoxicity and apoptosis were used to identify the lead compounds showing potent anti- cancer activity on various cell lines.

The pro- apoptotic activity was conclusively demonstrated with specific markers of apoptosis nmely; the externalization of the phosphatidylserine, ROS, caspases, cell cycle and Topoisomerase 1 inhibition.

5.1 BIOLOGICAL EVALUATIONS: RESULTS AND DISCUSSION

5.1.1 Investigation of cytotoxicity of crude extract, fractions and isolated compounds

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The cytotoxicity of a compound is believed to be an indication of its apoptotic potential. Cytotoxicity evaluations are generally used as a preliminary screening for plants and its constituents to investigate any possible biological activities especially anticancer activities.

The WST-1 cell proliferation assay is a colorimetric assay that is based on the cleavage of a tetrazolium salt viz., 4- [3- (4-Iodophenyl) -2- (4-nitrophenyl) -2H -5-tetrazolio] -1, 3-benzene disulfonate disulfonate by mitochondrial dehydrogenases to form formazan in viable cells (Berridge et al., 1996). The greater the number of viable, metabolically active cells, the greater the amount of formazan product produced following the addition of WST-1 and the quantification of the formazan dye produced by metabolically active cells which can be done by

measuring the absorbance of the dye solution with a scanning multiwell spectrophotometer (ELISA reader) at appropriate wavelengths.

Cells were plated in a tissue culture in 96 well plates as described in section **3.10.1** and treated with crude extract, fractions and isolated compounds for 24 h at a concentration of 0.125 – 2 mg/ml, 0.4 mg and 15.62 – 250 µg/ml for the crude extract, fractions and isolated compounds respectively. The cytotoxic activity against HeLa, HT-29, MCF-7 and KMST-6 cells as evaluated by the WST-1 assay for the crude extract of *P. pycnantha* leaves are presented in Figs. **5.1 - 5.2.** From the results obtained, the % viability of the cells decreased with increase in dose. The IC_{50} values for the extract on HeLa, HT-29, MCF-7 and KMST-6 cell lines are 620, 910, 460 and 1460 µg/ml respectively (Table **5.1**). This is an indication that the plant extract is more sensitive and selective for the cancer cells than the KMST-6 normal cell line ($IC_{50,}$ 1460 µg/ml).The reduced IC_{50} value of 460 µg/ml on the MCF-7 (breast cancer) cell line showed that the total plant extract can be used to kill breast cancer cells. The next sensitive cell line to the plant extract is HeLa (cervical cancer) with an IC_{50} value of 620 µg/ml, while the IC_{50} value of 910 µg/ml obtained for HT-29 (colon cancer) cell lines showed that the killing effect of this plant extract is weak on the cell line.

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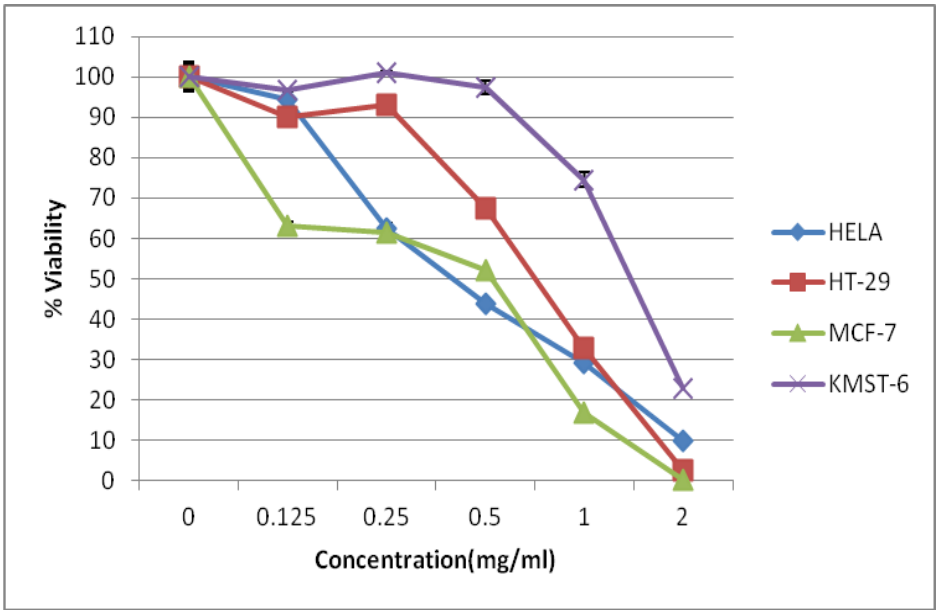

Figure 5.1. The percentage viability of crude extract of Pleiocarpa pycnantha leaves on Hela, HT-29, MCF-7 and KMST-6 cell line.

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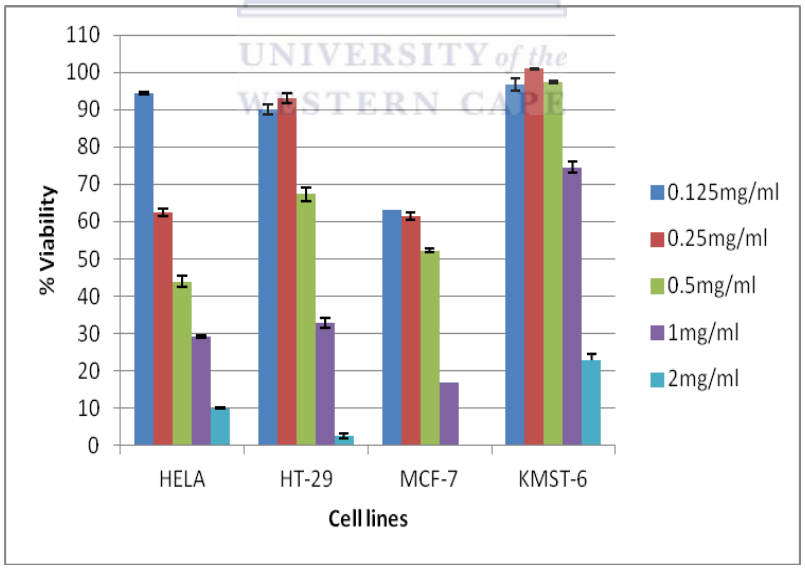

Figure 5.2. The dose response chart of percentage viability of crude extract of *Pleiocarpa pycnantha leaves* on HeLa, HT-29, MCF-7 and KMST-6 cell lines.

Table 5.1: **IC_{50} values for Pleiocarpa pycnantha ethanolic extract when treated for 24 h as determined by WST-1.**

	IC_{50} µg/ml	
Cell lines	Ethanolic extract	Selectivity Index
HeLa	620	2.4
HT-29	910	1.6
MCF-7	460	3.1
KMST-6	1460	-

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The results from the findings above showed that the ethanolic extract of *Pleiocarpa pycnantha* leaves displayed cytotoxic effects on MCF-7, HeLa and HT-29 cancer cell lines. However the activity was low with IC_{50} > 100 µg/ml on all the cell lines, which made us to screen the individual interesting fractions for their activities.Some of the fractions demonstrated potent activity which gave some justification to carry out further chemical investigations of such fractions as described in Section **3.7** with the scheme of extraction shown in Section **3.9.1**.

The fractions were screened using a single dose of 400 µg/ml representing a near average value for the IC_{50} and the result is presented in Fig. **5.3**.

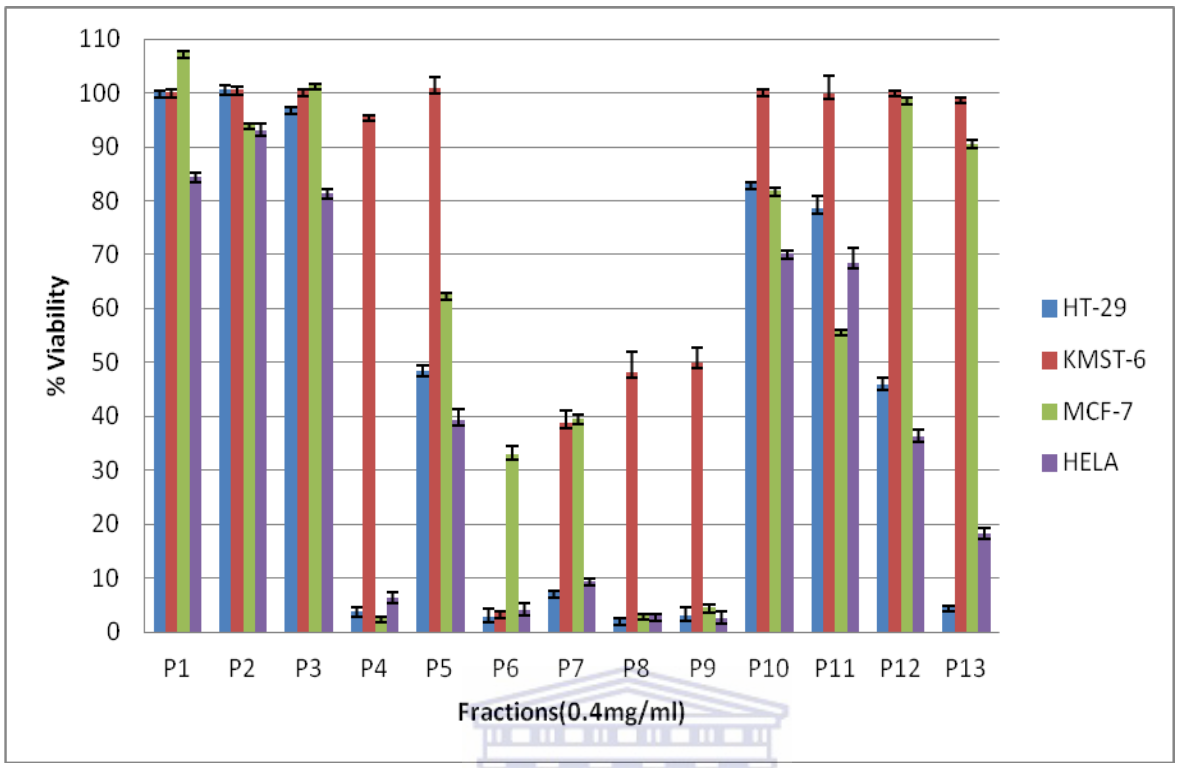

Figure 5.3. Comparative percentage viabilities of fractions obtained from *Pleiocarpa pycnantha* leaves on HeLa, HT-29, MCF-7 and KMST-6 cell lines.

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Fractions **P4**, **P5**, **P6**, **P7**, **P8**, **P9**, **P12** and **P13** displayed cytotoxic activity on selected cell lines **P11** was weakly cytotoxic while **P1**, **P2**, **P3** and **P10** were not active. Different criteria were used to select the fractions for further purification. The fractions; **P4**, **P7**, **P9** and **P12** were selected based on their biological activity while **P11** was selected based on its interesting chemistry and pro-apoptotic effect on all cell lines (data not shown). Of all the fractions selected for further purification to isolate the active constituents; fraction **P4** was outstanding in the sense that it killed all the cancer cell lines selectively without touching the normal cell lines.

The percentage viability at a concentration of 400 µg/ml on the normal cell, breast cancer, colon cancer and cervical cancer cell lines are : 2.75, 3.70, 6.42 and 95.9% respectively.

This finding seemed exciting to us because many of the anticancer drugs have been reported to interfere and destroy the normal cells as well albeit to a lesser extent. This led us to the next stage viz., that of isolating active constituents from the chosen extracts which may probably serve as 'leads' in phytomedicine.

On HeLa (cervical) cell lines as demonstrated in Fig.**5.3**, fractions **P4**, **P6**, **P7**, **P8**, **P9** and **P13** displayed significant cytotoxicity while fractions **P5** and **P12** were moderatively active.

The fraction that displayed the lowest % viability was **P9** (2.42), followed by **P8** (3.08), **P6** (4.09) ranking the first three, while the highest % viability fraction was **P1** (84.3).

Fractions **P4**, **P6**, **P7**, **P8**, **P9** and **P13** displayed outstanding % viability results on HT-29 (colon) cancer cell lines while **P5** and **P12** demonstrated average activity. The results further indicated that **P8** (2.39) had the least % viability being the most active followed by **P6** and **P9** with % viabilities of 2.68 and 3.15 respectively. The fraction that had the highest % viability (least active) was **P2** (100.15).

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On MCF-7 (breast cancer) cell lines, the results indicated that fractions **P4**, **P8** and **P9** displayed potent cytotoxicity while **P6**, **P7** and **P11** showed moderate cytotoxicity against the cell lines. Ranking from the 1st to the 3rd, the best cytotoxic fraction on the cell line was **P4** (2.72) followed by **P8** (3.22%) and **P9** (4.62%). The least cytotoxic fraction (least active) was **P1**(100.05%).

On KMST-6 (normal) cell line, as indicated in Fig. **5.3**, fraction **P6** (3.62%) was very cytotoxic and selective for the normal cell lines while **P7** (38.67%) and **P8** (48.07%) were also slightly cytotoxic on the normal cell lines. Others except **P9** (49.92%) were not selective for the normal cell lines.

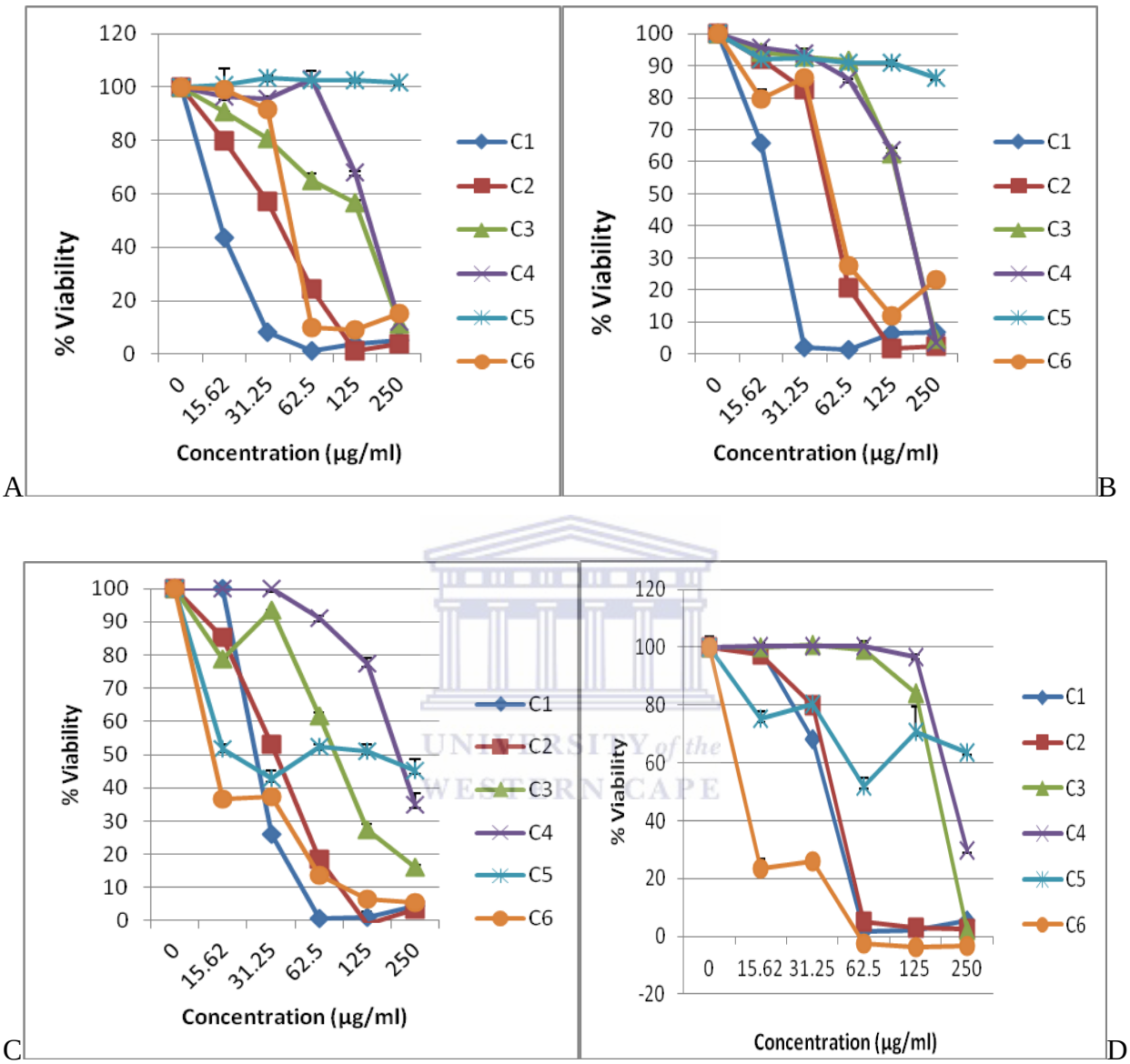

Figure 5.4. Effect of compounds C1-C6 on cell viability of (A) HeLa cells (B) HT-29 (C) MCF-7 (D) KMST-6 in a concentration dependent manner as measured by WST-1 assay.

The effects of isolated and purified compounds **C1-C6** on the viability of HeLa, HT-29, MCF-7 and KMST-6 cells are presented in Fig. **5.4** after treatment for 24 hours with concentration of compounds ranging from 15.62 - 250 µg/ml as determined by WST-1 assay.

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As shown in Fig. **5.4A**, compound **C1** is the most cytotoxic on HeLa cell lines with an IC_{50} value of 5.06 µg/ml, followed by **C2**, 33.19 µg/ml. Compound **C6** is fairly cytotoxic with an IC_{50} of 80.00 µg/ml, **C3** and **C4** is weakly cytotoxic with IC_{50} values of 130.47 and 160.95 µg/ml respectively, while **C5** is the least active with an IC_{50} value of >250 µg/ml, see Table **5.2** for IC_{50} values. The cytotoxicity results on HT-29 cell line (Fig. **5.4B**) followed a similar pattern as with the HeLa cell lines. Compound **C1** was the most cytotoxic on HT-29 cell lines with an IC_{50} value of 5.12µg/ml, followed by the fairly cytotoxic compounds **C2** (68.30 µg/ml) and **C6** (78.9µg/ml). **C3** and **C4** showed weak cytotoxicity at 144.53 µg/ml and 142.19 µg/ml respectively. **C5** did not show cytotoxicity at the highest concentration used in this assay as the IC_{50} was >250 µg/ml. On MCF-7 cells (Fig.**5.4C**), compound **C6** with an IC_{50} value of 5.46 µg/ml was the most cytotoxic while **C1** was the second with an IC_{50} value of 9.51 µg/ml and the third was **C5** with an IC_{50} value of 10.99 µg/ml.

It is noteworthy here that **C5 is selective for only MCF-7 cancer cells**. The selectivity index (SI) for **C5** was > 22 for MCF-7 cells, demonstrating that the activity of this compound is highly selective for MCF-7 adenocarcinoma cell line. **C2** (34.37 µg/ml) also displayed significant cytotoxicity while **C3** (114.06 µg/ml) and **C4** (204.30 µg/ml) are weakly cytotoxic.

On the non-cancerous KMST-6 cell line(Fig. **5.4D**), compounds **C3**, **C4** and **C5** were not cytotoxic on the normal cell lines with IC_{50} values of 160.94, 214.84 and >250 µg/ml respectively. **C1 (**50.77 µg/ml) and **C2** (61.32 µg/ml) are slightly cytotoxic while **C6** is highly cytotoxic (4.78 µg/ml) on KMST-6 cell lines. The IC_{50} values and the selectivity indices of all isolated compounds on the various cell lines are displayed in Table **5.2**.

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The use of plants and their extracts have been the basis of prevention and treatment of diseases from very early times in our documented history. Plant secondary metabolites and their semi-synthetic derivatives continue to play important roles in anticancer drug therapy.

The survey of the anticancer drug market between1981-2006 has revealed 47.1% of a total of 155 clinically approved drugs were either unmodified natural products, semi-synthetic derivatives or synthesized molecules based on natural compound pharmacophores (Newman and Cragg, 2007). Many of the anticancer drugs today had their origins from the preliminary cytotoxicity screening.

Triterpenoids exist abundantly in the plant kingdom, for example; ursolic acid (3β-hydroxy-urs-12-en-28-oic acid) is an ursane type triterpene found in all plant parts, but mainly in the leaves and presenting several important biological activities which includes; anti-inflammatory, antiviral, antioxidant and anti-cancer properties (da Silva et al., 2008;Tu et al., 2008).

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The anti-proliferative activity of ursolic acid has been reported in a wide variety of cancer cell lines (Neto, 2007). Ursolic acid hydroxycinnamate esters isolated from cranberry fruit has been evaluated for anti-tumor activity in a 60 tumor cell line panel through the National Cancer Institute's Developmental Therapeutics program (http://dtp.nci.nih.gov/about.html).

The cis- and trans- isomers of 3-O-p-hydroxycinnamoyl ursolic acid has been shown to inhibit tumour growth in vitro with the cis- isomer showing slightly greater activity in most cell lines with GI_{50} values ~20 µM in MCF-7 breast, ME 180 cervical, and PC3 prostate tumor cell lines (Murphy et al., 2003).

The anti-proliferative activity of hydroxycoumaroyl esters of ursolic acids was reported on A-549, HCT-15, MCF-7 and HT-1197 with IC_{50} ranging from 0.5-6.5 µg/ml from *Uncaria rhynchophylla* (Lee et al., 2000). Many plants from Apocynaceae family have been reported for

anticancer activity (Bonfil et al., 2002; Okouneva et al., 2003), including this report, which indicated the importance of their constituents as interesting source for the discovery of new anticancer agents.

Table 5.2: IC_{50} values for compounds C1-C6 and the crude extract as determined by WST-1 assay in µg/ml.

	Cell lines						
	HeLa		HT-29		MCF-7		KMST-6
	IC_{50}	SI	IC_{50}	SI	IC_{50}	SI	IC_{50}
C1	5.06	10.0	5.12	9.9	9.51	5.3	50.77
C2	33.19	1.8	68.30	0.9	34.37	1.8	61.32
C3	130.47	1.2	144.53	1.1	114.06	1.4	160.94
C4	160.95	1.3	142.19	1.5	204.30	1.1	214.84
C5	>250		>250		10.99		>250
C6	80.00	0.06	78.90	0.06	5.46	0.87	4.78

Selectivity Index (SI) = IC_{50} (KMST-6)/IC_{50} (HeLa) or any of the cancer cell lines used.

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5.1.2 Analysis of apoptotic effects of some representative fractions and isolated compounds

To analyze the inhibitory effects of fractions and isolated compounds, we investigated apoptosis. Cell deaths result from either a non-specific unregulated process of necrosis or may be due to a defined and programmed cellular death process known as apoptosis which is regulated by specific cellular signaling pathways.

We investigated whether the fractions and the isolated compounds were able to cause any general non-specific necrotic cell death or induce apoptosis of HeLa, HT-29, MCF-7 and KMST-6 using Apo-percentage dye^{TM} (Biocolor Ltd). This assay stains apoptotic cells with a red dye and the exposure of the membrane phospholipid phosphatidylserine (PS) allows the unidirectional uptake of the APOpercentage ^{TM} dye (Kurokawa et al., 1999). As a consequence, only cells that have undergone apoptosis i.e. externalization of phosphatidylserine; will be labeled by the dye, while the normal and the necrotic cells remain unlabelled.

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The effect of compounds **C1-C6** on HeLa cells as visualized under a Nikon inverted light microscope using a 20X objective and the photographs were taken using a Leica EC3 digital camera as displayed in Fig.**5.5**. The quantification of apoptosis of untreated control and compound **C1** on HeLa cell is shown in Fig.**5.6**.

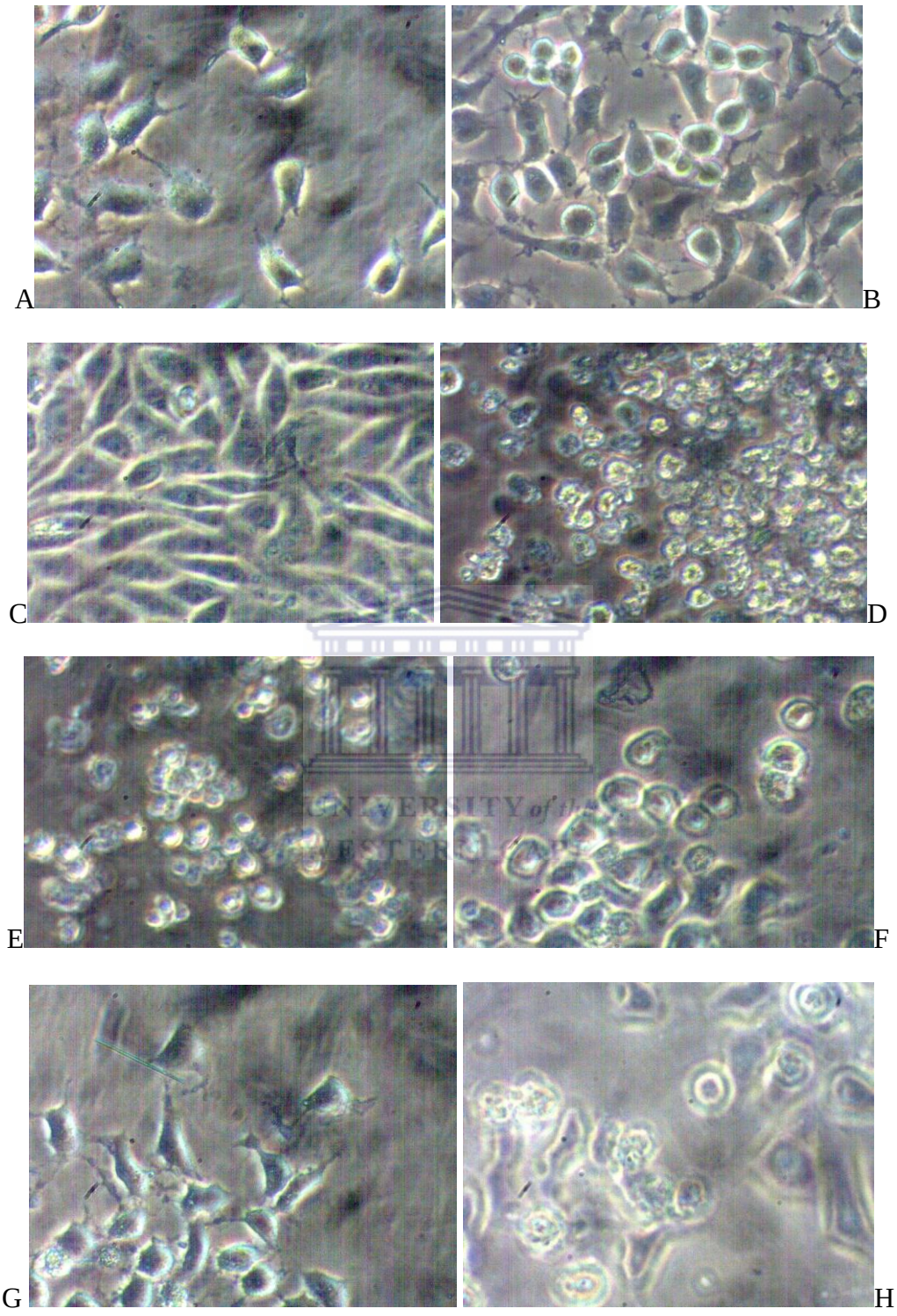
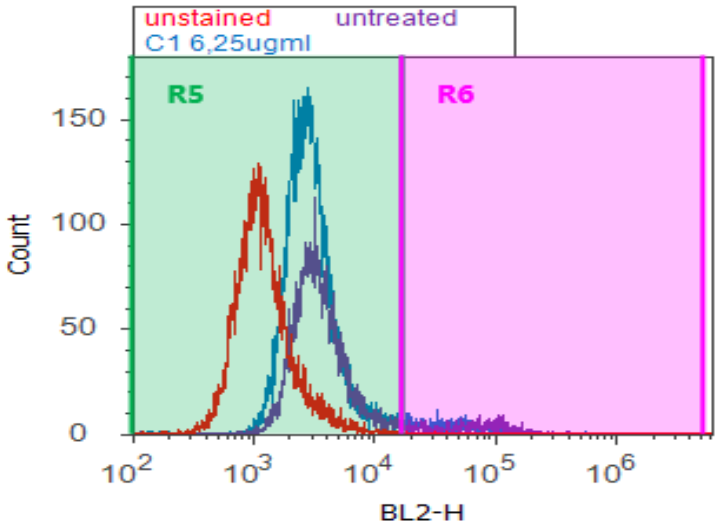
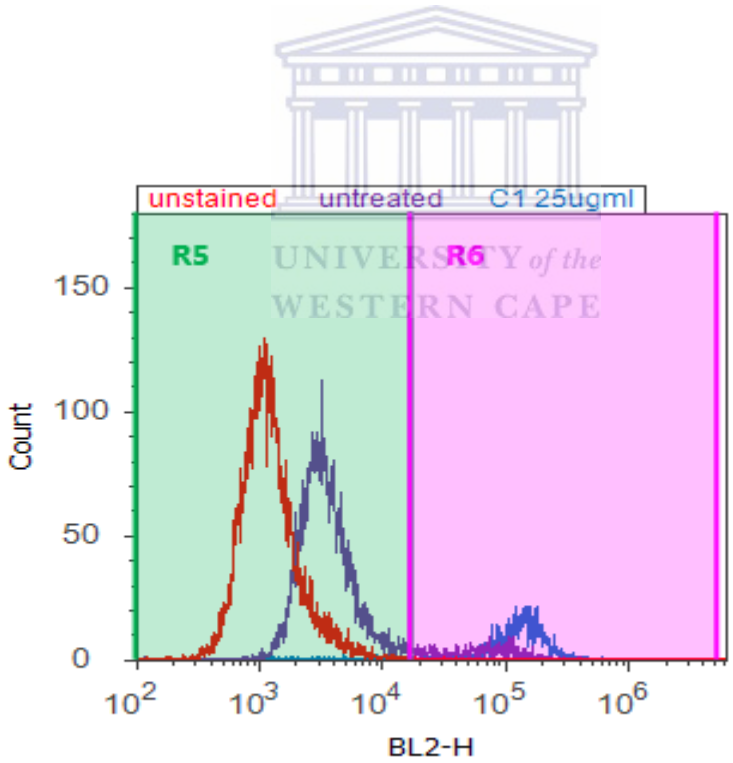


Figure5.5. Effect of compounds on HeLa cell morphology when treated for 24h A (untreated) B (50 µM camptothecin) C (12.5 µg/ml C1) D (12.5 µg/ml C2) E (12.5 µg/ml C3) F (12.5 µg/ml C4) G (12.5 µg/ml C5) H (12.5 µg/ml C6).



A



B

Figure 5.6. Flow cytometry diagram showing the quantification of apoptosis as measured by flow cytometer A (C1 6.25 µg/ml) B (C1 25 µg/ml) on HeLa for 24 h.

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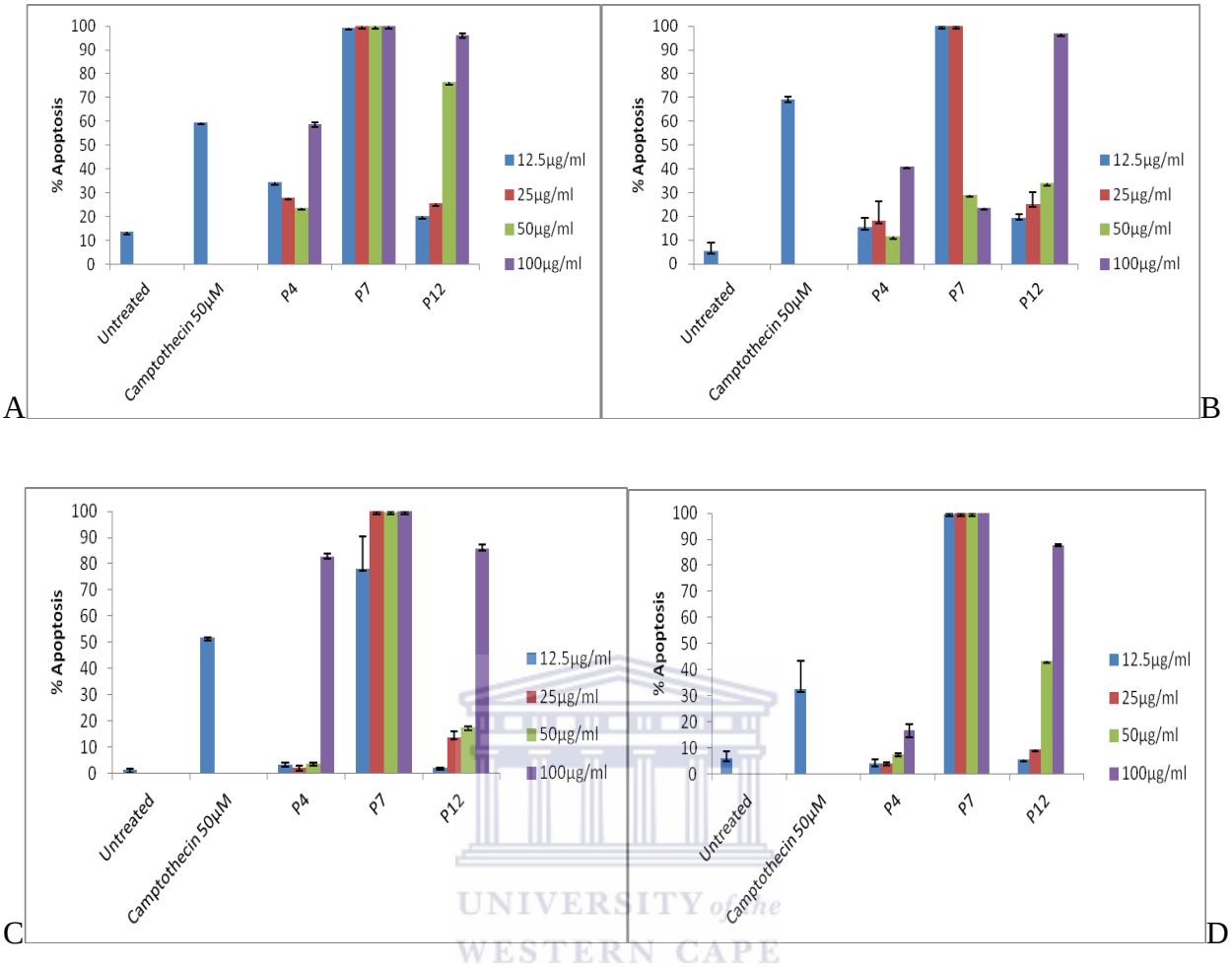

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Figure 5.7. **The apoptotic effect of different concentrations of fractionsP4, P7 and P12 on (A) HeLa (B) HT-29 (C) MCF-7 (D) KMST cells in a concentration-dependent manner as measured using Apopercentage™ assay after 24 h treatment.**

The effect of apoptosis induction of the fractions **P4**, **P7** and **P12** representing fractions where the compounds were isolated at a dose of 12.5-100 µg/ml for 24hours on HeLa, HT-29, MCF-7 and KMST-6 cell lines as described in section **3.10.6** are presented in Figure **5.7**. Camptothecin (50 µM) a renowned anticancer drug was used as a positive control.

All the fractions were apoptotic at the test concentration used on all cancer cell lines.

Fraction **P4** was apoptotic on cancer cell lines with maximum killing of ~ 60%, 40% and 82% on HeLa, HT-29 and MCF-7 at the highest dose of 100 µg/ml. This fraction was not selective for the normal cell even at the highest dose; the % apoptosis on KMST-6 was ~15% (Fig. **5.7D)**.

Fraction **P7** was a representative for **P8** and **P9** since ursolic acid was confirmed to be present in all 3 of these fractions. It was however shown to be non-selective as it kills both cancer and non cancerous cells in a similar faction. This is in agreement with cytotoxicity results.

Fraction **P7** was very active with maximum killing (~100%) at the lowest dose 12.5 µg/ml on HeLa, HT-29 and MCF-7 (~80%). On HT-29 cell lines(Fig. **5.7B)**, fraction **P7** seemed to have reached its maximum activity level at 25µg/ml, and the increased dosage of 50 and 100 µg/ml did not have a further effect on the cell, and so the percentage apoptosis decreased.

Fraction **P12** was used to represent **P11** and **P12** since coumaroloxy ursolic acids were confirmed to be present in both. Though not cytotoxic on most of the cell lines, it rather surprisingly displayed a very good dose-response on Apopercentage™ assay.

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It had a maximum killing at the highest dose of ~95%, 98% and 85% on HeLa, HT-29, and MCF-7 respectively. **P12** was also selective for the normal cell line at this same dose with a maximum of ~88% killing effect contrary to the cytotoxicity result. The cytotoxicity test is considered as a preliminary test for screening potential apoptosis inducers; however some previous reserch had demonstrated that cytotoxicity may not be such a good indicator of apoptotic potential (Essack, 2006 Masters Dissertation, UWC; Sagar, 2007 PhD Thesis, UWC; Kikuchi et al., 2011).

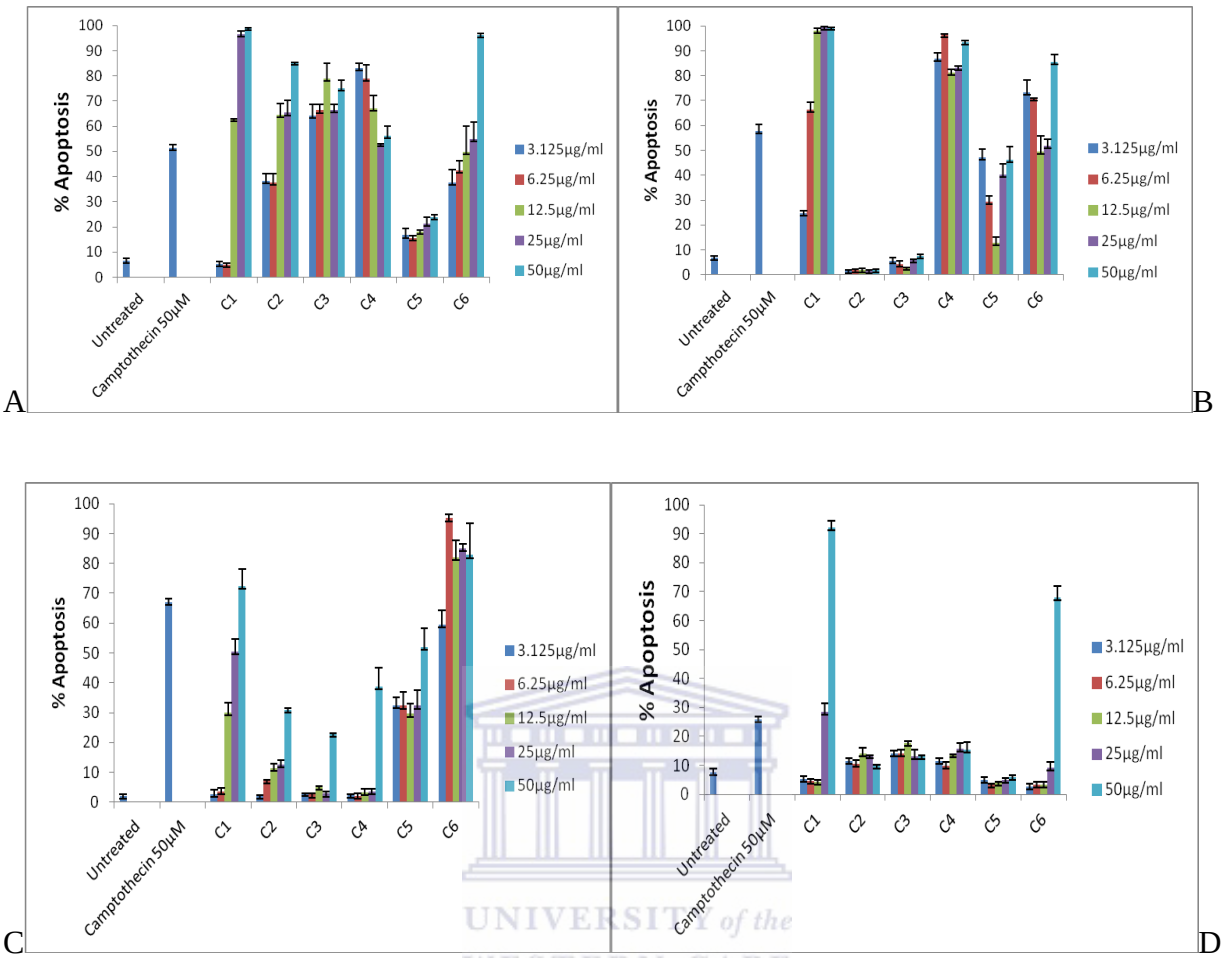

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Figure 5.8. Effect of apoptosis of C1-C6 on (A) HeLa (B) HT-29 (C) MCF-7 (D) KMST cell lines as determined by Apopercentage ™ assay for 24 h.

All the compounds **C1-C6** were also screened for apoptosis at a dose of 3,125-50.0 µg/ml on HeLa, HT-29, MCF-7 and KMST-6 cell lines for 24 hours as described previously.

The results of the induction of apoptosis of compounds **C1-C6** are presented in Fig. **5.8.**

The dose-response of all the isolated compounds for their induction of apoptosis on HeLa cell line is presented in Fig. **5.8A**. All the tested compounds displayed significant apoptotic potential with the exception of compound **C5** with ~23% apoptosis at the highest dose 50.0 µg/ml.

It should also be noted that, most of the compounds were more active than the positive control-Camptothecin on HeLa, except **C5**. Ursolic acid (**C1**) has been previously reported to suppress the proliferation of a number of tumor cells, induce apoptosis Shanmugam et al., (2011) and inhibit tumor promotion, metastasis and angiogenesis (Kiran et al., 2008).

The compounds **C2**, **C3** and **C4** were not cytotoxic on HeLa but were found to induce apoptotis on the same cell line; a similar occurrence was reported by Kikuchi et al., (2011). Hydroxycinnamate esters of ursolic acid from whole cranberry fruit and juice that inhibited the growth of several types of tumor cells in vitro, including MCF-7 breast, HT-29 colon, DU-145 prostate, H460 lung, ME180 cervical epidermoid and K562 leukemia cell lines was reported by Murphy et al., (2003).

As represented in Fig. **5.8B**, compounds **C1, C4** and **C6** were very active on HT-29, **C5** was moderately active while **C2** and **C3** were not active on HT-29 colon cancer cell lines. Compounds **C1** and **C4** were more active than Camptothecin. Compound **C4** was not cytotoxic on HT-29 but found to be highly apoptotic. This was synonymous to the findings reported by Kikuchi et al., (2011). On MCF 7 cells (Fig. **5.8C)**, compounds **C1** and **C6** were very active, **C5** was moderately active while **C2**, **C3** and **C4** were partially active on MCF-7 breast cancer cell lines. Compounds **C1** and **C6** actually compare well with Camptothecin activity. In Fig**.5.8D**, it can be clearly seen that compounds **C2**, **C3**, **C4** and **C5** were non toxic to the normal cell line KMST-6. At the highest concentration, the % apoptosis had a sharp rise for **C1**~90% and **C6** ~67% which were consistent with cytotoxicity results.

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5.1.3 Screening isolated compounds C1-C6 for specific markers of apoptosis

Apoptosis in mammalian cells occurs through one of two pathways viz., the cell surface death receptor pathway (extrinsic) or the mitochondria-initiated (intrinsic) pathway. The extrinsic pathway is initiated by cell surface receptor mediated activation of caspase-8 that subsequently transmits the death signal. Caspase 8 is an important initiator of apoptosis and the absence or down regulation of caspase 8 is known to cause resistance to apoptosis (Salvesen, 1999), whereas in the intrinsic pathway, it is marked by loss of mitochondrial integrity and activation of caspase-9. The loss of mitochondrial integrity, a critical event in the induction of mitochondria-mediated intrinsic apoptosis is measured in terms of the bax/bcl ratio, cytochrome-c release and loss of MMP (Kroemer et al., 2007). Loss of MMP and induction of mitochondria-mediated apoptosis could be induced by ROS which are small biochemically reactive oxygen containing molecules (Simon et al., 2000).

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Further in the intrinsic pathway, activated caspase-9 induces downstream caspases (caspase-3, 6 and 7). Caspases in turn cause cleavage of cellular proteins like, poly (ADP-ribose) polymerase (PARP) and activates Caspase-activated DNase (CAD) finally resulting in apoptotic cell death. Apoptotic cells are characterized by hall- marks such as exposure of phosphatidylserine on the outer leaflet of the plasma membrane (Anselmi et al., 2002; Fadok et al., 1998), phosphatidylserine in recognition of apoptotic cells by phagocytes and DNA fragmentation (Nagata, 2000).

5.1.3.1 Intracellular ROS measurement

Reactive oxygen species (ROS) result in oxidation of various cell constituents including DNA, lipids and proteins, consequently these oxidations may cause damage to the cellular substance leading to cell death which is the ultimate consequence. ROS have been implicated in a number of diseases including various forms of non-hormone dependent cancers, atherosclerosis, ischemic reperfusion injury, neurodegenerative diseases, chronic inflammatory disease, such as rheumatoid and psoriatic arthritis, and some factors underlying the aging process itself.

ROS might also play a role as signaling molecules and as such they may have a role in cell cycle progression (Boonstra, 2004). Hancock et al., (2001) also reported that ROS induce programmed cell death or necrosis, may suppress the expression of many genes, and possibly activate cell signaling cascades. Several anticancer agents, such as arsenic trioxide, doxorubicin, bleomycin, cisplatin, 5-Fu, and paclitaxel have been shown to induce ROS generation in cancer cells.

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To investigate whether the cytotoxic effects of isolated compounds were due to apoptosis, the cells were treated with 12.5 µg/ml of compounds for 4 hours and stained the nucleus with NucBlue^{TM} live cell staining dye for 20 mins and then with CM-H_2DCFDA molecular ROS probe for another 30 mins, the untreated HeLa cells showed the stained nuclei to be rounded and homogenously stained (Fig. **5.9**). The treated cancer cells showed different stained DNA nuclei from the control group, by presenting condensed chromatin and apoptotic bodies that are typical of the early and late stages of apoptosis except **C5** which did not induce ROS production.

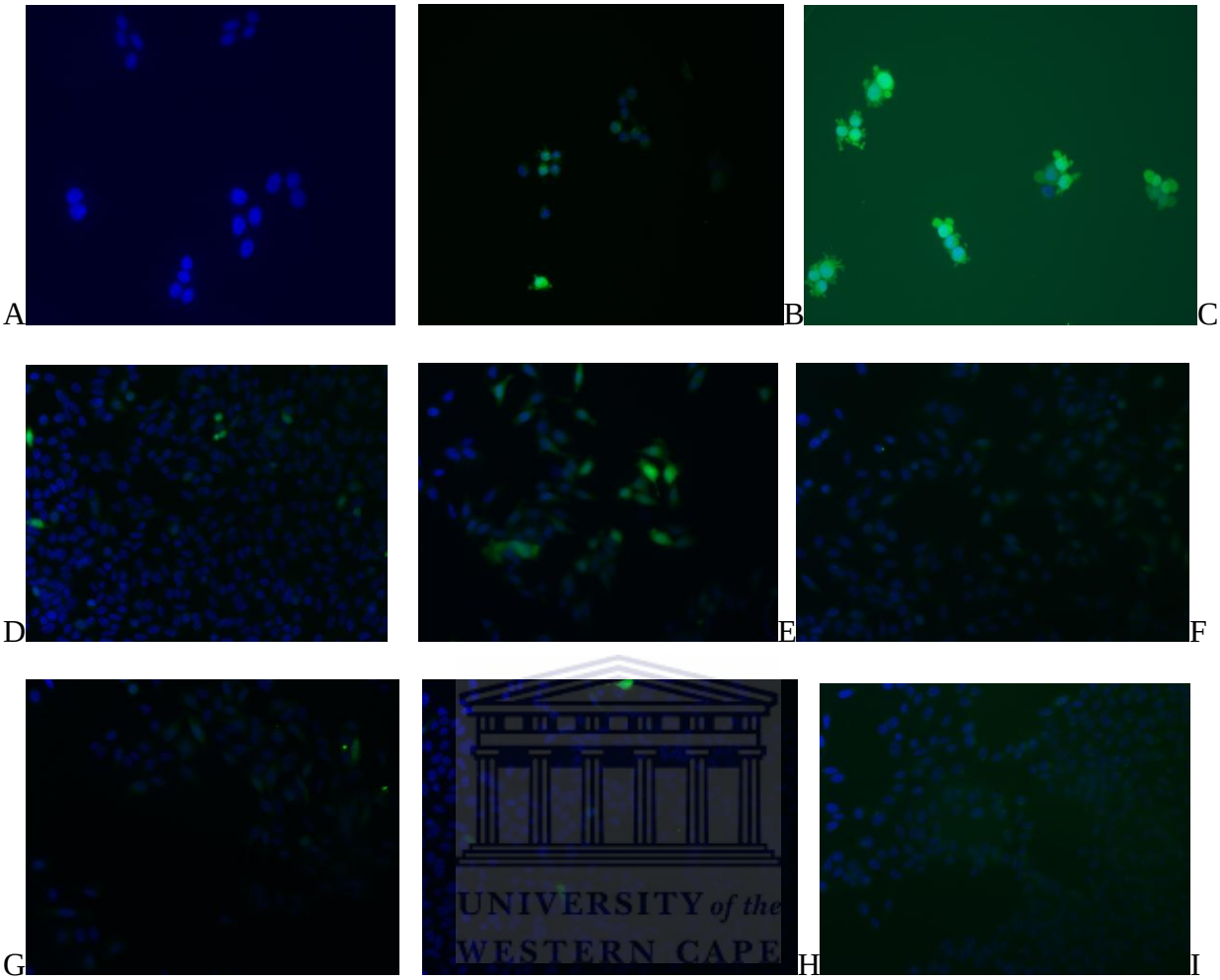

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Figure 5.9. Intracellular ROS production of C1-C6 (4 h) as determined by fluorescence imaging on HeLa A (untreated) B (0.05% H_2O_2) 30min C (0.05% H_2O_2) 1 h D (12.5 µg/ml C1) E (12.5 µg/ml C2) F (12.5 µg/ml C3) G (12.5 µg/ml C4) H (12.5 µg/ml C5) I (12.5 µg/ml C6).

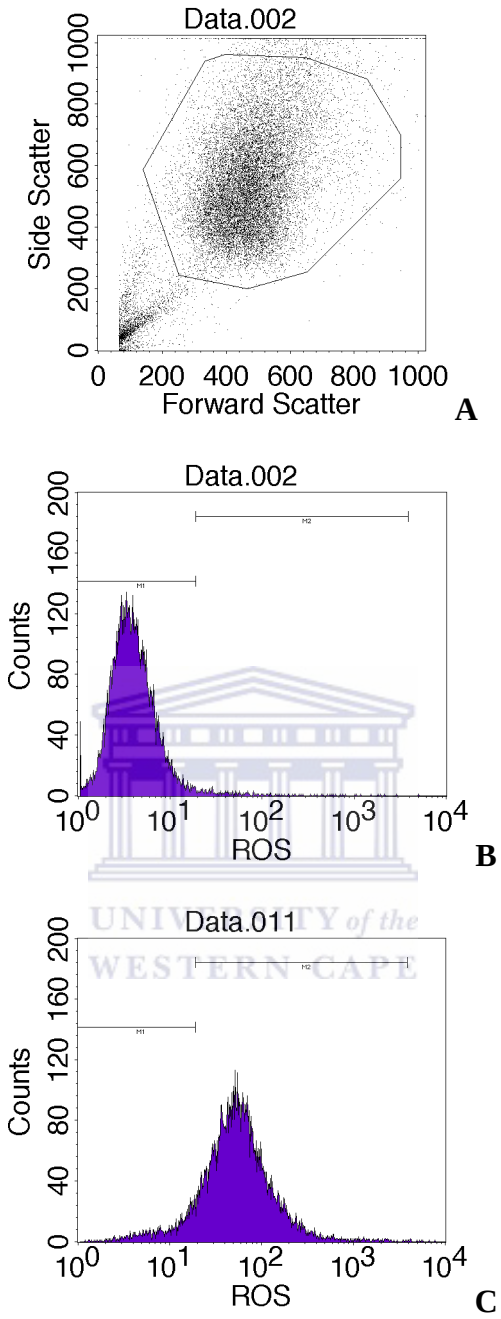

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Figure 5.10. Flow cytometry diagram showing the quantification of ROS as measured by flow cytometer A (Untreated dot plot) B (Untreated histogram plot) (C212.5 µg/ml) on HeLa for 24 h.

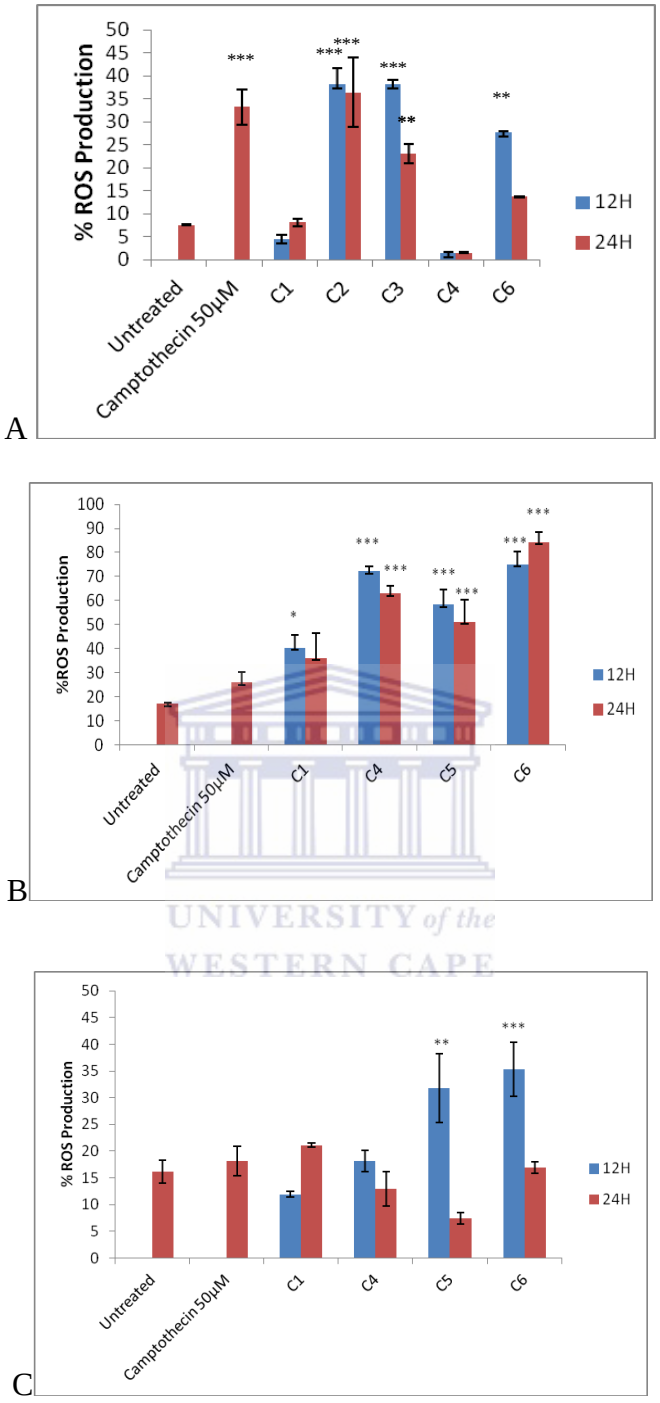

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Figure 5.11. Effect of some selected compounds ROS generation on (A)HeLa (12.5 µg/ml) (B) HT-29(25 µg/ml) (C) MCF-7(25 µg/ml) cell lines in a time-dependent manner. Data are mean ± SEM, ***P< 0.001,P< 0.01 and *P< 0.05 compared with control.**

To investigate the mechanism underlying **C1-C6** induced apoptosis, we measured their ability to generate intracellular ROS using the CM-H_2DCFDA molecular probe after 24 h of treatment as compared with the control (Fig. **5.10**). The measurement of intracellular ROS level for **C2, C3** and **C6** on HeLa cell lines showed a significant increase in ROS level after 12 and 24 h of treatment except **C6** that showed only for 12 h as compared with the control, a similar trend was also observed for 50 µM Camptothecin. However, in the case of **C1** and **C4**, there was no significant effect observed P>0.05 (Fig.**5.11A**).

The time-dependent ROS generation at 25 µg/ml dose of tested compounds **C1**, **C4**, **C5** and **C6** ROS production on HT-29 cell lines on CM-H_2DCFDA molecular probe is presented in Fig. **5.11B** after a 24 h treatment. All tested compounds increased ROS production,the highest ROS producer on HT-29 was **C6** while the least being **C1** when compared to untreated control.

The result showed a time-dependent ROS production especially at 12 h, when most of the cells are still active to interact with the ROS probe. The effect of 24 h treatment of **C1**, **C4**, **C5** and **C6** ROS production on MCF-7 cell lines on CM-H_2DCFDA molecular probe is presented in (Fig. **5.11C**). All tested compounds increased ROS production when compared to the untreated control with the highest ROS producer on HT-29 being **C6**. There was also a sharp decrease in the ROS production after 24 h in **C5** and **C6**. This may be due to the fact that most of the cells would have died at that time.

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5.1.3.2 Caspase 3/7 and 9 measurement

Caspases (cysteinyl-directed aspartate-specific proteases) are a family of enzymes that play a central role in the apoptotic process and result in the cleavage of protein substrates causing the disassembly of the cell. Caspase 3 and caspase 7 are “executioner caspases” that are activated

downstream in the apoptosis cascade by a sequence of intrinsic or extrinsic signals. Once activated, these enzymes cause degradation of many key cellular proteins and influence chromatin condensation and DNA damage during apoptosis. Activation of caspase-3/7 is thus a hallmark and confirmation of the apoptotic process (Boland et al., 2013). The Caspase-Glo 3/7 Assay System contains a single reagent solution which includes a luminogenic substrate for caspases 3 and 7, a thermostable luciferase, and buffer to induce cell lysis.

The presence of caspase 3 or 7 cleaves the luminogenic substrate which in turn leads to the release of the substrate for luciferase resulting in the production of light. The amount of fluorescent product generated is proportional to the amount of caspase-3/7 cleavage activity present in the sample (Scott, 2008).

The activation of caspases, which are key mediators of apoptosis was analyzed upon the exposure of various concentrations (6.25-50 µg/ml) of **C1-C6** to HeLa, HT-29 and MCF-7 cells for 24 hours and the caspases 3/7 and 9 were measured using a muti-well plate reader.

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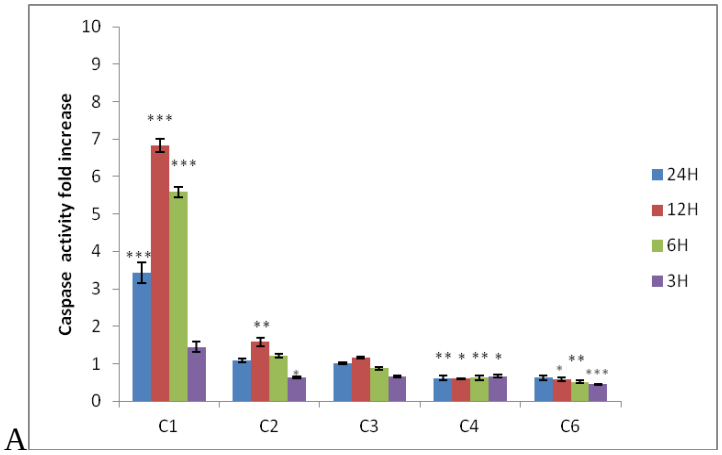
The results were compared to an untreated controlwhich was set arbitrarily at 1.0. It was observed that some compounds showed an increased caspase activity in a concentration and time-dependent manner (Fig.**5.12**).



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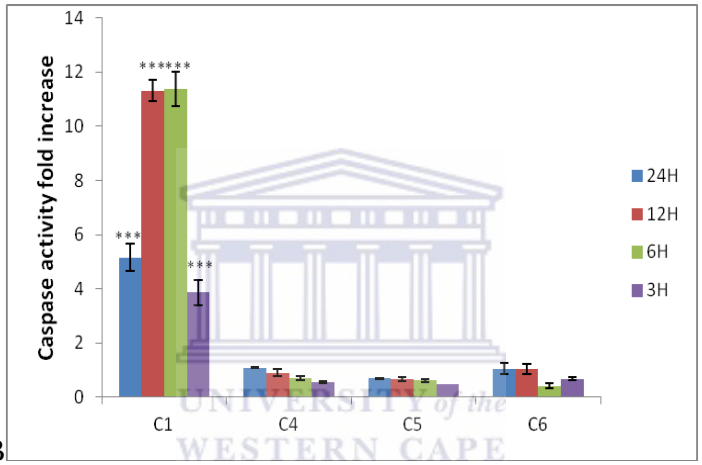

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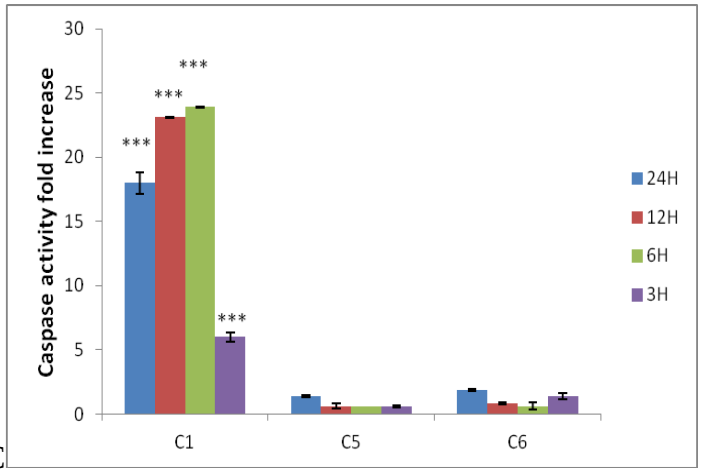

C

Figure 5.12. Measurement of caspase3/7 activity at 50µg/ml.Time-dependent induction of selected compounds on caspase-3/7 activation in (A) HeLa (B) HT-29 (C) MCF-7cell lines. Data are mean±SEM, ***P< 0.001, **P< 0.01 and *P< 0.05 compared with control.

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The result of time-depedent caspase3/7 activity on HeLa cell line is presented in Figure **5.12A.** Compounds **C1** and **C2** significantly activated caspase 3/7, P<0.001 and P<0.001 respectively when compared with control. Ursolic acid and derivatives have been reported to activate caspase 3 (Shao et al., 2011). Figure **5.12B** showed the result of time – dependent caspase 3/7 activity on HT-29 cell lines when treated with 50 µg/ml of **C1**, **C4**, **C5** and **C6** at various time intervals. Only **C1** significantly increased caspase 3/7 when compared with an untreated control. This is in agreement with the report of Andersson et al., (2003) that ursolic acid decreased proliferation by induction of apoptosis accompanied by activation of caspases-3, 8 and 9 in HT-29 colon cells.

The result of time – dependent caspase 3/7 activity on MCF-7 cell lines when treated with 50 µg/ml of **C1**,**C5** and **C6** at various time intervals indicated that **C1** significantly increased caspase 3/7 when compared with control (Fig. **5.12C**)**.**

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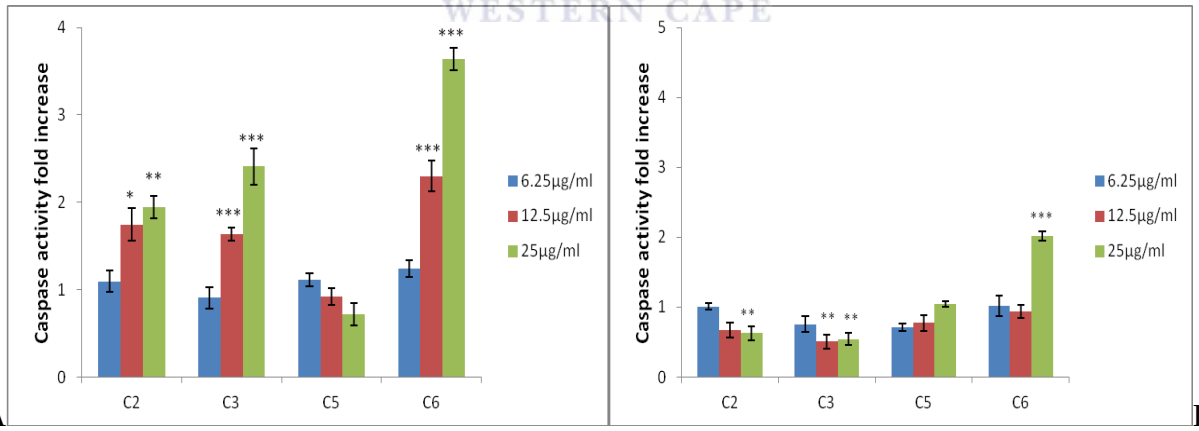

Figure 5.13. Measurement of caspase3/7 activity. Dose-dependent induction of caspase-3/7 of selected compounds in (A) HeLa (B) MCF-7 cell lines. Data are presented as mean±SEM, ***P< 0.001, **P< 0.01 and *P< 0.05 compared with control.

Compounds **C2**, **C3**, **C5** and **C6** were further investigated for their dose-response on caspase3/7 activity at different doses(6.25-25 µg/ml) to check their dependability for 24 h (Fig.**5.13**).

All compounds tested showed a significant increased caspase activity on HeLa in a concentration –dependent manner when compared with the control at doses 12.5 and 25 µg/ml with the exception of **C5** (Fig.**5.13A**). This is an indication that cell apoptosis took place, since caspase-3/7 activation is a hallmark and confirmation of the apoptotic process.

Further to this, compounds **C2**, **C3**, **C5** and **C6** were also investigated for their dose-response on caspase 3/7 activity at different doses(6.25-25 µg/ml) for 24 h on MCF-7 (Fig.**5.13B**). All the tested compounds did not show a significant increased caspase activity on MCF-7 ($P>0.05$) in a concentration –dependent manner when compared with the control with the exception of **C6** which was very significant even at 25 µg/ml ($P< 0.001$) . MCF-7 is deficient in caspase 3 and it is thus more likely that it is executioner caspase 7 that is being activated in this instance.

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MCF-7 has been characterized with caspase 3 gene mutation (Zhao et al., 2001). As described earlier in chapter **2**, Section **2.18** that the extrinsic and intrinsic pathways for apotosis commonly converge at caspase 3. This is one of the reasons why the presence of caspase 3 is recognized as the hallmark for apoptosis and accounts for the high resistance to apoptosis inducers displayed by the MCF-7 cell lines. Since compound **C5** is selective for MCF-7, it can be deduced that **C5** can induce an apoptotic pathway independent of caspase 3.

Based on the findings from this research, compounds **C2** and **C3** are selective majorly for HeLa, which in turn implies that they might have both caspase 3 dependent and independent modes of action, similarly **C1**, **C4** and **C6** which were non selective for a specific cell line.

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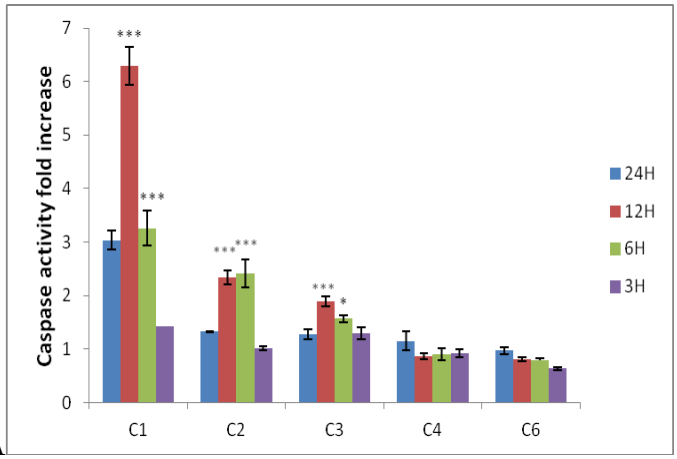

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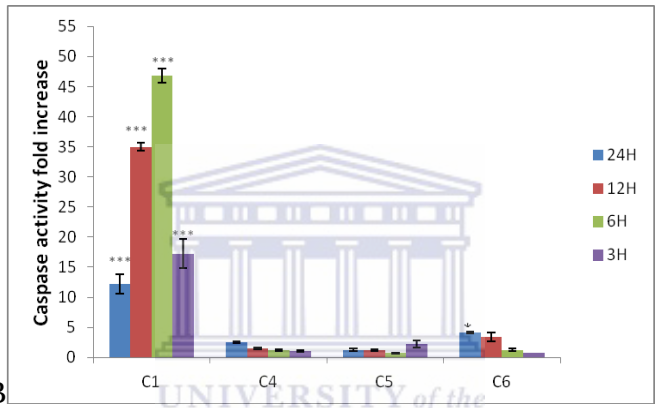

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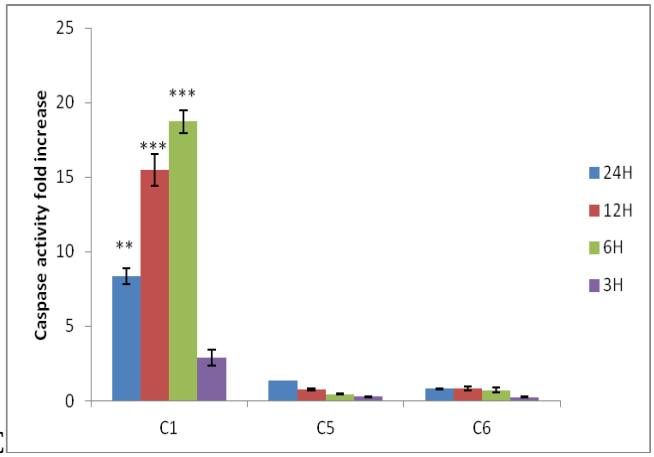

Figure 5.14. Measurement of caspase 9 activity at 50µg/ml. Time-dependent induction of caspase-9 on (A) HeLa (B) HT-29 (C) MCF-7 cell lines. Data are presented as mean±SEM,*P< 0.001, **P< 0.01 and *P< 0.05 compared with control.**

The occurrence of the apototic process was further confirmed by the presense of activation of caspase – 9 which triggers the proteolytic activation of executioner caspase3/7 and caspase 8 in a process in the cleavage of PARP, then subsequent DNA degragation and finally apoptotic death (Cecconi and Gruss, 2001; Cain et al., 2002).

Compounds **C1**, **C2**, **C3**, **C4** and **C6** at 50 µg/ml were tested for induction of caspase 9 activity on HeLa cell lines after treatments at different time intervals. It was observed that only **C1**, **C2** and **C3** significantly increased caspase activation at 12 h and 6 h of treatment as shown in Fig. **5.14A**. This may be partly due to the fact that the proteins would still be active and not denatured at these time points.

The result of caspase 9 activation on HT-29 are presented in Fig **5.14B**. Cells were exposed to 50 µg/ml of **C1**, **C4**, **C5** and **C6** at different time points (3, 6, 12 and 24 h).

Compound **C1** significantly increased caspase activation compared with the control. At $P< 0.05$, **C6** also displayed a slight increase in caspase activation within 24 h of treatment.

Ursolic acid's ability to induce apoptosis in many different cell types is likely to play a major role in its anti-proliferative activity. In HT-29 colon cells, ursolic acid has been reported to decrease proliferation by induction of apoptosis accompanied by activation of caspases-3, 8 and 9 (Andersson et al., 2003).

The result of the time dependent caspase 9 activation on MCF-7 cell lines is shown in Fig. **5.14C.** Cells were exposed to 50 µg/ml of **C1**,**C5** and **C6** at different time points (3, 6, 12 and 24 h). The level of caspase 9 activation were compared with the untreated control arbitrarily set at 1.0. The result showed that only **C1** showed a markedly increase in caspase 9 activation, although **C5** showed 1.3 fold increase, but was not significant at $P>0.05$.

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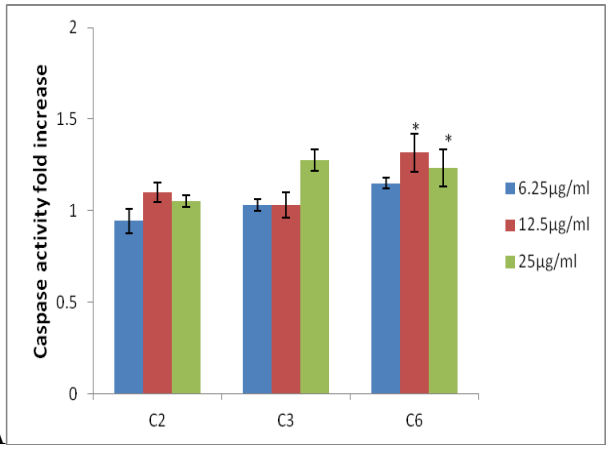

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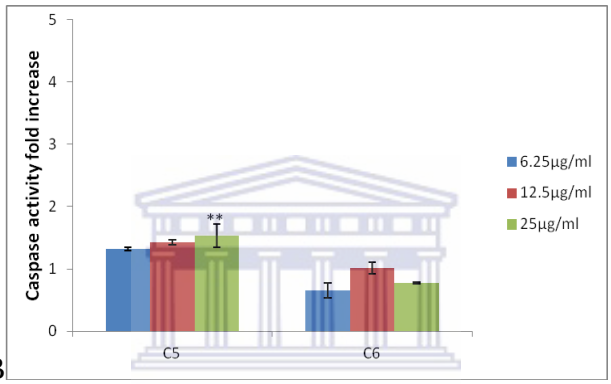

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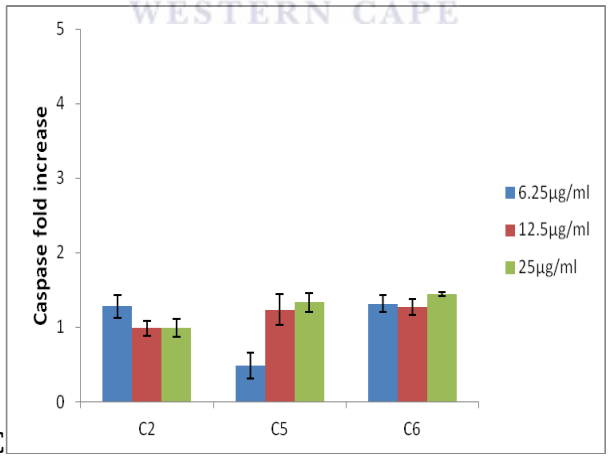

Figure 5.15. Measurement of caspase 9 activity. Dose-dependent induction of caspase-9 selected compounds in (A) HeLa (B) HT-29 (C) MCF-7 cell lines. Data are mean±SEM, **P< 0.01 and *P< 0.05 compared with control.

The investigation of different doses of **C2**, **C3** and **C6** on caspase 9 activation for 24 h showed that compounds **C2** and **C3** slightly increased caspase activity as compared to the control, but the differences were not that significant. The reason may be that at lower doses, **C2** and **C3** do not activate caspase while they do at higher concentration as shown in Fig. **5.14A**. It could also be there is a time dependent dynamic at play between the 6 and 12 h intervals. Of all the three compounds, only **C6** acivated caspase 9 at 24 h (12.5 and 25 µg/ml) $P< 0.05$ on HeLa (Fig **5.15A**).

We also investigated the effect of the dose-response of compounds **C5** and **C6** on caspase 9 activity on HT-29 for 24 h, in which case only compound **C5** increased caspase 9 activation by an ~1.8 fold increase at 25 µg/ml ($P< 0.01$) as shown in Fig. **5.15B**. The effect of dose-response of **C2**, **C5** and **C6** on MCF-7 cell line as shown below in Fig. **5.15C** indicated there was no significant difference observed for all compounds when compared with the control ($P>0.05$) at doses of 6.25, 12.5 and 25 µg/ml.

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5.1.3.3 Investigation of Topoisomerase inhibition

Topoisomerase are essential enzymes that control and modify the topological state of DNA. These enzymes act by sequential breakage and reunion of either one DNA strand

(topoisomerase I) or both DNA strands (topoisomerase II) .Topoisomerase-mediated strand passing leads to the reduction of DNA twists, as well as the relief of supercoiling, thereby allowing replication, transcription, and recombinant repair to take place (Burden and Osheroff, 1998; Pommier et al., 1998).

Topoisomerase inhibitors are agents designed to interfere with the action of topoisomerase enzymes (topoisomerase I and II) which control the changes in DNA structure by catalyzing the

breaking and rejoining of the phosphodiester backbone of DNA strands during the normal cell cycle (Ting et al., 2003).

In recent years, topoisomerases have become popular targets for cancer chemotherapy treatments (Rothenberg, 1997). It is thought that topoisomerase inhibitors block the ligation step of the cell cycle, generating single and double stranded breaks that harm the integrity of the genome and thus the introduction of these breaks subsequently leads to apoptosis and cell death.

The catalytic cycle of human topoisomerases consists of several distinct steps. Compounds such as camptothecin and etoposide interfere with the relegation step and stabilize the enzyme-DNA cleavable complex. These compounds are known as topoisomerase poisons because their action results in an alteration of topoisomerase function leading to DNA breakage (Capranico et al., 1997).

To further elucidate the mechanism of action of isolated compounds, we investigated whether our compounds are such poisons by measuring formation of topoisomerase-induced DNA-strand breaks. Figure **5.16** shows as expected, that camptothecin stabilized the topoisomerase I cleavable complex, resulting in the generation of open-circle plasmid DNA at 150 µM.

To our surprise, all the tested compounds inhibited topoisomerase I at different concentrations; viz **C1** (15 µg/ml and **C2**, **C3, C5** and **C6** (40 µg/ml).

Ursolic acid and other pentacyclic triterpenoids has been reported to inhibit topoisomerase I (Syrovets et al., 2000).

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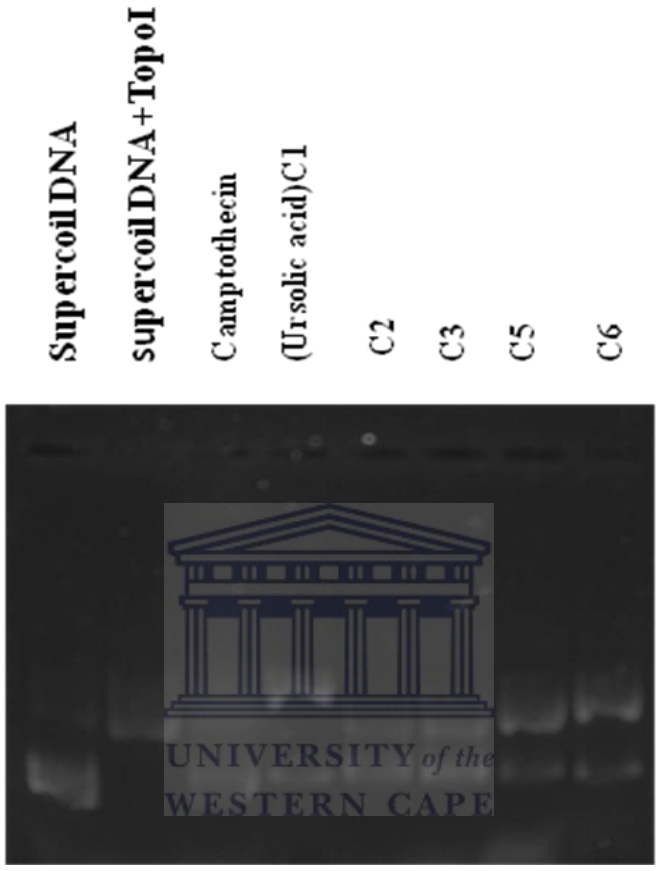

Figure 5.16. Inhibitory effects of isolated compounds on the catalytic activity of topoisomerase I. DNA relaxation assay showing the inhibitory effects of compounds C1, C2, C3, C5 and C6 on activity of topoisomerase I, control samples contained super coiled plasmid DNA (lane 1) and DNA incubated with 2 U of enzyme(lane 2) for 30 min at 37°C. One of three comparable experiments is shown.

5.1.3.4. Investigation of cell cycle distribution

Cell division cycle is a process of replicating DNA and dividing a cell in a series of coordinated events. At least two types of cell cycle control mechanisms are recognized; a cascade of protein phosphorylations that relay a cell from one stage to the next and a set of checkpoints that monitor

completion of critical events and delay progression to the next stage if necessary. The first type of control involves a highly regulated kinase family.

Kinase activation generally requires association with a second subunit that is transiently expressed at the appropriate period of the cell cycle; the periodic "cyclin" subunit associates with its partner "cyclin-dependent kinase" (CDK) to create an active complex with unique substrate specificity. Regulatory phosphorylation and dephosphorylation fine-tune the activity of CDK–cyclin complexes, ensuring well-delineated transitions between cell cycle stages (Collins et al., 1997).

A second type of cell cycle regulation, checkpoint control, is more supervisory and it is not an essential part of the cycle progression machinery.

Cell cycle checkpoints sense flaws in critical events such as DNA replication and chromosome segregation .When checkpoints are activated, for example by underreplicated or damaged DNA, signals are relayed to the cell cycle-progression machinery. These signals cause a delay in cycle progression, until the danger of mutation has been averted (Collins et al., 1997).

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The cell cycle checkpoints are the pathways which regulate the completion of specific events in one phase of cell cycle before entering the next phase during the eukaryotic cell cycle progression. These checkpoints also serve as sites where cells can arrest to repair any damage, responding to an exogenous cellular stress signal and making use of essential growth factors, hormones or nutrients. If the cells fail to repair the damage during arrest at checkpoints, it could activate the pathways leading to apoptosis and the defects in cell cycle checkpoints have been reported to result in gene mutations, chromosome damage and ultimately contribute to tumorigenesis (Schafer, 1998).

DNA damaging agents trigger checkpoints that produce arrest in G1 and G2 stages of the cell

cycle. The G1 and G2 phases of the cycle represented the “gaps” in the cell cycle that occur between the two obvious landmarks, DNA synthesis and mitosis. In the first gap, G1 phase, the cell prepares for DNA synthesis. S phase cells are synthesizing DNA and therefore have double DNA content. Cells can also arrest in S phase, which leads to a prolonged S phase with slowed DNA synthesis. The G2 phase is the second gap in the cell cycle during which the cell prepares for mitosis or M phase. Arrest in G1, allows repair before DNA replication, whereas arrest in G2, allows repair before chromosome separation in mitosis (Poot et al., 1995).

The cells which are actively undergoing cell cycle are targeted in cancer therapy as the DNA is relatively exposed during cell division and hence susceptible to damage by drugs or radiation, gaining insight into the mechanisms and alterations by which components of the apoptotic machinery contribute to pathogenic processes should allow the development of more effective, highly specific and therefore better-tolerable therapeutic approaches. These includes; targeted activation of pro-apoptotic tumour suppressors or alternatively the blockade of antiapoptotic oncogenes in the case of cancer, whereas for the treatment of premature cell death during neurodegeneration the inhibition of pro-apoptotic key components such as the caspases might be promising (Reed, 2002).

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To determine the possible role of cell cycle arrest in compounds **C1-C6**, HeLa, HT-29 and MCF-7 cells were treated with different concentrations of the compounds.

Cell cycle distribution was observed by flow cytometric analysis after staining of the DNA with propidium Iodide (PI) and the results are shown below in Fig.**5.19-5.21** while the measurement as determined by flow cytometry was demonstrated in Fig. **5.17-5.18**.

The result on the effect of compounds on cell cycle distribution on HeLa cells showed that at the concentrations used in this assay, compounds **C2**, **C3**, **C5** and **C6** caused a significant change in cell cycle phases G1 and S when compared with the control at P<0.001 (Fig.**5.22**).

C1 did not show any remarkable diferrence in comparison with the control, this could be as a result of the low concentration. On HT-29 cells, only **C2** and **C3**, significantly affected the cell cycle at the S phase P< 0.05(Fig.**5.22**). On MCF-7 cells, the treatment with compounds **C2**, **C3** and **C5** resulted in a significant change (P<0.001) in the number of cells in G1 and S phases while there was no significant difference observed for **C1** and **C4** in any of the phases when compared with control (Fig.**5.22**). Ursolic acid has been reported to induce apoptosis and growth inhibition at the G1 phase of the cell cycle in some cancer cells (Heo et al., 2002).

In this research we have been able to further confirm the ursolic acid ability to induce apoptosis.

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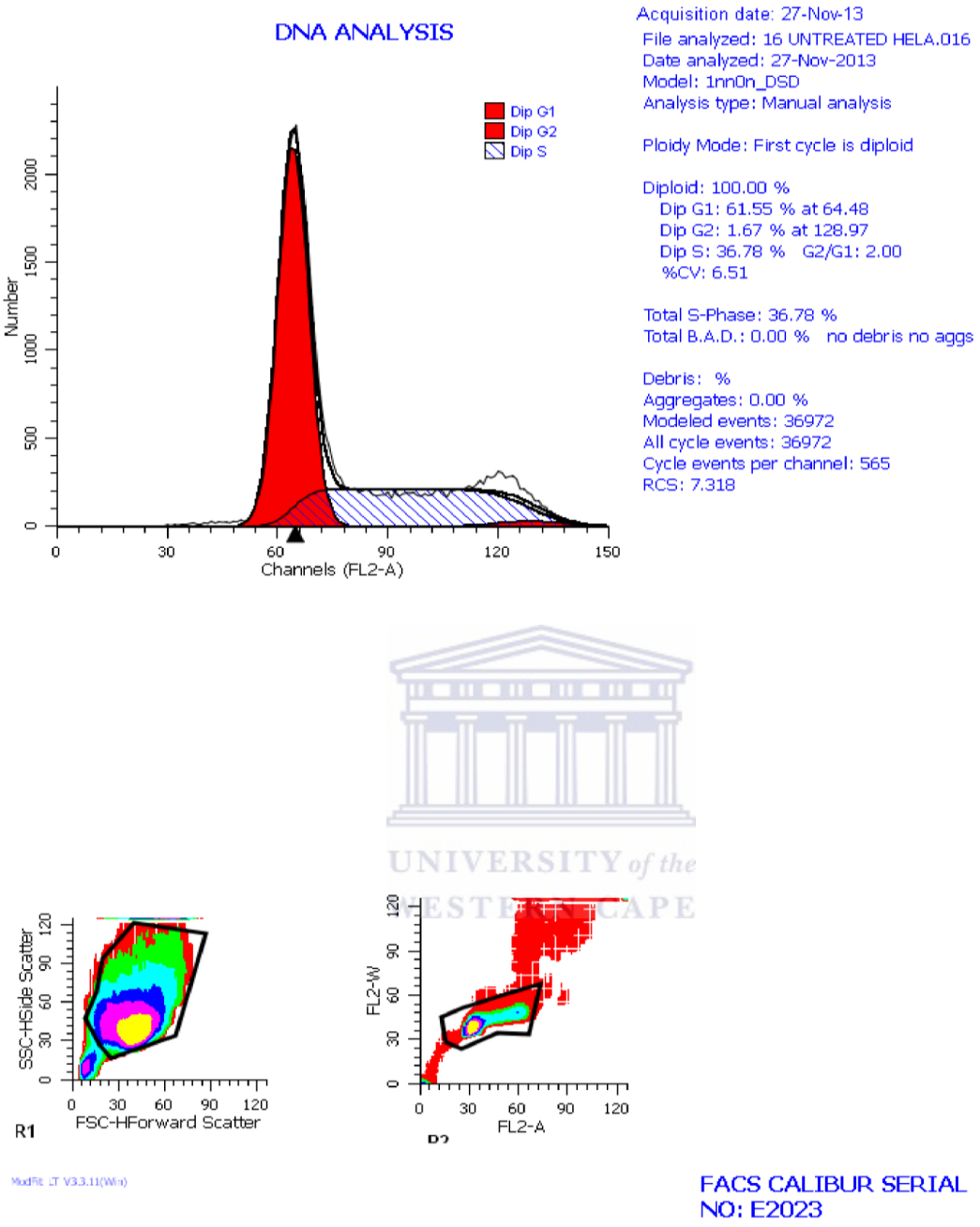

Figure 5.17. Cell cycle distribution phase of untreated control on HeLa cells.

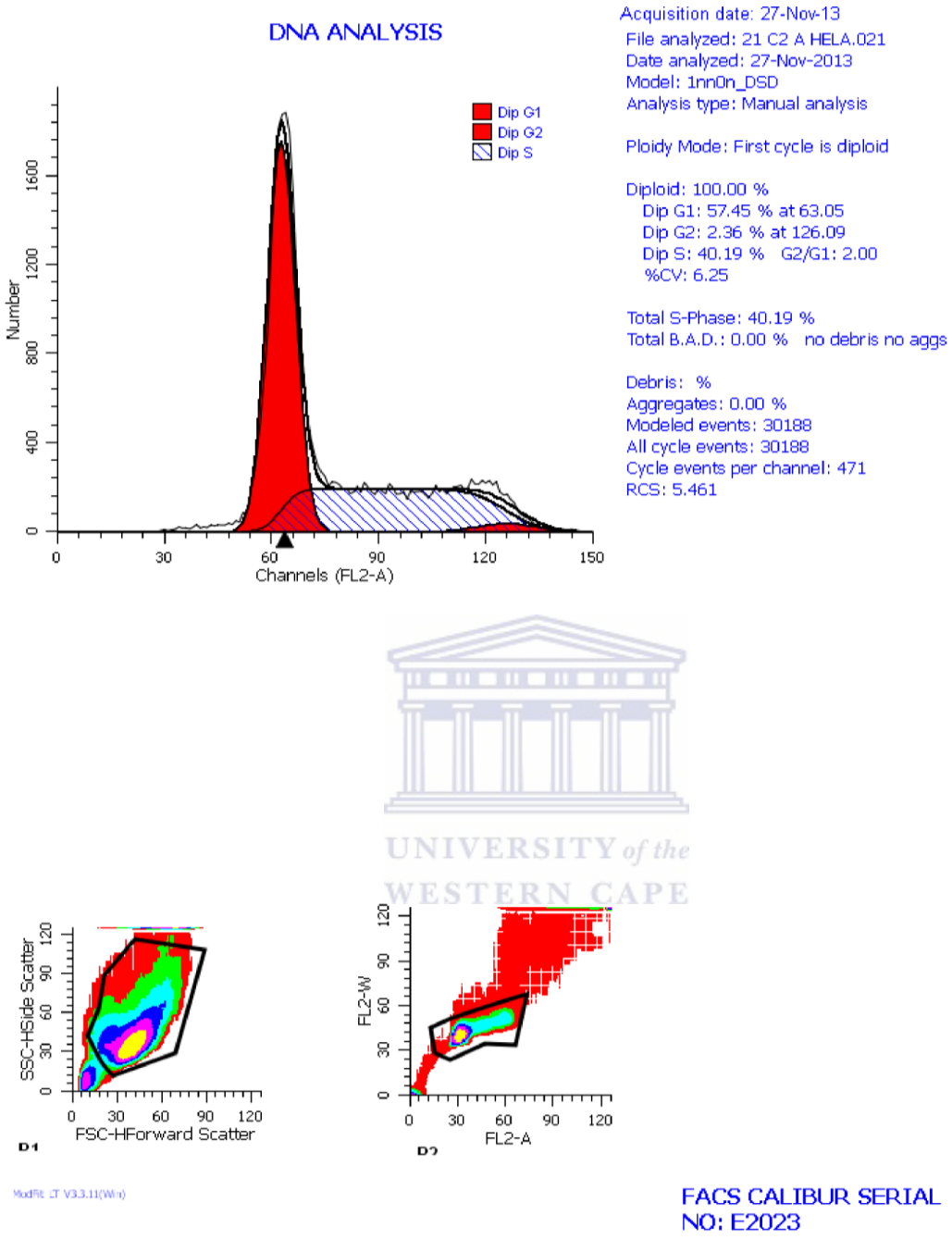

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Figure 5.18. Effect of C2 (12.5 µg/ml) on cell cycle distribution phase on HeLa cells as measured by flow cytometry.

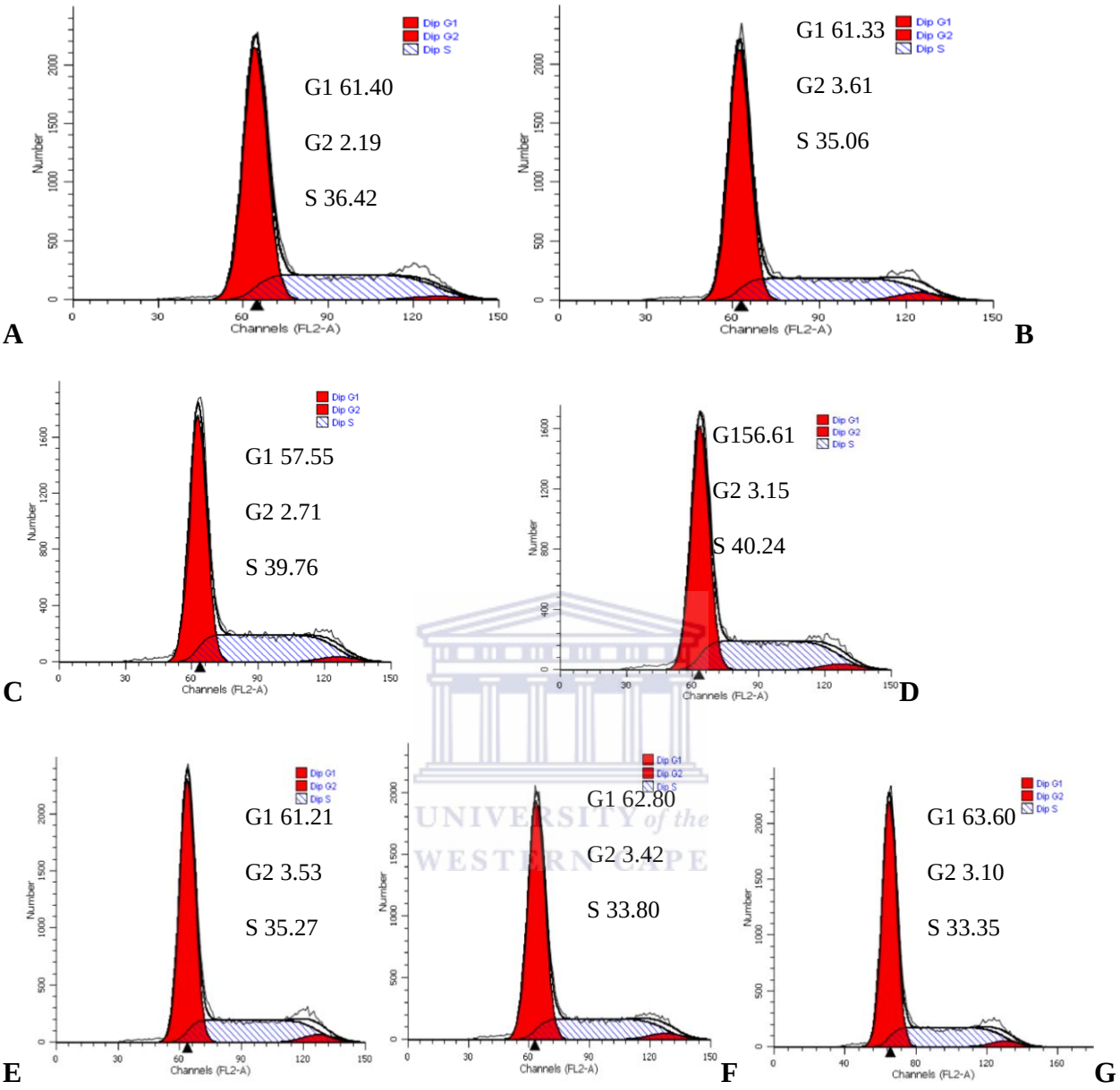

Figure 5.19. Effect of compounds on cell cycle phases of HeLa cells at 24h (A). Untreated control (B). C1 (6.25 µg/ml) (C). C2 (12.5 µg/ml) (D). C3 (12.5 µg/ml) (E). C4 (12.5 µg/ml) (F). C5 (12.5 µg/ml) (G) C6 (6.25 µg/ml). Data are expressed as mean±SEM as determined by flow cytometry.

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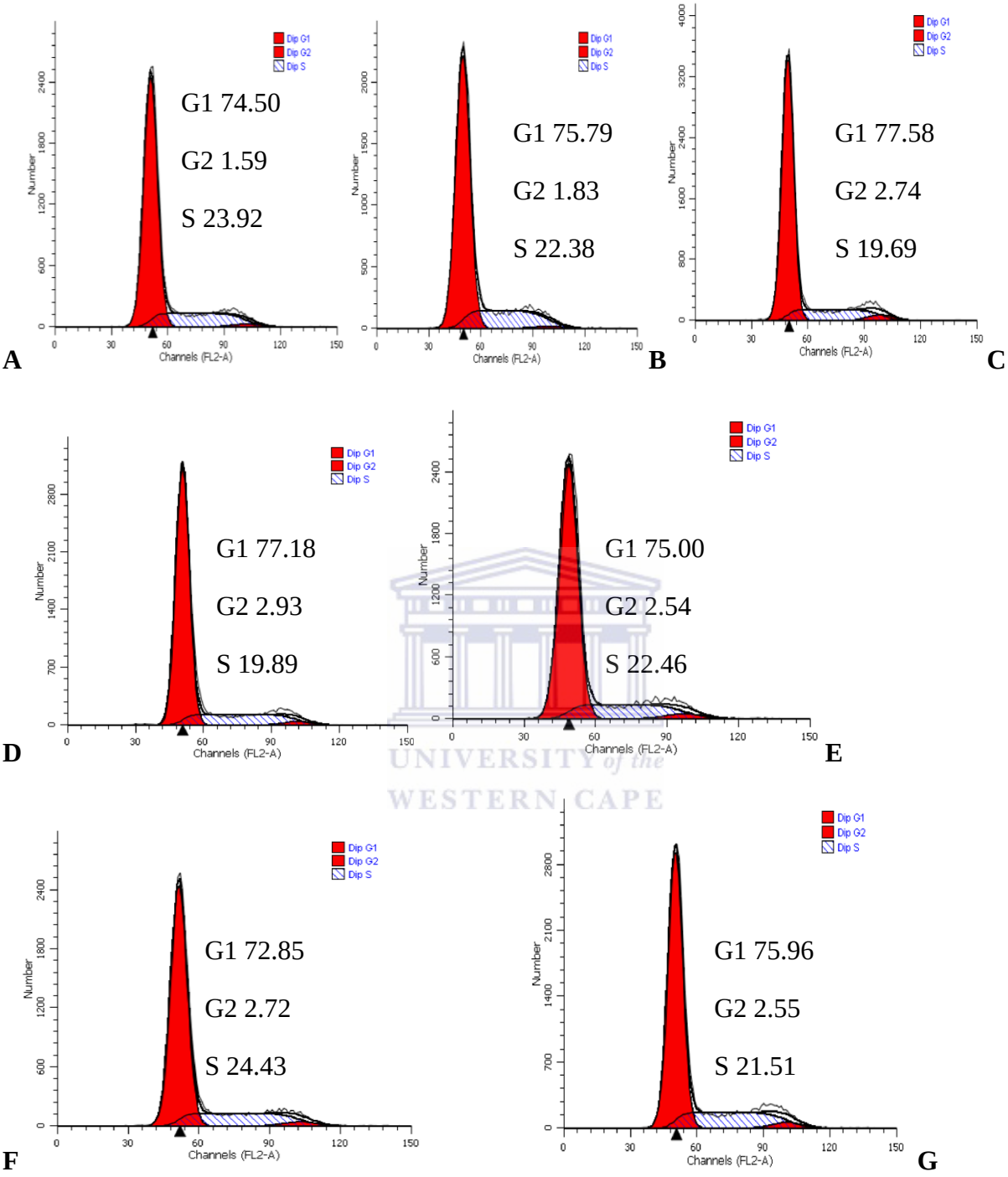

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Figure 5.20. Effect of compounds on cell cycle phases of HT-29 cells at 24 h (A). Untreated control (B). C1(6.25 µg/ml) (C). C2 (12.5 µg/ml) (D). C3 (12.5 µg/ml) (E). C4 (12.5 µg/ml) (F). C5 (12.5 µg/ml) (G) C6. (6.25 µg/ml). Data are expressed as mean±SEM as determined by flow cytometry.

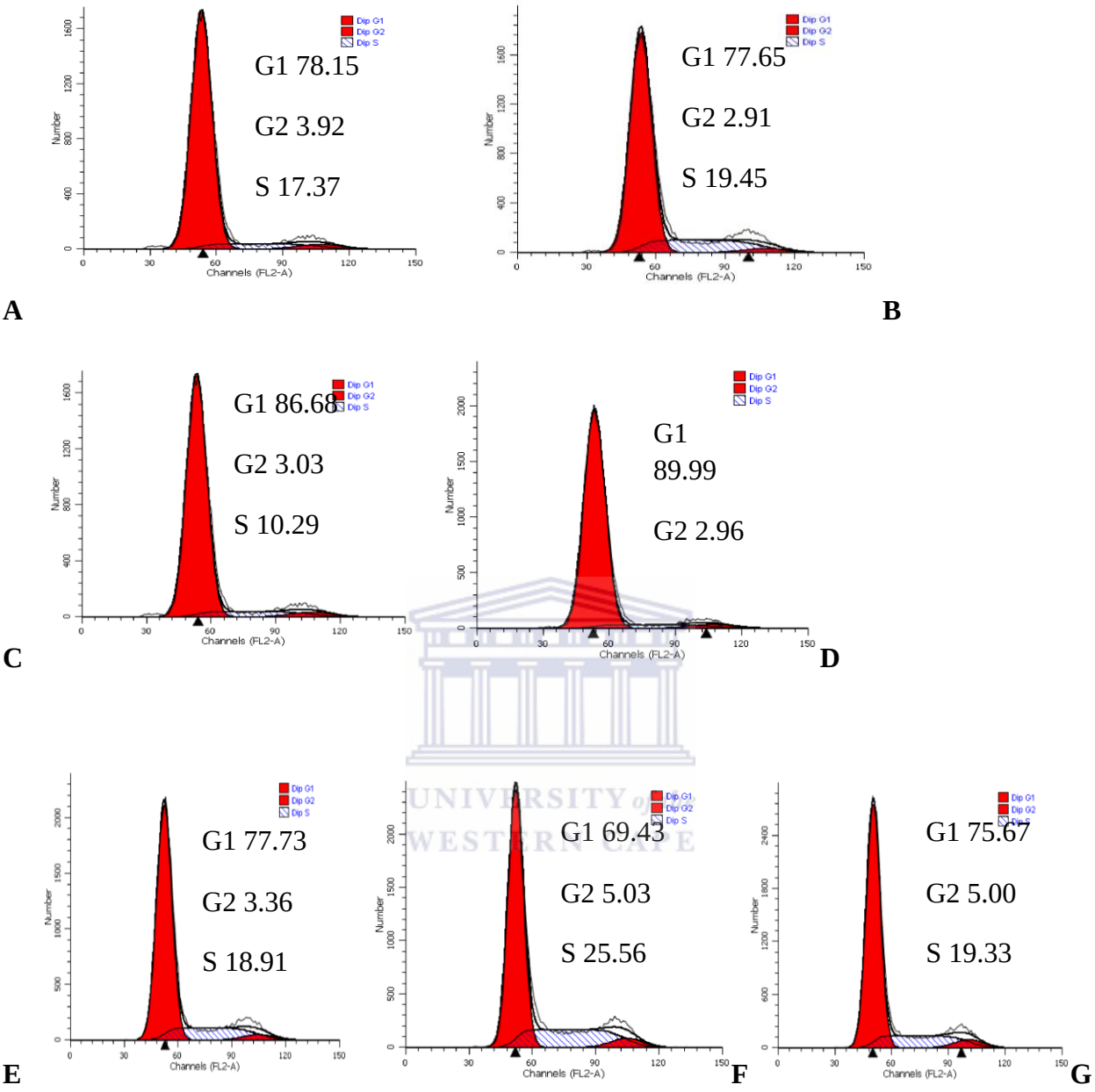

Figure 5.21. Effect of compounds on cell cycle phases of MCF-7 cells at 24 h (A). Untreated control (B). C1 (6.25 µg/ml) (C). C2 (12.5 µg/ml) (D). C3(12.5 µg/ml) (E). C4 (12.5 µg/ml) (F). C5 (12.5 µg/ml) (G). C6 (6.25 µg/ml). Data are expressed as mean±SEM as determined by flow cytometry.

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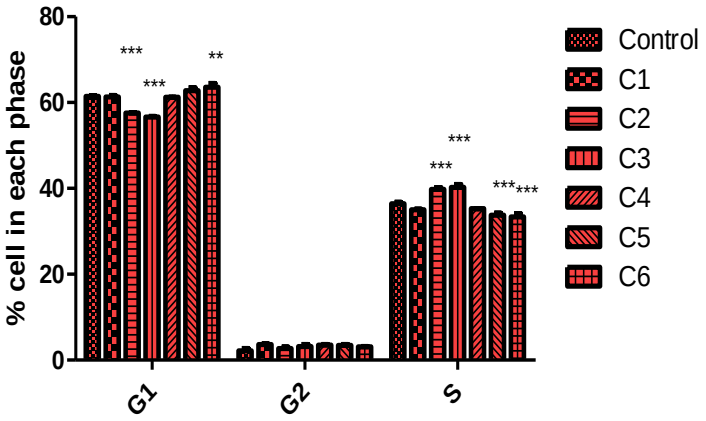
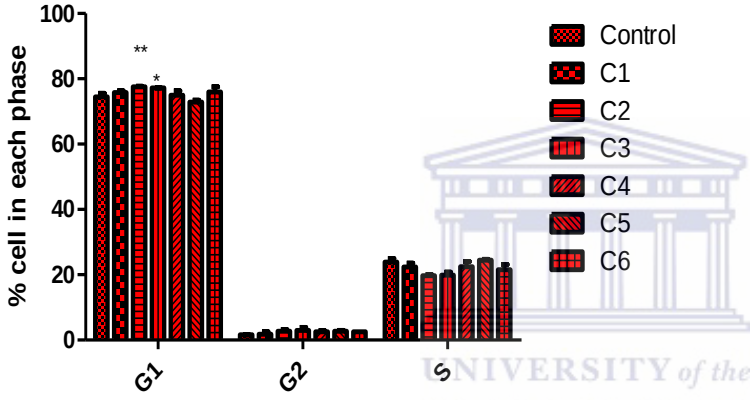


A



B

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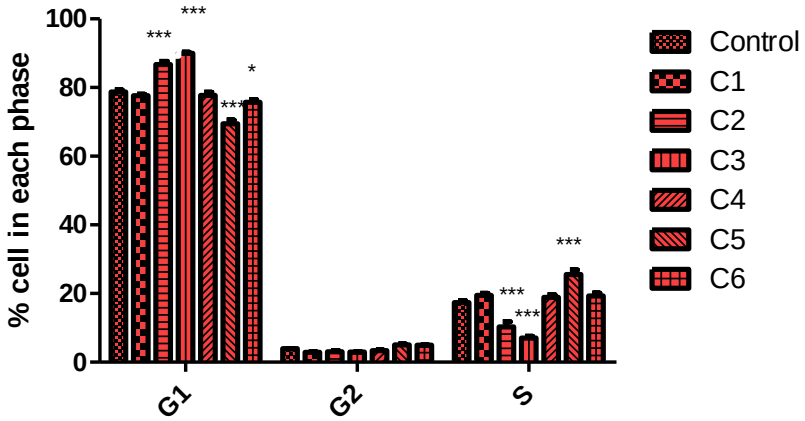

C

Figure 5.22. Dose-response effect of compounds on cell cycle phases at 24 h (A). HeLa cells (B). HT-29 cells (C). MCF-7 cells. Data are expressed as mean±SEM ***P < 0.001, **P < 0.01 and *P < 0.05 when compared with control.

The result on the effect of compounds on cell cycle distribution on HeLa cells further showed that at the concentrations used in this assay and when compared with the control; compounds **C2**, **C3** caused a significant increase in the number of cells in S phase while **C6** showed an increase in G1 phase at P<0.001 (Fig.**5.22A**). This implies that **C2**, **C3** arrested the cell cycle at S phase while **C6** arrested at G1 phase.

On HT-29 cells, there was no significant difference between the number of cells in the control and the treated at G1 phase, this may be that the cells managed to repair the damaged at the first check point in the cell cycle. Only **C2** and **C3**, caused a slight decrease in the number of cells at the S phase (Fig.**5.22B**).

On MCF-7 cells, the treatment with compounds **C2** and **C3** resulted in increase of cells in G1 phase and a decrease in S phase while on **C5**, a decrease was observed in G1 phase and an increase in S phase. There was no significant difference observed for **C1** and **C4** in any of the phases when compared with control (Fig.**5.22C**). Our results suggests that on MCF-7 cells, compounds **C2** and **C3** arrested cell cycle at the G1 phase while **C5** arrested at the S phase.

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It has been reported that some anticancer agents cause growth inhibition by interfering with the process of cell cycle (Schwartz and Shah, 2005; Chakravarti et al., 2012; Shao et al., 2011).

CHAPTER 6

6.0 CONCLUSION AND RECOMMENDATIONS

To the best of our knowledge, this is the first report in the literature on the isolation of triterpenes and the bioactivity guided isolation of novel cytotoxic and apoptotic compounds from *Pleiocarpa pycnantha* leaves as anticancer agents. This is the first significant biological activity reported for the plant and the first report on isolation of ursolic acid and derivatives from *Pleiocarpa* genus. It may also be of interest to know that this is the first report on the isolation of seco-triterpenes from the Apocynaceae family; thus making it an important contribution to scientific knowledge.

The GC/MS and the phytochemical screening results revealed the presence of triterpenes, saponin, glycosides, essential oils and others were mostly saturated fatty acids and methyl esters, the GC-MS result is not shown in the thesis. Flavonoids and alkaloids were notably absent. This study successfully attempted to relate specific constituents present in these plants with their widespread ethno medicinal uses.

Pleiocarpa pycnantha leaves were extracted with 95% ethanol, to mimic the preparation of herbs and decoctions in some African countries. The fractionation of the plant material was carried out using standard chromatographic techniques. A total of eight (8) compounds were isolated from the plant, while only 5 of them, from which two were novel were characterized and their structures were unambiguously established by spectroscopic methods including infrared and ultraviolet spectroscopy, high resolution mass spectrometry, one- and two-dimensional nuclear magnetic resonance experiment and single X-ray crystallography.

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Two of the remaining three isolates were inconclusive in terms of structural elucidation due to their very low quantity and the fact that **C4** was a mixture of two compounds which we were unable to separate.

The total (crude) extract and isolated compounds were tested for cytotoxicity using WST-1 assay on HeLa (cervical), HT-29 (colon), MCF-7 (breast) and KMST-6 (normal/non cancerous) cell lines. The crude extract was cytotoxic on MCF-7 (breast) and HeLa (cervical) cell lines with IC_{50} value of 314.9 µg/ml and 446.2 µg/ml respectively, while it slightly affected the viability of HT-29 colon cancer cell line (IC_{50} 703.0 µg/ml). The extract did not significantly affect the viability of the normal (KMST-6) cell line, (IC_{50} 1904.0 µg/ml).

Fraction P4 displayed significant cytotoxicity with % viability on breast, cervical and colon cancer cell lines being 2.747± 0.08, 6.423± 0.99 and 3.704± 0.83 respectively at 0.4 mg/ml while on normal cell line is 95.867± 1.91. The highest cytotoxicity was displayed by ursolic acid **C1** with IC_{50} of 5.06, 5.12 and 9.51 µg/ml on HeLa, HT-29 and MCF-7 respectively. Compounds C**5** and C**6** displayed cytotoxicity on MCF-7 cell lines with IC_{50} values 10.99 and 5.46 µg/ml respectively. Compound **C2** also displayed moderate cytotoxicity with IC_{50} of 33.20, 34.37 and 68.34 µg/ml on HeLa, MCF-7 and HT- 29 respectively.

The induction of apoptosis using Apo-percentage ^{TM} assay on all cell lines was tested on fractions P4, P7 and P12 representing fractions for isolated compounds to predict the mechanism of action. All tested fractions showed evidence of apoptosis with P7 highly selective for the normal cell line KMST-6. Fraction P4 was apoptotic on all cancer cell lines with maximum killing of ~ 60%, 40% and 82% on HeLa, HT-29 and MCF-7 at the highest dose of 100 µg/ml. This fraction was not selective for the normal cell even at the highest dose; the % apoptosis on KMST-6 was ~15%. Fraction P12, though not cytotoxic on most of the cell lines, displayed a

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very good dose-response on Apopercentage^{TM} assay. It had a maximum killing at the highest dose of ~95%, 98% and 85% on HeLa, HT-29 and MCF-7 respectively. Fraction **P12** was selective for the normal cell line at this same dose with maximum of ~88% killing effect, contrary to cytotoxicity result.

We also tested the apoptotic potential of all isolated compounds on all the cell lines. All the tested compounds displayed significant apoptotic potential on HeLa with the exception of compound **C5** with ~23% apoptosis at the highest dose 50.0 µg/ml, most of the compounds were more active than the positive control-Camptothecin on HeLa, except **C5**. On HT-29, **C1**, **C4**, **C6** were very active, **C5** was moderately active while **C2** and **C3** were not selective for HT-29 colon cancer cell lines. Compounds **C1** and **C4** were more active than Camptothecin. **C4** was not cytotoxic on HT-29 but found to be highly apoptotic; a similar occurrence was reported by Kikuchi et al., (2011). On MCF-7 cell lines, compounds **C1**, **C6** were very active; **C5** was moderately active while **C2**, **C3** and **C4** were partially active on MCF-7 breast cancer cell lines. Compounds **C1** and **C6** could compare well with Camptothecin activity. Finally on apoptosis results; **C2**, **C3**, **C4** and **C5** were non toxic to the normal cell line KMST-6 while at the highest concentration, the % apoptosis had a sharp rise for **C1**~90% and **C6** ~67%, this was consistent with cytotoxicity result. Based on the results obtained above, we further screened isolated compounds for specific markers of apoptosis in order to understand their mechanism of action.

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All compounds were tested for their ability to induce ROS production on different cell lines, depending on selectivity: **C2**, **C3** at 12.5 µg/ml caused a significant increase in ROS production on HeLa within 12-36 h, $P<0.05$ when compared with control.**C1** and **C6** also increased ROS production as shown by fluorescence imaging. There was no significant difference between **C4**

and control P>0.05 as observed in our experiments, however **C5** was not tested since the fluorescence imaging did not indicate ROS production.

On HT-29, compounds **C1**, **C4**, **C5** and **C6** were analyzed for ROS at 12 and 24 h, all compounds significantly increased ROS production, P<0.05 at 25 µg/ml in a time-dependent manner, except **C1** which was only significant at 12 h. On MCF-7 cell lines; **C1**, **C4**, **C5** and **C6** were selected at 25 µg/ml for 12 and 24 h, based on our results, only **C5** and **C6** significantly increased ROS production at 12 h.

The activation of caspases which are key mediators of apoptosis was analyzed on exposure of compounds to the selected cell lines for this research. Caspases 3/7 and 9 activities were measured on time and dose-dependent basis, for a period of 3, 6, 12 and 24 h (50 µg/ml) and 6.25-25 µg/ml respectively for 24 h. All compounds were tested for their time-response on caspase 9 activity on HeLa; **C1** gave a maximum fold increase of ~6.2, **C2** ~ 2.2 and **C3**~1.8 at 12 h, 6 h and 12 h respectively. Of all the compounds tested for dose – response, only **C6** (25 µg/ml) activated caspases 9 with~1.2 fold increase when compared to control within 24 h period. For the time-dependent assay on HT-29 caspase 9 activation, **C1** gave a maximum fold increase of ~47 fold (6 h) and **C6** ~ 2.4 (24 h) while in the dose-dependent results, it was observed that only **C5 (**25 µg/ml) had a significant fold increase of ~ 1.5 in 24 h. On MCF-7, only **C1** had a significant fold increase ~18 fold within 6 h at P<0.05 when compared with control.

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We further confirmed the apoptotic process by investigating the executioner caspases 3/7 which is the hall mark of apoptosis. Based on the results of our experiment for the time-dependent activation of caspases 3/7 on HeLa, **C1** and **C2** increase significantly the caspase activity

with~27 and 1.8 fold at 6 h and 12 h respectively, when compared with control. On the other hand, for the dose-response activation of caspases 3/7 at 25 µg/ml, the maximum fold increase obtained for **C2**, **C3** and **C6** are ~2.0, 2.2 and 3.6 respectively. On HT-29, the time-dependent result showed that only **C1** significantly increase caspase activity by 11.5 fold at 6 and 12 h.

MCF-7 is deficient in caspase 3, so it most likely that it is caspase 7 that is being activated.**C1** activated caspase7 in a time-dependent manner with ~24 fold increase in 6 h, while **C6** at 25 µg/ml gave a 2fold maximum increase in 24 h. It should be noted that **C1**was not tested for dose-response caspase activity because it is a known compound.

The result of the cell cycle analyis showed that on HeLa cells, compounds **C2** and **C3** arrested cell cycle at S phase while **C6** arrested at G1 phase. On MCF-7 compounds **C2** and **C3** arrested cell cycle at G1 phase while **C6** arrested at the S phase.

To further confirm the apoptotic process, all isolated compounds except **C4** were tested for topoisomerase I inhibition, all the compounds inhibited Topo I at different doses when compared with Camptothecin a well known Topoisomerase I inhibitor. This research project has thus further confirmed the mechanism of action of ursolic acid **C1** and thus indicates the mechanism of action for **C2**-**C6** on HeLa, HT-29 and MCF-7 cell lines.

In conclusion, the phytochemical study of the ethanolic extract from *Pleiocarpa pycnantha* leaves has led to the isolation of a new taraxerane-type triterpenoid; in addition to three known compounds. 2, 3-seco triterpenoids are rare in nature and thus compound **C5** represents the first example of this type to be isolated from Apocynaceae family.

Our studies suggest that the pure isolated compounds present in *Pleiocarpa pycnantha* demonstrate cytotoxic activity against cervical, breast and colon cancer. The present study also

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identified apoptosis as the mechanism underlying **C1**-**C6** induced growth inhibition in HeLa, HT-29 and MCF-7 cell lines and that the apoptosis is mediated by cell cycle arrest, caspase activation, ROS production and topoisomerase inhibition.

Although, we have not been able to get a substantial ethno botanical evidence for the use of this plant as anticancer agent, but the identification of ursolic acid and pleiocarpamine already identified as anti cancer agents triggered this research. Thus, based on our findings, we therefore propose that the total crude extract and components could be used in management of cervical, colon and breast cancer. However, these results warrant in our view, further investigations especially on other possible mechanisms of action and the assessment of their anti tumor therapeutic efficacy in vivo in experimental models.

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