In vitro cytotoxic effects of selected Nigerian medicinal plant extracts on cancer cell lines

By

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Declaration

I, Lucinda Baatjies 209081697, hereby declare that the dissertation for MSc is my own work and that it has not previously been submitted for assessment or completion of any postgraduate qualification to another University or for another qualification.

Lucinda Baatjies

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β:	Beta
γ:	Gamma
AIF:	Apoptosis-inducing factor
APC:	Anaphase-promoting complex
Apo 3:	Apoptosis antigen-3
Apo 3L:	Apoptosis antigen-3 ligand
ATM:	Ataxia telangiectasia mutated kinase
ATR:	ATM-rad3-related proteins
Bcl-2:	B-cell CLL/Lymphoma 2
BID:	BH3-interacting domain death agonist
BH3:	Bcl 2-homology domain 3
BRCA 1:	Breast cancer 1, early onset gene
CDKs:	Cyclin-dependent kinases
cFLIP:	cellular FLICE-inhibitory protein
CKI:	CDK Inhibitors
CO_2:	Carbon dioxide
DEDs:	Death effector domains
DIABLO:	Direct inhibitor of apoptosis protein (IAP)-binding protein with low PI
DISC:	Death-induced signal complex
DMSO:	Dimethyl Sulphoxide
DNA:	Deoxyribonucleic acid
DP 1:	Death receptor-3
EDTA:	Ethylenediaminetetra acetate
EF2:	Elongation factor 2
FADD:	Fas-associated death domain
FANCD2:	Fanconi anemia complement group D2
FasL:	Fas ligand
FasR:	Fas receptor
FBS:	Foetal bovine serum
FITC:	Flourescein isothiocyanate
FLICE:	FADD-like-IL-1β-converting enzyme
FLIP:	FLICE-like inhibitory protein

FS:	Forward-scatter
G:	Gap
GADD45:	Growth arrest and DNA damaging inducible gene
GAPDH:	Glyseraldehyde-3-phosphate dehydrogenase
HDAC:	Histone deacetylases
HU:	Hydroxy urea
hTERT:	Human telomerase reverse transcriptase
M:	Mitosis
mAb:	Monoclonal antibody
Mdm 2:	Murine double Minute-2
MPTPs:	Mitochondrion permeability transition pores
Mre 11:	Meiotic recombinant 11 homolog A
MTT:	3-(4, 5-dimethylthiazolyl-2)-2, 5 diphenyltetrazoliumbromide
Myt 1:	Myelin transcription factor 1
NBSI:	Nijmegen breakage syndrome gene
NLS:	Nuclear location signal
PARP:	poly (ADP-ribose) polymerase
PBMC:	Peripheral Blood Mononuclear Cell
PBS:	Phosphate buffered saline
PBSA:	Phosphate buffered saline (without Ca^{2+} and Mg^{2+}; containing EDTA)
PCNA:	Proliferating cell nuclear antigen
PEST:	Segment rich in proline (P), glutamic acid (E), serine (S) and threonine (T) residues
PI:	Propidium Iodide
PS:	Phosphatidylserine
R:	Restriction point
Rb:	Retinoblastoma protein
ROS:	Reactive oxygen species
RNA:	Ribonucleic Acid
S:	Synthesis
Smac:	Second mitochondria-derived activator of caspases
SS:	Side-scatter
TAM:	Traditional African Medicine
tBID:	truncated BID
TGF β:	Transformimg Growth Factor β

TNF:	Tumour Necrosis Factor
TNFR:	TNF receptor
TRAIL:	Tumour necrosis factor-related apoptosis-inducing ligand
UV:	Ultraviolet
WHO:	World Health Organization

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Summary

Cancer is a disease that imposes a heavy burden on public health and poses a challenge to science. The World Health Organization estimates that 80% of people in developing countries of the world rely on traditional medicine for their primary health needs, and about 85% of traditional medicine involves the use of plant extracts. This is particularly true in Africa where a large percentage of the population depends upon medicinal plants for health care. Therefore, detailed screening and evaluation of bioactive substances for chemotherapeutic purposes of African plants are urgently warranted. Furthermore, this will serve to validate the efficacy and safety of African traditional medicine.

The current study investigated the *in vitro* cytotoxic effects of 17 ethanolic extracts of the following 16 plants used in traditional anticancer medicine in Nigeria: *Sapium ellipticum* leaves, *Sapium ellipticum* stembark, *Combretum paniculatum, Celosia trigyna, Pupalia lappacea, Justica extensa, Hedranthera barteri* leaves, *Alternanthera sessilis, Ethulia conyzoides* leaves, *Lannea nigritana* stembark, *Combretum zenkeri* root, *Combretum molle* leaves, *Adenanthera parvoniana*, *Lannea acida*, *Cyathula achyranthoides*, *Drymaria cordata*, *Cyathula prostrata*, against HeLa cancer cells. Five of the most promising extracts (*Sapium ellipticum* leaves, *Combretum paniculatum, Celosia trigyna, Drymaria cordata, Cyathula prostrata)* were selected for further screening against HT29 and MCF-7 cancer cells. Of the five, the first two were investigated further based on their activities in the screening phase.

The *S. ellipticum* leaf extract yielded IC_{50} values of 88.60 ± 0.03 and 93.03 ± 0.03 µg/ml against HeLa and MCF-7, respectively. The toxicity was also evaluated on normal cells and an IC_{50} of 77.66 µg/ml was obtained for peripheral blood mononuclear cells (PBMCs). The IC_{50} values for proliferating and confluent Chang liver cells were both >125 µg/ml. These results suggest that the extract may be selective for specific cell types.

Bio-assay guided fractionation of the *S. ellipticum* ethanolic extract yielded two active fractions; chloroform and ethyl acetate. Two compounds isolated from the chloroform extract were screened against the three cancer cell lines and found to be inactive. Three compounds were isolated from the ethyl acetate fraction and revealed IC_{50} values < 62.5 and < 31 µg/ml against MCF-7. Unfortunately these two compounds soon lost activity before any further work could be done on them and work was continued with the crude extract.

Cell cycle analysis with propidium iodide staining revealed that *S. ellipticum* leaf crude extract has a dual effect, inducing G1/G0 and G2/M arrest of HeLa, HT29 and MCF-7 cells after 48 hours. Using the Annexin V-FITC/PI assay, the plant extract was found not to cause an increase in apoptosis when compared to the vehicle control cells. These results conclude that *S. ellipticum* does not cause apoptosis but acts as an inhibitor of proliferation.

Due to *C. paniculatum's* potential cytotoxicity, the leaf crude extract was further fractionated into three fractions. All three were active against the three cancer cell lines with the highest activity seen in the ethyl acetate fraction. Two pure compounds with a dose-dependent cytotoxic effect were isolated from the ethyl acetate fraction and identified as pheophorbide a (Cpe 4-4), and pheophorbide a methyl ester (Cpe 3-4). Pheophorbide a and pheophorbide a methyl ester had comparable activities against HeLa and MCF-7 cells but the former was more active on HT29 cells. It was also more cytotoxic to normal PBMCs and cell cycle analysis results revealed that 62.5 μg/ml of the methyl ester was a more potent inducer of cell cycle arrest in G1/G0 phase. No increase was observed in the sub-G1 (apoptotic) peak for any of the two compounds and it would appear that reduction in viable cell numbers compared to control cells is due to inhibition of proliferation rather than cytotoxicity.

A frequent characteristic of cancer cells is elevated telomerase activity which overrides normal replicative senescence due to telomere shortening. Telomerase inhibition is an attractive anticancer target and U937 cells were used to investigate possible effects of the 17 plant extracts on the levels of the catalytic subunit of telomerase (hTERT). Seven of the plant extracts gave promising results when levels of hTERT protein were lower than the control cells at 12.5 and 125 μg/ml after 48 hours of exposure. *S. ellipticum* showed a decrease in the level of hTERT protein at the higher concentration at both 24 and 48 hours. By quantifying one or more reference proteins in the same cells in which hTERT levels were measured, one would be able to validate the results and observe any possible non-target effects. β-actin and GAPDH were included as reference proteins but neither gave satisfactory results.

Finally, three more extracts (*Uvaria chamae* and *Lecaniodiscus cupanioides* leaf (LL) and root extracts (LR) were screened for cytotoxic activity as part of an ongoing collaboration with the University of Lagos. *U. chamae* showed low activity against HT29 but >50% inhibition against HeLa and MCF-7 at 125 μg/ml. LR showed very low inhibitory effect

against all three cancer cell lines. LL was active and an ethyl acetate fraction was found to hold the best promise for future investigation.

Chapter 1: Literature review

1.1 Cancer today

Cancer, often believed to be a disease of the so called developed world, has become a global health challenge [1]. It is the second largest cause of death world-wide [2, 3] after heart disease [4] and the incidence of cancer is increasing rapidly all over the world [1]. Increase in population size as well as continuing, globalization trends, are instrumental in the global increase of cancer [1]. Cancer has a profound toll on human suffering, with substantial economic cost to society [2]. A total of 559 888 cancer deaths were recorded in the United States in 2006, accounting for about 23% of all deaths [5]. In 1997, a world-wide total of 6.2 million deaths were due to cancer (out of a total of 52.2 million deaths). Cancers of the lung (1.1 million), stomach (765 00), colon and rectum (525 000), liver (505 000), and breast (385 000) were the leading cause of deaths from cancer [6].

What is cancer and how is it defined? For many people cancer was a death sentence, but today it is a curable disease for some and a chronic illness for most [7]. At first it was believed that cancer arise when cell growth exceeds the rate at which cells die, so that the cells grow at an uncontrollable rate [8]. However cancer is now being described as the product of malfunctions within the regulation of the cell cycle. These malfunctions thus allow injured or mutated cells which are normally killed to progress through the cell cycle, accumulating mutations [9].

Cancer is a disease that involves dynamic changes in the genome. It is a multistep process characterized by a progression of changes on cellular and genetic level leading to the uncontrolled growth of abnormal cells within the body [10]. These cells can also acquire the ability to invade surrounding normal tissue boundaries and metastasize to distant organs [11].

During normal growth and differentiation, cell proliferation is strictly controlled to maintain homeostasis [12]. Homeostasis within a cell is regulated by the balance between proliferation, growth arrest and apoptosis [9]. During cancer development the genomes of the cancer cells acquire mutant alleles of proto-oncogenes, tumour suppressor genes and other genes that control cell proliferation [13].

What then are the proto-oncogenes and the tumor suppressor genes? During the 1970s, scientists discovered two families of genes that play major roles in the genesis and spread of

cancer, namely the oncogenes and tumor suppressor genes [4]. Proto-oncogenes are regulatory genes that code for proteins that function to drive the cell cycle forward [14, 15]. These proteins play a central role in receiving and processing of growth-stimulating signals from the extracellular environment. The growth-stimulating signals bind to their receptors on the cell surface. The receptors convey a stimulatory signal to proteins in the cytoplasm to the nucleus where transcription is initiated to generate proteins that will move the cell through its growth cycle [15, 16].

Oncogenes, the mutated form of proto-oncogenes, cause the proteins involved in these growth-promoting pathways to be overactive [16]. Oncogenes behave in a dominant way, whereby one of the two alleles of the genes needs to be altered for a biological effect to occur [13]. In contrast to proto-oncogenes and oncogenes that code for growth-stimulating proteins, tumour suppressor genes code for inhibitory proteins that normally act to prevent cell growth or even promote programmed cell death (apoptosis) [13,17]. These genes are recessive; both alleles must be mutated before an effect is seen [13].

Apart from the coordination action of proto-oncogenes and tumor-suppressor genes cells have at least three other systems that can help them avoid runaway cell division. They have a DNA repair system, detecting and correcting errors in DNA. Should this system fail, the error (now a mutation) becomes a permanent feature in that cell and its descendants. A second cellular back-up system prompts the cell to commit suicide (apoptosis) if damaged beyond repair. This observation suggests that tumours arise from cells that have managed to evade apoptosis. A third back-up system limits the number of times a cell can divide [15]. The ends of chromosomes are capped with a protective DNA sequence (telomeres). These telomeres progressively become shorter after each cycle of cell division. When telomeres reach a threshold length an internal signal is triggered, that causes the cell to stop dividing [18].

Cancer cells have the ability to activate cell growth independent from growth stimuli due to mutations of intracellular signal pathways [9]. Research has revealed a small number of molecular, biochemical, and cellular traits - acquired capabilities - shared by most cancers [10]. The classical features of most cancer cell genotypes are their capacity to generate their own mutagenic signal, their insensitivity to anti-growth signals, evasion of apoptosis, ability to proliferate without limits, sustained angiogenesis and invasion and metastatic properties [7, 19, 20].

The majority of human cancers result from exposure to environmental carcinogens [3]. A minority of cancers are known to be hereditary [6]. Possible causes of the malfunctions within the regulation of the cell cycle are viruses, chemical carcinogens, chromosomal rearrangements, spontaneous transformations, oncogenes and tumour suppressor genes [3].

Since cancer is being described as a malfunction within the regulation of the cell cycle, the next section will give an overview of the function and regulation of the cell cycle.

1.2 The cell cycle

The cell cycle is a highly ordered and tightly regulated process that results in the duplication and transmission of genetic information from one cell generation to the next [21]. It is a sequence of events by which a growing cell duplicates its components and divides them into two nearly identical daughter cells, so that each daughter cell receives all the genetic information to repeat the process [22]. In order to replicate, a cell must first produce DNA, secondly manufacture sufficient cellular organelles and all that is needed for cell duplication. Finally, equal partition of the DNA and cytoplasm must occur to form two daughter cells [3]. The cell cycle process begins with a stimulus, which is the release of growth factors from neighbouring cells. These factors bind to receptors on the cell membrane. The binding of ligands stimulates a series of events inside the cell where the signals are conveyed to cytoplasmic proteins, which in turn signal the release of transcription factors within the nucleus. These signal transductions push the cell through the cell cycle [9]. To ensure that the molecular events are sequential and correctly orientated a significant amount of feedback control is in place. Failure to control the cell cycle carries with it a high price [3].

Morphologically the cell cycle can be subdivided into the interphase and mitotic (M) phase (Figure 1.1). The M phase includes prophase, metaphase, anaphase and telophase. The interphase includes the first gap phase (G1), synthesis phase (S) and the second gap phase (G2) [23, 24].

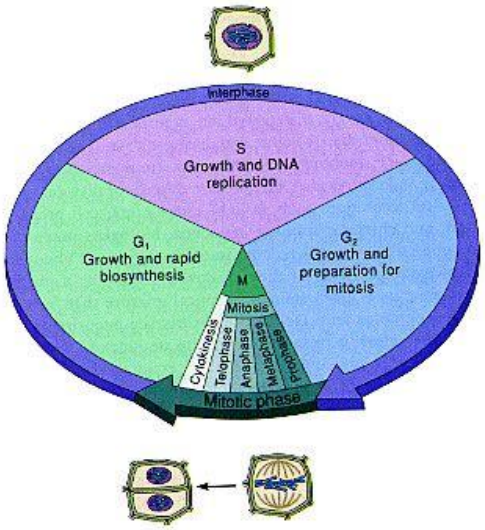


Figure 1.1: The stages of the cell cycle. The cell cycle can be subdivided into the interphase (G1, S and G2) and the mitotic phase (prophase, metaphase, anaphase and telophase) [25].

1.2.1 Interphase

Interphase describes the phase in the cell cycle where there is active preparation for cell division. Most adult mammals spend 90% of the total time of cell division in this phase [26]. There are three stages of interphase in which cells that are committed to replication; they move from G1 (first stage) into S phase (second stage) and finally into G2 (third phase) before duplication in the M phase [9].

1.2.1.1 G0 phase

The daughter cells from the previous cycle can either transit to the quiescent state, where they can remain in G0 indefinitely or return to the G1 phase of the cell cycle upon appropriate stimulation [27]. Quiescent cells are viable cells found in the G0 phase of the cell cycle where there is minimal protein synthesis. A cell may remain in this state for many years, but can re-enter the cell cycle at G1, following binding of a growth factor to the extracellular receptor [28].

1.2.1.2 G1 phase

During G1 phase a decision is made either to proliferate or enter G0 [27]. During this phase a cell is subject to stimulation by extracellular growth factors and mitogens. In response to these stimuli the cell passes through G1 [21]. G1 represents a phase in which a cell synthesizes a series of mRNAs and proteins that are necessary for DNA synthesis in the S phase [28].

1.2.1.3 S phase

The S phase involves duplication of the entire genome [29]. This phase is of particular importance, because replication of chromosomes and segregation (M phase) ensures that each daughter cell receives a full complement of hereditary material [30].

1.2.1.4 G2 phase

The G2 phase represents a time where there is active protein synthesis and preparation for mitosis [29]. During this phase cells can repair errors that occurred during DNA duplication, preventing propagation of these errors to daughter cells [31].

1.2.2 Mitotic phase

Mitosis involves five phases, based on the state of the chromosomes and spindles. Cytokinesis is the final physical cell division that follows telophase [32]. M phase is marked by the generation of bipolar mitotic spindles, segregation of sister chromatids and cell division [21].

1.3 Cell cycle regulation

To ensure survival and propagation of accurate copies of the genome on to subsequent generations, control mechanisms are in place to avoid inappropriate cell proliferation [33, 34]. Different cellular proteins regulate the transition from one cell cycle phase to another. Cyclin–dependent kinases (CDKs), a family of serine/threonine protein kinases are the key regulatory proteins which are activated at specific points of the cell cycle [24]. CDKs form

distinct complexes with cyclin proteins at specific phases of the cell cycle (Figure 1.2) and thereby drive the cell from one stage of the cell cycle to another [28]. CDK 1, 2, 4 and 6 seem to be important players in cell cycle control. CDK1 is responsible for initiating mitosis at the G2/M transition. CDK 2, 4 and 6 are involved in passage through the restriction point and consequent initiation of S phase [27].

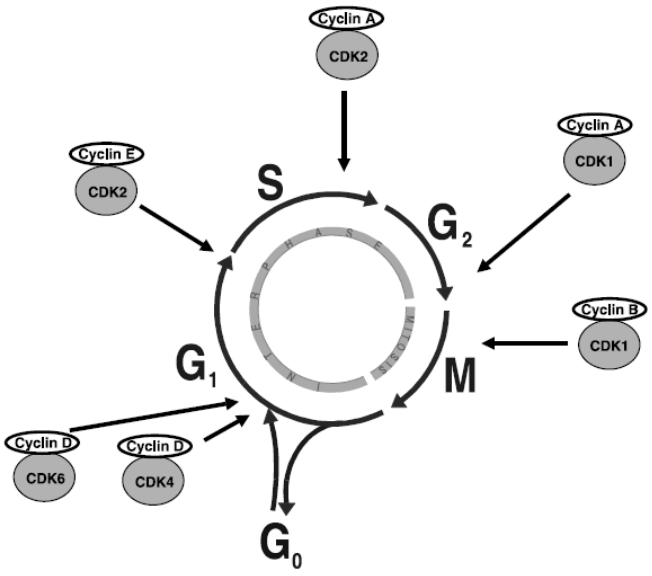


Figure 1.2: The cell cycle and cyclins. The site of activity of regulatory cyclin/CDK complexes is also indicated. [24]

CDK protein levels remain stable during the cell cycle, in contrast to their activating proteins, the cyclins [24]. Cyclins perform multiple regulatory functions [23] and activated cyclin/CDK complexes function during distinct stages of the cell cycle (Table 1.1) [21]. Cyclin A and B contain a destruction box and cyclins D and E contain a PEST sequence (segment rich in proline (P), glutamic acid (E), serine (S) and threonine (T) residues): these are protein sequences required for efficient ubiquitin-mediated cyclin proteolysis at the end of a cycle phase [23, 24]. Within each phase of the cell cycle the level of cyclins independently increase or decrease due to regulated degradation [21]. Thus, before a cell enters the next phase, the appropriate cyclin of the previous phase is degraded and the cyclin of the next phase synthesized [23]. The D type cyclins (D1, D2 and D3) bind to CDK 4 and to CDK 6, and mediate the progression through G1 [24, 33]. Cyclin D is synthesized as long as the growth stimuli persist [24]. Cycin E associates with CDK2 to regulate progression from G1 into S phase [24, 31, 35]. Cyclin B in complex with CDK1 further regulates mitosis [24]. Cyclins B contain nuclear localization signals (NLS) that target the CDKs to the nucleus.

These nuclear localization signals are lacking in CDKs [24]. Cyclin A gets activated during the S phase transition and binds to CDK1 and CDK2 [33, 35]. Once bound to the CDKs, the cyclins are able to guide the CDKs to appropriate substrates and activate catalytic activity [36].

Table 1.1: Cyclin CDK complexes are activated at specific points of the cell cycle.

CDK	Cyclin	Cell cycle phase activity
CDK 1	Cyclin D1, D2, D3	G_1 phase
CDK 6	Cyclin D1, D2, D3	G_1 phase
CDK 2	Cyclin E	G_1/S phase transition
CDK2	Cyclin A	S phase
CDK 1 (cdc2)	Cyclin A	G_2/M phase transition
CDK 1 (cdc2)	Cyclin B	Mitosis
CDK 7	Cyclin H	CAK, all cell cycle phases

CAK, CDK activating kinase [24]

The positive regulatory activities of the cyclins and CDK on the cell cycle are constrained by CDK inhibitors (CKIs) [28]. CKIs have been assigned to one of two families (Table 1.2) based on their structures and CDK targets [37]. They can be divided into the INK4 proteins, which specifically inhibit the catalytic subunits of CDK 4 and CDK 6 and the Cip/Kip proteins whose actions affect the activities of cyclin D-, E-, and A-dependent kinases [24, 28].

There are four INK4 family members: $p16^{INK4a}$, $p15^{INK4b}$, $p19^{INK4d}$, and $p18^{INK4c}$, which specifically inactivate CDK 4 and CDK 6 in the G1 phase. These CKIs form stable complexes with the CDK enzyme, before cyclin binding, preventing association with cyclin D.

The Cip/Kip family members include: $p21^{Waf/Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$. These inhibitors inactivate CDK-cyclin complexes. Thus, CKIs can bind to CDK alone or to the CDK-cyclin complex and regulate CDK activity [24, 37]. By binding to and inhibiting the proliferating cell nuclear antigen (PCNA), p21 inhibits DNA synthesis. The expression of *p21* is under

transcriptional control of p53 tumour suppressor gene. Activation of p15 and p27, contributes to growth arrest in response to transforming growth factor β (TGY-β). CDK inactivating kinases Wee1 and Myt 1 protect the cell from premature mitosis and 14-3-3 group of proteins regulates the intracellular trafficking of different proteins [24].

Table 1.2: Cyclin dependent kinase inhibitors (CKI) bind to CDK alone or to the CDK-cyclin complex and regulate CDK activity. P19 (ARF) is also encoded by the INK 4 locus, but has no known CKI acitivity

CKI family	Function	Family members	
INK4 family	Inactivation Of G_1 CDK (CDK4, CDK6)	$_P$15	(INK4b)
		$_P$16	(INK4a)
		$_P$18	(INK4c)
		$_P$19	(INK4d)
Cip/Kip family	Inactivation of G_1 cyclin-CDK Complexes and Cyclin B-CDK1	$_P$21	(Waf1, Cip1)
		$_P$27	(Cip2)
		$_P$57	(Kip2)

[24]

1.4 Cell cycle progression

Cell cycle progression is an organized process that controls cell growth, cell proliferation, and ontogenesis and is well connected to the regulation of DNA damage repair [33]. It requires a series of phosphorylation and dephosphorylation events carried out by protein kinases and phosphatases [38]. Multiple mechanisms exist to regulate the progression of the cell cycle after its initiation (Figure 1.3) [27]. During the G1 phase cells respond to extracellular growth or mitogenic stimulation that ultimately determine whether cells will make the decision to replicate DNA and divide, or alternately, exit the cell cycle into a quiescent state (G0) [39]. Stimulation by growth factors or mitogenic factors to enter the cell cycle from G0 to G1 phase requires continuous mitogenic stimulation during the first two-thirds of G1 phase to be driven to the "restriction point" R [36, 39, 40]. There is an increase in the expression of the D cyclins (D1, D2 and D3) as the cell moves out of G0 into G1 [21]. Once synthesized, the D-type cyclins associate with CDK 4 and CDK 6 [40]. Formation of

the cyclin-CDK complexes results in the phosphorylation of CDKs whereby activated CDKs, phosporylate the retinoblastoma (Rb) protein [21]. Phophorylation of pRb by CDK 4/6 leads to disruption of the complex with histone deacetylase (HDAC) and the release of the transcription factor E2F-1 and DP-1 allowing E2F to transcribe a number of responder genes required for passage through R [21, 23, 24]. pRb is maintained in its hyperphosphorylated state throughout the remainder of the cell cycle and cyclin E/CDK 2 participates in maintaining this hyperphosphorylated state [24].

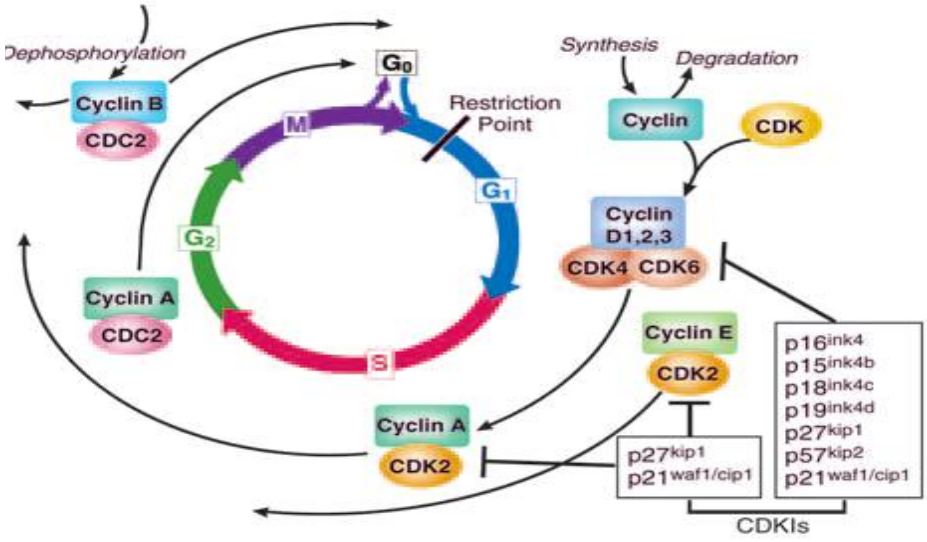


Figure 1.3: The cell cycle. The cell cycle is divided into four phases (G1, S, G2, and M). Progression through the cell cycle is promoted by cyclin-dependent kinases (CDKs), which are regulated positively by cyclins and negatively by CDK inhibitors (CDKIs). The restriction point is the point at which cells progress through the cell cycle independent of external stimuli [35].

pRb is the gatekeeper of the cell cycle (Figure 1.4). As the cell progresses through late G1, cyclin E increases. Transition from G1 to S phase requires the cyclin E/CDK 2 complex [21]. The latter complex phosphorylates its inhibitor p27; inducing its proteasome-dependent degradation [24]. Increased expression of cyclin A occurs at G1/S transition and persists through S phase. DNA synthesis proceeds through binding of cyclin A to CDK 2. Increase of cyclin A and cyclin B complexes with CDK 1 allows progression of the cell through mitosis [21].

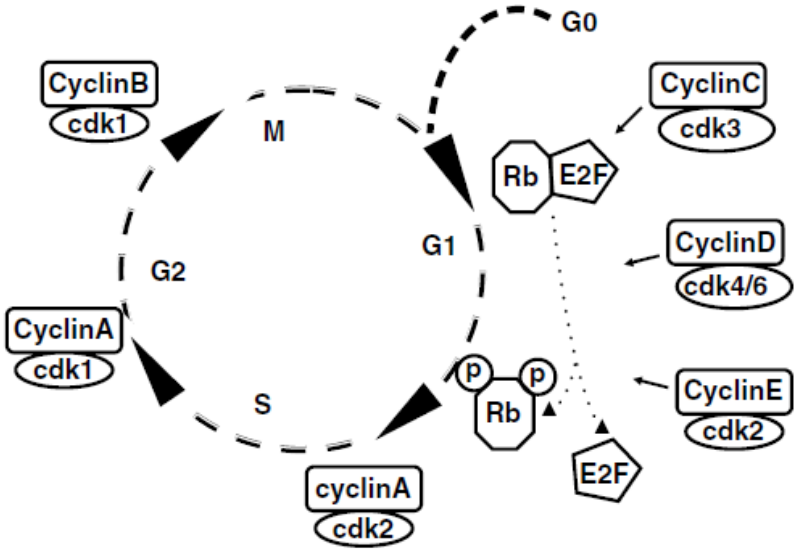


Figure 1.4: Rb and cell cycle machinery. Rb and Rb-p represent the unphosphorylated and the phosphorylated forms of the retinoblastoma protein. In G0 and early G1, Rb physically associates with E2F factors and blocks their transactivation domain. In late G1, Rb-p releases E2F, allowing the expression of genes that encode product necessary for S-phase progression [36].

1.5 Cell cycle checkpoints and cell cycle arrest

Under normal circumstances the cell cycle proceeds without interruptions. However, most normal cells have the capacity to arrest proliferation when damage occurs in G1, S and G2 of the cell cycle, and then resume when the damage has been repaired [41]. The arrest or delay of the cell cycle progression is mediated by a network of signalling pathways, the quality control points of the cell cycle referred to as checkpoints [34, 42]. When checkpoints are activated they relay signals to the cell cycle progression machinery, which in turn cause a delay in cell cycle progression, until the danger of the mutation has been rectified [43]. Checkpoints are biochemical signalling pathways that sense various types of structural defects in DNA, or chromosome activities and induce a multifaceted cellular response that activates DNA repair and delays cell cycle progression. Potentially hazardous cells with irreparable DNA damage are eliminated by checkpoints through permanent cell cycle arrest or cell death [44].

Cell cycle checkpoints mediate cell cycle arrest which is produced by a variety of factors that may be intrinsic or extrinsic and may affect several different checkpoints [23]. Cell cycle arrest is essential because it allows time to repair damage before DNA synthesis [45].

Checkpoints are activated by under replicated DNA, damaged DNA or faulty mitotic spindle alignment and these signals are relayed to the cell cycle progression machinery [33, 41]. Arrest in G1 allows time for repairs before DNA replication whereas arrest in G2 allows time for repairs before chromosome separation in mitosis [23].

The biochemical pathways that delay progression, or transition between major cell cycle phases are discussed in the next sections [34].

1.5.1 The restriction point

The G1/S transition is a highly regulated and important transition in the cell cycle [42]. The point of no return, following which a cell is committed to enter the cell cycle, is defined as the restriction point (R) [24]. This is a critical point late in G1 phase where mammalian cells make the decision to begin DNA replication, becoming committed to entering the S phase and to completing the cell cycle even in the absence of growth factors [39, 42]. Evidence that has been accumulated over the past twelve to fifteen years has shown that the Retinoblastoma protein pRb is the molecular device that serves as the R point switch [36, 40, 46]. Cyclin D/CDK 4/6 complex initiates pRb phosphorylation. Subsequent phosphorylation of pRb occurs at the hand of cyclin E/CDK 2 complexes. The formation of cyclin E/CDK 2 complex in late G1 phase acts as a positive feedback loop on the pRb pathway already initiated by cyclin D/CDK 4 [42]

1.5.2 G1 checkpoint and G1 arrest

During G1, cells make critical decision about their fate. They check whether their environment favours proliferation and whether their genome is ready to be replicated [47]. Damage of the genome occurring during the G1 phase of the cell cycle causes rapid cell cycle arrest [40]. Arrest in response to DNA damage in the G1 phase of the cell cycle has been found to depend heavily on the action of p53 a product of a tumour suppressor gene, which is known to be mutated in more than 50% of human cancers [41]. DNA damage activates the checkpoint transducing kinasesmataxia-telangiectasia-mutated/ataxia and rad3 related (ATM/ATR) and DNA protein kinase (DNA PK) family of protein kinases which leads to the post-translational stabilisation of normally labile p53 protein [24, 40]. Induced p53 acts as a

transcription regulatory factor that causes the expression of several responder genes, including p21, growth arrest and DNA damage inducible gene (GADD45) and Murine Double Minute-2 (Mdm2). It can also act as a transcriptional repressor by interfering with the binding of basal transcription factors to the TATA motif which may account for some of the ability of p53 to interfere with neoplastic processes [41, 48]. Accumulation of p21, results in the inhibition of cyclin E/CDK2 and cyclin D/CDK 4/6 complexes and hypophosphorylation of pRb [47]. Another important regulator of the G1/S cyclin/CDK complexes is p16, known to inhibit cyclin D/CDK 4/6 complexes probably acting as an inhibitor of pRb phosphorylation [41]. p21 mediates p53-dependent G1 arrest by inhibiting the activity of CDKs, which phosphorylate the pRB. In its hypophosphorylated form pRB sequester the E2F transcription factor, preventing transition from G1 to S phase [49].

1.5.3 S phase checkpoint and S phase arrest

Less is known about the S phase DNA checkpoint [24, 48]. It serves to protect the genome from spontaneous damage during replication [50] and operates like a surveillance camera [51]. Stalled replication forks that are processed to Holiday junctions and double-strand breaks are one source of spontaneous damage. Checkpoint activation caused by DNA damage or stalled replication forks inhibit ongoing DNA synthesis, giving time for DNA repair [50]. The G1/S, S and G2/M checkpoints share some components. ATR, the primary S phase checkpoint kinase, plays a role in both damage sensing and DNA replication [52]. It gets activated by stalled replication forks (caused by hydroxyurea (HU)) and lesions (caused by ultraviolet (UV) light) [50]. In contrast to ATR, ATM only sense DNA double–strand breaks caused by ionizing radiation [50, 52]. Many S phase checkpoint genes, such as ATM, Nijmegen Breakage Syndrome gene (NBSI), Meiotic recombinant 11 homolog A (Mre11), Breast Cancer 1, Early Onset gene (BRCA 1), Chk 2 and Fanconi Anemia Complement Group D2 (FANCD 2) are mutated in human cancers [50].

1.5.4 The G2/M checkpoint and G2/M arrest

To ensure accurate segregation of chromosomes, cells in G2 phase of the cell cycle prevent mitosis in the presence of DNA damage [34, 53]. Signal transduction cascades link the

detection of DNA breaks to inhibition of CDK 1, and thus to the inhibition of mitosis, the induction of DNA repair and of apoptosis [54]. CDK 1 is maintained in its inhibited form, through inhibitory phosphorylation or by sequestration of components of the cyclin B/CDK 1 complex outside the nucleus [24]. Thus, regulation of cyclin B/CDK1 complex involves activating phosphate by CDK-activating enzyme and inhibitory phosphates in the roof of the active site by Wee 1 [53]. Agents that trigger the G2 checkpoint response suppress cyclin B/CDK 1 activation at the G2/M border [41]. After DNA damage, activation of cyclin B/CDK 1 is prevented through ATM/ATR and Chk1/Chk2 inhibition or Cdc25c phosphatase that normally activates Cdk1 at the G2/M border [34]. p53 also plays a role in the regulation of the G2/M checkpoint. DNA damage dependent increase of p53 leads to an increase of p21 and of 14-3-3 σ. p53 also mediates the dissociation of cyclin B/CDK1 complexes by induction of GADD45 [24, 34].

1.5.5 Mitotic spindle checkpoint and arrest

The spindle checkpoint is a specific checkpoint that arrests cells in mitosis (anaphase) until all chromosomes is attached properly to the spindle [41, 55]. It is activated by a lack of attachment and/or tension at kinetochores and in response inhibits chromosome segregation [56]. It serves to inhibit cell cycle progression until all chromosomes are appropriately bound to microtubules at their kinetochores. Kinetochore attachment is recognized as a key regulator of initiation of mitosis. A single unattached kinetochore is sufficient to inhibit mitosis [27]. Progression through mitosis is dependent on the degradation of cyciln B on the anaphase-promoting complex (APC)/cyclosome [35, 41]. The assembly of the bipolar spindle by the centrosome is vital to the prevention of genetic infidelity between daughter cells [35]. Mitotic arrest deficient (Mad) and budding uninhibited by benomyl (Bub) proteins are activated when defects in microtubule attachment occur and inhibit the Cdc 20 subunit of the APC resulting in the prevention of metaphase-anaphase transition [24].

One theme emerging in drug discovery is to develop agents that target the cell cycle checkpoint that is responsible for the control of cell cycle progression [53].

1.6 Apoptosis and necrosis

1.6.1 Apoptosis

Fundamental aspects of the life cycle of a eukaryotic cell are cell growth, differentiation and cell death [57]. Programmed cell death involves genetically determined elimination of cells which is an important aspect of organism development through its role in maintaining a balance between cell growth and death [58]. Apoptosis is a form of programmed cell death which enables an organism to eliminate old, unwanted and abnormal cells through an orderly process of cellular disintegration that avoids eliciting inflammation [59, 60]. It plays an essential role in controlling cell number in many developmental and physiological settings [61].

Kerr and colleagues (1972) proposed the term apoptosis which described a specific morphological pattern of cell death observed as cells were eliminated during embryonic development, normal cell turn over in healthy adult tissue, and atrophy upon hormone withdrawal [60]. Its morphological characteristics include chromatin condensation, nuclear fragmentation, plasma membrane blebbing and cell shrinkage. Ultimately there is the formation of apoptotic bodies (small membrane surrounded fragments) that are phagocytosed without inflicting an inflammatory response (Figure 1.5) [58, 60, 62, 63]. This is achieved by an energy- and caspase dependent phosphatidylserine (PS) exposure on the outer leaflet of the plasma membrane [60].

The exposure of PS is a biochemical feature that results in early phagocytic recognition by adjacent cells, resulting in quick phagocytosis with minimal compromise to surrounding tissue [58]. Collapse of the membrane lipid asymmetry, [64] leads to the expression of negatively charged PS from the inner to the outer cell membrane [58, 60, 65]. In healthy cells PS is localized on the inner leaflet of the plasma membrane [60]. Annexin V is a PS-binding protein that interacts strongly and specifically with PS residues and can be used for the detection of apoptosis [58] via flow cytometry when fluorescently conjugated [60]. In vitro, apoptotic bodies may lose their integrity and proceed to secondary apoptotic necrosis due to absence of phagocytosis [60].

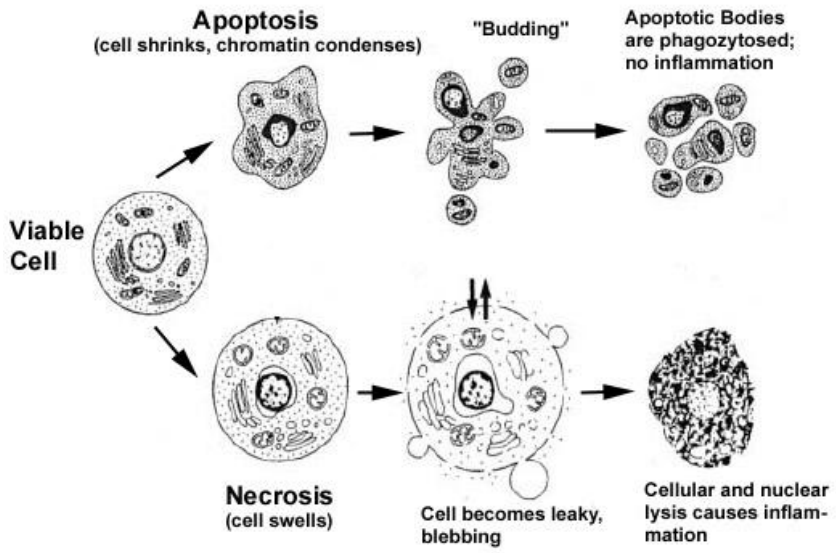


Figure 1.5: Hallmarks of the apoptotic and necrotic cell death process. Apoptosis includes cellular shrinking, chromatin condensation and margination at the nuclear periphery with the eventual formation of membrane-bound apoptotic bodies that contain organelles, cytosol and nuclear fragments and are phagocytosed without triggering inflammatory processes. The necrotic cell swells, becomes leaky and finally ruptures and releases its contents into the surrounding tissue resulting in inflammation. [63].

The progress of apoptosis is regulated in an orderly way by a series of signal cascades. The caspase-cascade system plays a vital role in the induction, transduction and amplification of intracellular apoptotic signals [66]. The name caspases are derived from cysteiene-dependent aspartate –specific protease [63]. They are characterized by a cysteine containing active site with aspartate substrate specificity [67]. All caspases have a conservative pentapeptide active site QACXG (X cab be R, Q or D) [66, 63] and they specifically cleave their substrate after Asp residues [63]. Members of the caspase family differ significantly in their physiological roles, even though they have similarity in amino acid sequence and structures [60].

Caspases are synthesized as inactive proforms, but can become activated by proteolytic processing at conserved Asp residues [68, 69]. They contain an N-terminal pro-domain followed by the region that forms a two subunit catalytic effector domain [60]. The N-terminal of the pro-domain in pro-caspases contains the highly diverse structure required for caspase activation. They are capable of auto activating as well as activating others [66]. Two main groups of caspases are categorized; initiator caspases (caspase -8, -9, -10, and -12), which cleave other caspases, or executioner caspases (caspase -3, -6, and -7) which cleave various cellular proteins [67].

There are two major pathways through which the apoptotic caspase family protease can be activated [66].

1.6.1.1 Extrinsic pathway (death receptor- mediated pathway)

The extrinsic pathways that initiate apoptosis involve transmembrane receptor-mediated interactions [58]. These receptors contain different death effector domains (DEDs) [33]. These death domains play a critical role in transmitting the apoptotic signals (after ligation with specific ligands) to the intracellular signalling pathways (Figure 1.6) [58, 67]. The best characterized ligands and corresponding death receptors include FasL/FasR, TNF-α/TNFR1, Apo3L/DR3, Apo 2L/DR4 and Apo 2L/DR5 [58]. Upon ligand binding, death receptors, such as CD95 (Apo-1/Fas), aggregate and form membrane-bound signalling complexes [70]. Binding of Fas ligand (FasL) to Fas receptors (FasR) result in the binding of adaptor protein Fas-associated death domain (FADD). Ligand binding to tumor necrosis factor (TNF) receptors result in the binding of the adaptor protein TNFR-associated death domain (TRADD) with recruitment of FADD and RIP [58, 70]. This results in the formation of death induced signal complex (DISC) which in turn leads to the auto catalytic activation of pro-caspase-8 and triggering of the proteolytic caspase cascade [33, 58]. Activation of the initiator caspase-8 propagates the apoptosis signal by direct cleavage of downstream effector caspases such as caspase-3 and -7 [68, 71].

Alternatively, active caspase -8 can activate the mitochondrion-mediated pathway by cleaving the Bcl2-homology domain 3 (BH3) of the protein BH3-interacting domain death agonist (BID) in the cytosol, into its active form trauncated BID (tBID). tBID will trigger the activation of the mitochondrion pathway [33, 66]. The release of cytochrome c, apoptosis-inducing factor (AIF) and other molecules from the mitochondria leads to the induction of apoptosis [66]. Death receptor-mediated apoptosis can be inhibited by a protein called cFLIP which will bind to FADD and pro-caspase-8, rendering them ineffective [33].

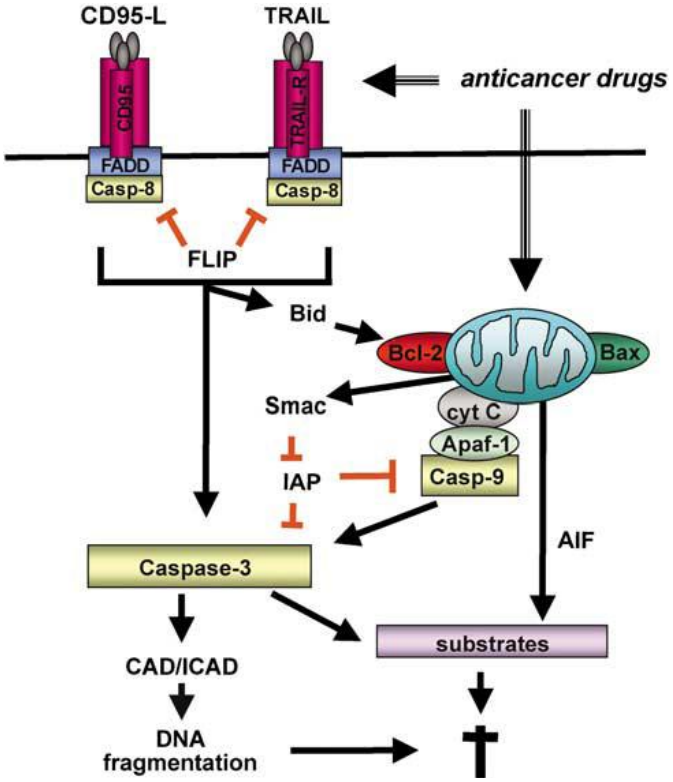


Figure 1.6: Apoptosis signalling pathways. Apoptosis pathways can be initiated through different entry sites, for example, at the plasma membrane by death receptor ligation (receptor pathway) or at the mitochondria (mitochondrial pathway). Stimulation of death receptors of the tumor necrosis factor (TNF) receptor superfamily such as CD95 (APO-1/Fas) or TNF-related apoptosis-inducing ligand (TRAIL) receptors by CD95 ligand (CD95-L) or TRAIL results in receptor aggregation and recruitment of the adaptor molecule Fas-associated death domain (FADD) and caspase-8. Upon recruitment, caspase-8 becomes activated and initiates apoptosis by direct cleavage of downstream effector caspases [68].

1.6.1.2 Intrinsic pathway (mitochondrion-mediated pathway)

Mitochondria are well known for their life sustaining production of ATP, but these organelles can also function as engines of cell death. This is due to the release of cytochrome c (activator of Apaf1/caspases), Smac [IAP (inhibitors of apoptosis protein) inhibitor] and AIF (endonuclease activator) [69]. Mitochondria are regarded as an important player mediating intrinsic death signals, and could serve as a novel target for chemotherapy [72]. The intrinsic pathway that initiates apoptosis involves a diverse array of non-receptor mediated stimuli. These stimuli produce intracellular signals that act directly on targets within the cell cycle and are mitochondrial initiated events (Figure 1.7) [58].

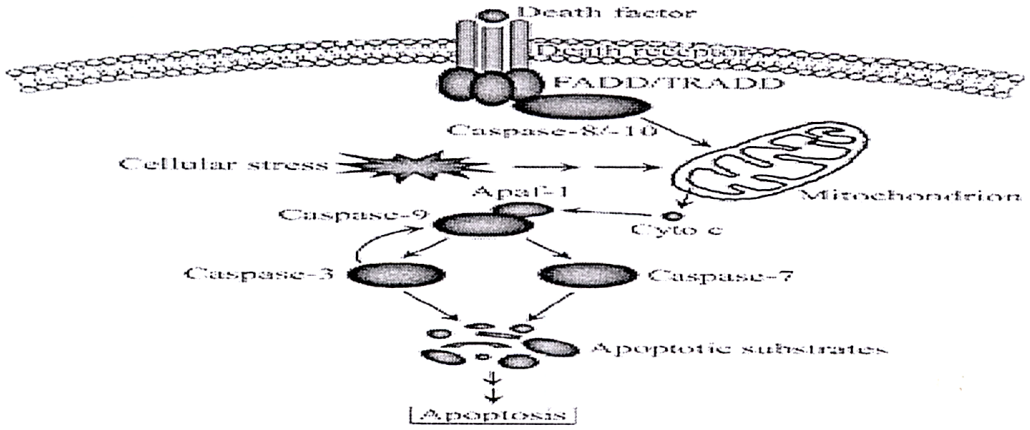


Figure 1.7: Typical mitochondrion-mediated and caspase dependent apoptotic pathway [66].

This pathway is used extensively in response to diverse forms of cellular stress (e.g. DNA damage) [70]. These stressors promote the activation of pro-apoptotic proteins in the cytosol, which will induce the opening of mitochondrion permeability transition pores (MPTPs) [66, 70]. The release of cytochrome c from the intermembrane space into the cytosol triggers caspase-3 activation through formation of the cytochrome c/Apaf-1/caspase-9-containing apoptosome complex [58, 68, 70]. Clustering of pro-caspase-9 leads to activation, whereas Smac/DIABLO [direct inhibitor of apoptosis protein (IAP) - binding protein with low PI] and Omi/HtrA2 are reported to promote apoptosis by inhibiting IAP [58, 68]. A second group of pro-apoptotic mitochondrial proteins (AIF, Endonuclease G) has been found to be released from the mitochondria that can cause apoptosis by DNA fragmentation and subsequent chromosomal condensation [58]. There is considerable cross-talk between the intrinsic and extrinsic pathway [33] with links at different levels [68].

1.6.2 Necrosis

Necrosis (non-apoptotic, accidental cell death) is another mechanism of cell death [58]. It is considered a toxic process that is energy-independent [58]. This is the result of exposure to exogenous agents resulting in the loss of osmotic balance [62]. Rapid loss of cellular membrane potential distinguishes necrosis from apoptosis [73]. The influx of cations, which

poison respiration, results in the swelling of the endoplasmic reticulum and mitochondria. Ca^{2+} may activate Ca^{2+} dependent degradative enzymes to disrupt cellular membranes and randomly degrade DNA. The result is bursting of the cell and releasing of lysosomal and degradative enzymes that attack and severely injure or kill adjacent cells [62]. Morphological changes that occur with necrosis includes, cell swelling; formation of cytoplasmic blebs; swollen or ruptured mitochondria; disaggregation and detachment of ribosomes; disrupted organelle membranes; swollen and ruptured lysosomes and eventually disruption of cell membranes (Figure 1.5) [58].

1.7 Telomeres and telomerase

Normal somatic cells divide for a limited number of times and eventually stop dividing through the process termed cellular senescence or replicative senescence [74- 76]. A major mechanism of cellular senescence involves shortening of telomeres. Telomeres are essential protein-DNA complexes forming capping structures at the end of eukaryotic chromosomes that protect chromosome ends from fusion and degradation [77-79]. Human telomeres contain long stretches of DNA tandem repeats TTAGGG [74, 80] which terminate in 3' protruding single stranded DNA overhang [80] primarily because the lagging strand of DNA synthesis is unable to replicate the extreme 3' end of the chromosome [74]. This is due to the fact that DNA-dependent DNA polymerase cannot completely replicate the ends of chromosomes so that more and more terminal DNA is lost with each cycle of DNA replication [79]. Critically short, dysfunctional telomeres eventually induce cells to senesce or die [75]. This end replication problem of the lagging strand and shortening of telomeres can be overcome by the expression of telomerase [81].

Telomerase is a ribonucleoprotein enzyme [81-83] that uses its own RNA as template to add the hexanucleotide to the ends of replicating chromosomes [74, 83]. Its catalytic core consists of two major components, the RNA subunit (hTER) which provides the template and the catalytical subunit (hTERT). Other telomerase-associated proteins-hsp 90, p23, TEP1 and others may play a role in biogenesis and regulation [76, 82].

The absence or low telomerase activity in most human somatic cells prevents conventional DNA polymerase to replicate the ends of chromosomes resulting in replicative senescence [84]. In contrast to normal cells telomerase activity is detectable in the majority of cancer

tissue [74, 77]. Eighty to ninety percent of human tumours possess telomerase activity [85]. The nearly universal expression of hTERT in human cancers, its important role in promoting tumour proliferation and its restricted expression in normal tissue makes hTERT an attractive anti-tumour target [79, 85]. The gene for hTERT of telomerase was discovered in 1997 and soon thereafter targeted as well. The disruption of the telomeric structure and/or functions by drugs interacting with this substrate of the telomerase enzyme evolved into a promising way for telomerase inhibition [76].

1.8 Cancer therapies

For many years surgery, radiation therapy, chemotherapy and hormone therapy have been the mainstays of cancer treatment [86]. Cancer therapy, for example, chemotherapy, γ-irradiation, immunotherapy or suicide gene therapy, primarily exert their anti-tumour effect by triggering apoptosis in cancer cells [68]. Chemotherapy has been a mainstay anticancer treatment for many decades [87]. However chemotherapy is highly inadequate due to the lack of cytotoxic drug specificity [87, 88] and the consequent generation of toxic side effects when high doses are administered [87]. During anticancer treatment it is difficult to protect the healthy cells from side effects by the drugs administered [89].

The ideal situation would be that chemotherapeutic drugs should specifically target only cancerous cells by inducing a cytotoxic and/or cytostatic effect with minimal damage to normal cells. Systemic toxicity due to lack of specificity, rapid metabolism and both intrinsic and acquired drug resistance has decreased the effectiveness of chemotherapy [71]. Despite many therapeutic advances in the understanding of the process in carcinogenesis, overall mortality statistics are unlikely to change until there is a reorientation of the concepts for the use of natural products as chemo preventive agents. Natural or semi synthetic compounds may be used to block, reverse, or prevent the development of invasive cancers [3].

1.9 Cancer incidence in developing countries and use of medicinal plants as alternative and complementary therapeutic agents

The global increase in cancer especially in developing countries is a serious concern as documented in reports of the World Health Organization (WHO) [90]. It was estimated by the WHO that cancer will be the leading cause of death in the world by 2010 [91]. In 2008, approximately 72% of cancer deaths occurred in low and middle income countries [91, 92]. A challenge facing the developing countries in cancer control is poverty [91] and priority for cancer care is rated low [90]. The high cost of treatment and lack of healthcare insurance system for the poor is a fundamental problem in cancer control in developing countries [92].

According to the WHO, 80% of the world's population, primarily those of developing countries, rely on plant derived medicine for their health-care [93, 94]. Traditional African Medicine (TAM) serves as major African Socio-cultural heritage servicing over 80% of the population in Africa, in existence for several hundred of years, was once believed to be primitive and wrongly challenged with animosity [95]. The fact that the people do not have scientific insight to explain and predict the curative action of plants is one of the reasons why medicinal plants are often associated with witchcraft and superstition. The people may not understand the scientific rationale behind their medicines, but they know from personal experience that some medicinal plants can be highly effective if used at therapeutic doses [93]. Thus, the major challenge for the usage of medicinal plants is scientifically based evidence, quality standards and regulations that needs to be developed at the same pace as the demands for the medicine [95].

Plant drugs fell into disfavour with the rise of the modern pharmaceutical industry in the early 20^{th} century [96]. Since a large majority of the developing countries of the world were unable to provide healthcare to the populations using the imported orthodox health facilities, the WHO are supporting, promoting and assisting the development of traditional medicine to help move the African health agenda forward [95]. For the maintenance of good health in developing countries, traditional medicine and medicinal plants are used [97].

Plants have a long history of use in the treatment of cancer, it has played an important role as source of effective anticancer agents and it is significant that over 60% of currently used anticancer agents are derived in one or another form from natural sources [98]. The search for anticancer agents from plant sources started in the 1950's with the discovery and

development of the vinca-alkaloids, vinblastine and vincristine from *Vinca rosa* L (Periwinkle) [98, 99]. Epiphodophyllotoxin analogs (VP-16 and VM-26) are other drugs derived from medicinal plants [99].

Most of the current drugs are synthesized against the backbone of one or another natural product [3]. Anticancer drugs, such as paclitaxel was originally isolated from the taxol extract of the bark of the Pacific yew, *Taxus brevifolia*. Paclitaxel is used to treat drug-refractory ovarian cancer and metastatic breasts cancer, small and non- small lung cancer [99]. Paclitaxel acts by inhibiting microtubule depolymerisation and promotes tubulin assembly and inhibits all proliferation [3, 100]. Paclitaxel's structure was elucidated in 1971 and was clinically introduced to the US market in the early 1990s.

Numerous types of bioactive compounds have been isolated from plant sources [94]. Africa is a rich source of medicinal plants where researchers can use ethnomedical data to increase their chance of finding plants with anticancer activities [97].

Chapter 2: Aim and objectives of study

Cancer is one of the most prominent human diseases which have stimulated scientific and commercial interest in the discovery of new anticancer agents from natural sources. In Africa the majority of people use plant based traditional medicine, where traditional healers have been treating various cancers and cancer-related conditions for ages [101].

Due to its large geographical spread, Africa is one of the continents with the richest biodiversity in the world, however little effort has been devoted to the development of chemotherapeutic agents from these plants. Therefore, detailed evaluation of the constituents and screening of bioactive substances for chemotherapeutic purposes of African plants are urgently warranted. Furthermore, this will serve to validate the efficacy and safety of African traditional medicine.

This study aims to investigate the *in vitro* anticancer activity of previously identified Nigerian medicinal plants with the intension of providing scientific evidence for the use of African traditional medicine as economical alternative for community healthcare. The plants that were investigated are listed as follows: *Sapium ellipticum* leaves, *Sapium ellipticum* stembark, *Combretum paniculatum, Celosia trigyna, Pupalia lappacea, Justica extensa, Hedranthera barteri* leaves, *Alternanthera sessilis, Ethulia conyzoides* leaves, *Lannea nigritana* stembark, *Combretum zenkeri* root, *Combretum molle* leaves, *Adenanthera parvoniana, Lannea acida, Cyathula achyranthoides, Drymaria cordata, Cyathula prostrata, Uvaria chamae* and *Lecaniodiscus cupanioides* (Table 2.1). The extracts were screened for cytotoxicity against human cancer cell lines as well as Chang liver cells and peripheral blood mononuclear cells (PBMCs). The extracts were further screened for effects on levels of the catalytic subunit of the telomerase enzyme, hTERT. Based on the screening results, a few extracts were selected for fractionation and active compound isolation and the mechanism of cell death was investigated. The fractions and isolated compounds that were investigated further are listed in Table 2.2. Some of the active extracts were identified for further studies by other students in the research group. These will be pointed out in later chapters.

Plant selection was based on their frequency in recipes for the management of cancer from an ethnobotanical survey of traditional medical practitioners in South-Western Nigeria. This survey was performed by Dr AA Sowemimo, a research collaborator from the University of Lagos. As part of our ongoing and growing collaboration with Dr Sowemimo, we were first

supplied with 17 extracts prepared from 16 Nigerian plants. After screening of these 17 extracts was complete, she prepared and supplied three more extracts of the plants *U. chamae* and *L. cupanioides* to be investigated for *in vitro* anticancer activity. Results for the first 17 extracts are reported in Sections 4.1, 4.2 and 4.3. Cytotoxicity screening of the additional two plants is reported in Section 4.4.

Table 2.1: List of plants screened for anticancer activity

Extract number	Name of Plant	Part used	Medicinal Uses	Ref.
1a	*Sapium ellipticum* (Krauss.) Pax. (Euphorbiaceae)	Leaves	Wounds, sore eyes and abdominal swelling	102
1b	*Sapium ellipticum* (Krauss.) Pax. (Euphorbiaceae)	Stembark	Purgative, eczema, scurvy and stomatitis	102
2	*Combretum paniculatum* Vent. (Combretaceae)	Leaves	Flower: -inflammation, root:tumours	103
3	*Celosia trigyna* L. (Amaranthaceae)	Whole plant	Malaria, fever, intestinal worms, headache, nose inflammation, pain during pregnancy, uterus pain.	104
4	*Pupalia lappacea* (L.) A. Juss (Amaranthaceae)	Whole plant	Treatment of boils, chronic and fresh wounds, piles, enema for fever and malaria.	105, 106
5	*Justica extensa* T. Anders (Acanthaceae)	Whole plant	Leaf: arthritis, rheumatism, pain; small-pox, chicken-pox, measles, Leaf, flower: diarrhoea, dysentery Leaf, root: paralysis, epilepsy, convulsions, spasm; pulmonary troubles; skin, mucosae Leaf-sap: cutaneous, subcutaneous parasitic infection Plant: fish-poisons Root: febrifuges	107
6	*Hedranthera barteri* (Hook.f.) Pichon (Apocynaceae)	Leaves	Treatment of painful tumour, dizziness, pain inflammation.	108, 109
7	*Alternanthera sessilis* L. DC.	Whole plant	Treatment of infected wounds, sprains, burns, eczema, carbuncle, acute conjunctivitis, snakebites, dysentery, diarrhoea, acne and pimples.	110
8	*Ethulia conyzoides* Linn. F. (Asteraceae)	Leaves	Anthelminthic for round worms and abdominal disorders.	111

Table 2.1 continued...

Table 2.1: List of plants screened for anticancer activity

Extract number	Name of plant	Plant part used	Medicinal Uses	Ref.
9	*Lannea nigritana* (Scott Elliot) Keay SB. (Anacardiaceae)	Stembark	Bark: diarrhoea, dysentery, pain. Bark root: pulmonary troubles, skin, mucosae. Bark-sap: paralysis, epilepsy, convulsion, spasm. Seed: laxatives. Twig: stomach troubles.	112
10	*Combretum zenkeri* Engl.& Diels (Combretaceae)	Root	Purgative and vermifuge, malaria, menstrual pain, worm treatment and oedemas.	113
11	*Combretun molle* R.BR (Combretaceae)	Leaves	Leaves: chest complaints anthelmintic, inhalant, snake bite. Root: induce abortion, treat constipation, leprosy, headaches, stomach pains, fever, dysentery, general pains.	114
12	*Adenanthera parvoniana* L. (Mimosaceae)	Fruits	Bark, leaves, seeds: arthritis, gout, burning sensation, vomiting, fever, haemorrhoids, and diarrhoea.	115
13	*Lannea acida* A. Rich (Anacardiaceae)	Stembark	Eye treatments, anal haemorrhoids, diarrhoea, dysentery, oral treatments, stomach pains, rheumatism, and gonorrhoea.	116,117
14	*Cyathula achyranthoides* (Kunth.) Moq. (Amaranthaceae)	Whole plant	Flowers and leaves: applied to dog bites, relieve headaches, fever.	118
15	*Drymaria cordata* (Linn.) Willd. (Caryophyllaceae)	Whole plant	Antidote, appetizer, depurative, emollient, febrifuge, laxative and stimulant, sinus, boils.	119,120
16	*Cyathula prostrata* (Linn.) Blume (Amaranthaceae)	Whole plant	Root: fever, oitment for scrotum to enable dilatation	121,122

Table 2.1 continued.

Table 2.1: List of plants for anticancer activity

Extract number	Name of plant	Plant part used	Medicinal Uses	Ref.
U	*Uvaria chamae* P. Beauv (Annonaceae)		Root bark: respiratory catarrh, piles, menorrhagia, epistaxis, haematuria and Haemolysis, abdominal pains.	123
LL LR	*Lecaniodiscus cupanioides* Planch ex Benth (Sapindaceae)	Leaves Root	Treatment of wounds and sores, abdominal swelling caused by liver abscess, fevers, measles, hepatomegaly and burns	124

Table 2.2: List of selected plant fractions and isolated compounds screened for anticancer activity.

Plant crude extract	Fractions	Isolated Compounds
Sapium ellipticum **(Leaves)**	SPE- Petroleum ether	
	SB - Butanolic	
	SAQ – Aqueous	
	SEA – Ethyl acetate	SV 2-2a
		SV 2-2b
		SV_2-3-3
	Scf - Chloroform	SCL V2-2
		SCL V2-3
Combretum paniculatum **(Leaves)**	Cpl - Aqueous	
	Cpl - Petroleum ether	
	Cpl - Ethyl acetate	Cpe 4-4
		Cpe 3-4
		Cpe 4-9
Lecaniodiscus cupanioides **(Leaves)**	LL(Aq) - Aqueous	
	LL(B) - Butanolic	
	LL(Etb) - Ethyl acetate A	
	LL(Eta) - Ethyl acetate B	
	LL(H) - Hexane	
	LL(Cl) - Chloroform	

Chapter 3: Materials and methods

3.1 Plant material and extraction

All tested plants were collected from the Olokemeji Forest Reserve and from the Campus Obafemi Awolowo University, Ile-Ife, Nigeria in July 2006. They were authenticated by comparison with corresponding herbarium specimens by Mr Daramola at the Forestry Research Institute, Ibadan, Nigeria (FRIN) where voucher specimens were also deposited. The plants were dried in a hot air oven at 40^0C, ground to powder and stored in amber coloured bottles. One hundred grams of each powdered plant material was macerated with 80% ethanol at room temperature. The resulting extracts were filtered and concentrated to dryness *in vacuo* at room temperature. The respective plant parts used are as stated in Table 2.1.

Fractions of selected extracts were prepared at the University of Lagos and compounds isolated at the Peking University, Beijing, China. The method was described in a journal article recently accepted for publication (Addendum 2).

3.1.1 Plant extract preparation for screening

Plant extract stock solutions (100 mg/ml) were prepared fresh on the day of the experiment. The extracts were dissolved in dimethyl sulfoxide (DMSO) (Saarchem Unilabs) at 100 mg/ml to make a stock solution. The test concentrations were obtained by diluting the stock solution with RPMI 1640 containing 25 mM Hepes and 2 mM glutamine (Lonza), supplemented with 10% foetal bovine serum (FBS) (Gibco). The final concentration of DMSO to which cell cultures were exposed never exceeded 0.25% and a vehicle control was included in all experiments.

3.2 Growth and maintenance of cell lines

Cancer cell lines HT29 (colon cancer), HeLa (cervical cancer), MCF-7 (breast cancer) and U937 (histiocytic lymphoma) and a non-cancerous cell line (Chang Liver) was taken from liquid nitrogen stocks. The cells were thawed in a water-bath (37°C) and transferred to 10 cm culture dishes (Nunc) for adherent cell lines and a 50 ml suspension flask (Nunc) for U937 suspension cultures. The 1 ml thawed cell stock was diluted with 9 ml pre-warmed RPMI 1640 medium containing 25 mM Hepes and 2 mM L-glutamine (Lonza) supplemented with

10% FBS (Gibco). The cells were incubated for approximately 4 h in a 37°C humidified incubator with 5% CO_2 to allow them to attach. The growth medium was removed and fresh growth medium was added to remove DMSO present in the freeze medium. The suspension cell culture (U937) was centrifuged at 300 x g for 5 minutes, the pellet resuspended in 10 ml pre-warmed growth medium and transferred to a 50 ml flask and incubated in a 37°C humidified incubator with 5% CO_2. Cells were viewed using an inverted phase contrast microscope to assess the degree of confluency and to confirm the absence of bacterial and fungal contaminants. When a confluence of ~80% was reached, cells were sub-cultured. U937 cells were maintained at a density between $1x10^5$ and $1x10^6$ cells/ml [125].

3.2.1 Sub-culture of cells

When cells reached 80% confluency they were sub-cultured. The growth medium was aspirated and the cells were washed twice with 5 ml phosphate buffered saline containing no Mg^{2+} or Ca^{2+} (PBSA). One milliliter 0.25% (w/v) trypsin (Roche) was added to the plate to detach cells from the surface. Within a few seconds of allowing the trypsin to cover the surface of the plate, it was aspirated. The dish was incubated at 37°C for 10 min. The cells were resuspended in 1 ml growth medium. To determine the amount of viable cells, 20 µl of the cell suspension was transferred to an eppendorf tube containing 20 µl of trypan blue [0.4% (w/v) in PBS]. The cell suspension was counted on a Neubauer haemocytometer. The cell suspension was replated into new culture dishes at a ratio of 1:5 and incubated [125].

3.3 Determination of cytotoxic activity

3.3.1 Screening of extracts, fractions and compounds using the MTT assay

Traditionally the determination of cell growth is done by counting the viable cells after staining with a vital dye. The yellow MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, a tetrazole) is reduced to a purple formazan product in the mitochondria of living cells. The absorbance of this coloured compound can be quantified after solubilisation in DMSO and by measuring the absorbance at a certain wavelength (usually between 500 and 600 nm) by a spectrophotometer. This reduction takes place only when mitochondrial reductase enzymes are active, and therefore conversion can be directly related to the number of viable (living) cells [126].

Cells in an exponential growth phase were trypsinized, counted using a Neubauer haemocytometer and diluted to a density of 30 000 cells/ml. The cells were seeded into a 96-well plate at 6 000 cells/well in 200 µl aliquots in RPMI 1640:10% FBS. Plates were incubated at 37°C for 24 hours to allow cells to attach. The plant extracts, fractions and isolated compounds were screened for cytotoxicity using concentrations ranging from 1–250 µg/ml. Cisplatin served as a positive control in concentrations ranging from 10 – 100 µM. Following the initial 24 hour incubation period, the growth medium was removed and 200 µl aliquots of the plant extracts, isolated compounds or cisplatin were added and the plates incubated for a further 48 hours. After the 48 hour incubation period the medium was replaced with 200 µl MTT (Sigma) (0.5 mg/ml in RPMI 1640: 10% FBS). The MTT was removed after a further 4 hour incubation at 37°C and the purple formazan product dissolved in 100 µl/well DMSO. Plates were agitated for 60 seconds and absorbance measured at 540 nm on a multiwall scanning spectrophotometer (Multiskan MS, Labsystem). All incubation steps were carried out in a 37°C humidified incubator with 5% CO_2.

3.3.2 Screening of extracts, fractions and compounds on peripheral blood mononuclear cells (PBMCs)

This is an important control mechanism to test the cytotoxic effects of the plant extracts on normal healthy cells.

3.3.2.1 PBMC isolation, seeding and treatment

Raw blood was collected from a healthy donor using heparinised Vacutainer® CPT™ cell preparation tubes (Becton Dickinson). Within 30 minutes of collection, blood was centrifuged at 1 800 x g for 30 minutes at 20°C. The tube was inverted and the top layer containing plasma together with the PBMCs was transferred to a sterile 15 ml falcon tube. Centrifugation followed at 300 x g for 15 minutes, followed by aspiration of the plasma. The pellet was resuspended by grating the tube over a round-bottom plate. Ten milliliters RPMI 1640:10% FBS was added and centrifuged at 300 x g for 15 minutes and the supernatant discarded. This wash step was repeated twice. The pellet was resuspended and diluted to the desired cell density. PBMCs were seeded at 100 000 cells/ml in 100 µl aliquots in round bottomed 96-well plates. One hundred microliters of cisplatin concentrations ranging from 1 - 100 µM and 100 µl of selected plant extracts with concentrations ranging from 8 – 250 µg/ml

were added to the PBMCs and incubated for 48 hours at 37°C. Viability was determined using the Cell Titre Blue assay as described in the next section.

3.3.2.2 Cell Titre Blue assay

Cell Titre Blue Viability assay uses the dark blue indicator dye resazurin to measure the metabolic capacity of cells – an indicator of cell viability. Viable cells retain the ability to reduce resazurin into resorufin (pink), which is highly fluorescent. Non-viable cells rapidly lose metabolic capacity, and do not reduce the indicator dye and thus do not generate a fluorescent signal.

Following the 48 hour incubation period, 40 µl of the Cell Titre Blue reagent was added to the plates. Plates were covered with tin foil and incubated for a further 4 hours at 37°C. Flourescence was read at an excitation wavelength of 560 and an emission wavelength of 590 nm using a Fluoroskan Ascent FL Fluorometer (Thermo Labsystem, Finland). The IC_{50} of the cisplatin and the selected plant extracts was determined using GraphPad Prism 4 program [127].

3.3.3 Dose response curves

Dose response curves for cisplatin and any of the plant extracts that gave more than 50% growth inhibition over the tested concentration range, were prepared. This was done in order to obtain the IC_{50} values of the cisplatin and the selected plant extracts, using the log-dose response curve in the GraphPad Prism 4 program. One of the extracts with the lowest IC_{50} value was selected for further investigations and in those assays, the cells were treated with an extract concentration equal to the IC_{50} value.

3.4 hTERT immunodetection

Increasing data suggest that hTERT can serve as target for widely applicable immunotherapy against cancer. The critical role of telomerase in tumour growth and development suggests that hTERT down regulation may have a deleterious effect on tumour growth [128].

For detection and quantification of hTERT levels in treated and control cells, cells were fixed and permeabilised using the IntraPrep™ kit (Beckman Coulter) before immunodetection

using flow cytometry. A primary anti-hTERT antibody and a FITC-conjugated secondary antibody were used to detect the target protein.

3.4.1 hTERT screening

U937 cells were seeded at 100 000 cells/well in 500 µl aliquots in a 24 well plate. An equal volume of cisplatin or selected plant extract was added to the plates giving a final cisplatin concentration of 50 µM or extract concentration of 12.5 and 125 µg/ml. After the treatment exposure period of 48 hours cells were transferred to polypropylene flow cytometry tubes (Beckman Coulter), centrifuged at 300 x g for 5 minutes and the supernatant discarded and the pellet washed twice with PBS. Fifty microliters IntraPrep™ 1 reagent was added, vortexed and incubated for 15 minutes at room temperature. The cells were washed with 1 ml PBS, centrifuged at 300 x g for 5 minutes and the supernatant discarded. Fifty microliters of IntraPrep™ 2 reagent was added and incubated for 5 minutes at room temperature. Cells were washed in 1 ml PBS supplemented with 0.5 % bovine serum albumin Fraction V (BSA) (Roche) and centrifuged at 300 x g for 5 minutes at room temperature and the supernatant discarded. Cells were resuspended in 100 µl PBS-BSA and allowed to incubate for 10 minutes at room temperature. Two microliters of primary antibody (primary rabbit anti-human hTERT antibody from Santa Cruz Biotechnology, Inc.) was added to the positive control, negative control and treated cells but not to the isotype control and incubated for 60 minutes at room temperature. Cells were washed with 1 ml PBS-BSA and centrifuged at 300 x g for 5 minutes and the supernatant discarded, leaving approximately 100 µl in the tube. Two microliters of the secondary antibody [Goat Anti-Rabbit IgG fluorescein isothiocyanate (FITC)-conjugate from Santa Cruz] was added to all the tubes except the isotype control which received 5 µl of the isotype antibody (Rabbit IgG-FITC from Beckman Coulter) and incubated for 30 minutes at room temperature. Cells were washed in 1 ml PBS-BSA, centrifuged at 300 x g for 5 minutes and the supernatant discarded. Three hundred microliters PBS-BSA was added to the tubes and the samples analyzed by flow cytometry using a Beckman Coulter® FC 500 Flow Cytometer (Miami FL, USA).

3.4.2 GAPDH and β-Actin reference proteins

To better explain biological end experimental variations it is best to include specifically selected internal reference control(s) for data normalization. The expression of the reference control(s) should be constant across all samples and different phases of the cell cycle [129].

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin were selected as reference proteins to verify any variations of hTERT levels in the different cell lines under the various plant treatments.

The measurement of GAPDH and β-actin followed the same method that was used for hTERT screening except for the use of different primary antibody GAPDH (14C10) Rabbit mAb and β-actin (13E5) Rabbit mAb (Cell Signalling), respectively.

3.5 Cell cycle analysis

The sequence of cell cycle events can be identified as follows: Initiating from a quiescent or resting phase (phase G0), cell growth and preparation of chromosomes for replication takes place in phase G1. The cell continues with synthesis of DNA (S phase) and is followed by preparation for cell division (phase G2). Finally mitosis (M phase) occurs and is perpetuated with newly divided cells. Flow cytometry offers a rapid method for measuring the DNA content of cells and provides a convenient research tool to monitor cell cycle status and regulation. An exponentially growing population of cells will have a DNA content distribution containing an initial peak of G0/G1 cells, a valley of S phase, and a second peak containing G2/M cells. Measurement can be performed and is based on the ability of nuclear dyes, such as DAPI and PI, to bind selectively and at a characteristic stoichiometric ratio to DNA under appropriate staining conditions. The DNA Prep Kit from Beckman Coulter was used as per manufacturer's recommendations [130].

Hela, HT29 and MCF-7 cancer cells were seeded in 10 cm culture dishes at 1.2 x 10^6 cells/dish, in 10 ml complete medium and left to attach for 24 hours at 37°C in a humidified incubator and 5% CO_2. After attachment, medium was aspirated and the treatment added. Enough culture dishes were prepared for 6, 16, 24 and 48 hours of treatment. At these incubation times, the medium was transferred to polypropylene flow cytometry tubes, the tubes centrifuged at 300 x g for 5 minutes and the supernatant discarded. This was done to collect cells that may have detached during incubation. The adherent cells were washed twice with phosphate buffered saline (Ca^{2+} and Mg^{2+} free) (PBSA), trypsinized for 10 minutes and resuspended in 1 ml sheath fluid (Beckman Coulter) and transferred to the polypropylene flow cytometry tubes already containing the detached cells. The cells were centrifuged at 300 x g for 5 minutes, the supernatant discarded and the pellet was gently vortexed. The Coulter®

DNA Prep™ reagent kit (Beckman coulter) was used for DNA cell cycle analysis. One hundred microliters Lysis buffer (0.1% NaN_3, non-ionic detergents, saline and stabilizers) was added to each tube and incubated for 5 minutes at room temperature. Five hundred microliters of propidium iodide (PI) was added and incubated in the dark for 15 minutes at 37°C. Samples were analyzed using a Beckman Coulter Cytomics FC500 (Miami FL, USA).

3.6 Apoptosis detection: Annexin V-FITC/PI staining

In most viable eukaryotic cells, the negatively charged phosphatidylserine (PS) is located in the cytosolic leaflet of the plasma membrane lipid bilayer. PS translocation from the inner to outer leaflet is an early event during apoptosis [131]. Annexin V is a PS-binding protein that interacts strongly and specifically with PS residues and can be used for the detection of apoptosis [58] via flow cytometry when fluorescently conjugated [60]. Apoptotic cells can be directly detected through their staining with fluorochrome-conjugated Annexin V. Dual staining with Annexin V and PI enables one to differentiate between necrotic and apoptotic cell death. PI is able to penetrate necrotic cells through their compromised plasma membranes and these cells will therefore stain positive for PI. Dead cells (in the later stages of apoptosis or necrosis) are stained with both Annexin V and PI, whereas viable cells cannot be stained with either.

U937 cells were seeded at 100 000 cells/ml in a 24-well plate treated with DMSO (0.25%, v/v), ciplatin (5 and 50 µM) or *S. ellipticum* leaf extract (125 µg/ml) and incubated for 24 hours at 37°C in a humidified incubator and 5% CO_2. Following the incubation period cells were transferred to polypropylene flow cytometry tubes and centrifuged at 500 x g for 5 minutes at room temperature. Cells were washed in ice-cold Dulbecco's Modified Eagle's Medium (DMEM) and centrifuged at 500 x g for 5 minutes at 4°C. The protocol of the Annexin V-FITC/PI Apoptosis Detection kit (Beckman Coulter) was used. The supernatant was discarded and the pellet resuspended in ice-cold 1X binding buffer. Annexin V-FITC (1 µl) and PI (5 µl) were added to each tube. Tubes were gently mixed and incubated on ice for 15 minutes in the dark. Control tubes for setting up the flow cytometry protocol contained, cells with Annexin V-FITC only, PI only and combination of Annexin V-FITC and PI. Samples were read on a Beckman Coulter Cytomics FC500 (Miami FL, USA).

3.7 Data analysis

For cytotoxicity screening results with fractions and isolated compounds, quadruplicate results of treated cells were compared to vehicle control cells using the two-tailed Student's t-test assuming equal variances. For flow cytometry experiments, a minimum of 10 000 cells were analysed per sample. For cell cycle analysis and Annexin V-FITC/PI assays, duplicate samples were prepared and analysed. For screening of hTERT levels, only single samples were run.

Chapter 4: Results

4.1 Cytotoxic screening using MTT assay and Cell Titre Blue assay

The MTT assay was used to determine cell viability for adherent cells (HT29, MCF-7, HeLa and Chang liver cells) and the Cell Titre Blue assay for PBMCs that are cultured in suspension.

4.1.1 Cytotoxic screening of 17 medicinal plant extracts against HeLa cells

The potential cytotoxicity of 17 medicinal plants was evaluated in a pre-screen against HeLa cervical cancer cells using the MTT viability assay. The crude extracts were initially pre-screened against HeLa cells using two concentrations (250 and 500 µg/ml). From the results of this pre-screen, extracts were selected for further screening against HT29 and MCF-7 cells.

The MTT cytotoxicity results are as shown in Figure 4.1 with extract numbers corresponding to those in Table 2.1. Cisplatin at 10 and 100 µM caused 49.25 ± 3.33% and 88.19 ± 0.60% (SEM, n=4) inhibition, respectively. In consideration of the cytotoxicity, the extracts could be

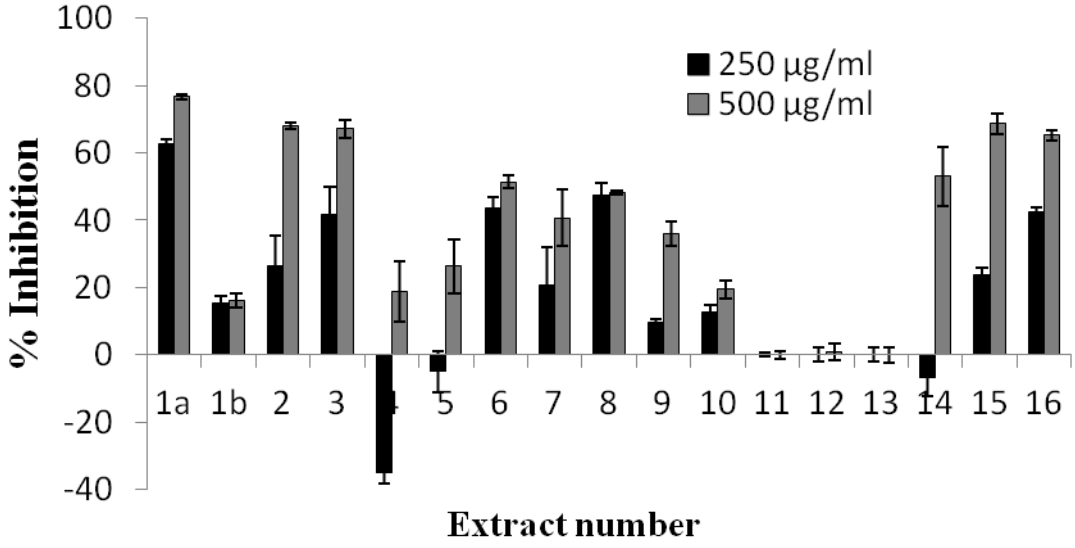


Figure 4.1: Screening results of seventeen extracts, prepared from sixteen plants, on HeLa cervical cancer cells. Results represent the mean ± standard error of the mean of quadruplicate determinations. Cisplatin as positive control at 10 and 100 µM caused 49.25 ± 3.33% and 88.19 ± 0.60% (SEM, n=4) inhibition, respectively (not shown on graph).

classified into four categories according to their relative toxicities. Firstly, potentially cytotoxic were *S. ellipticum* leaves, *C. paniculatum* leaves, *C. trigyna*, *D. cordata* and *C.*

prostrata showing over 50% activity at 500 µg//ml. Secondly, moderately cytotoxic were *E. conyzoides* leaves, *H. barteri* leaves and *C. achyranthoides* showing between 40-50% activity at 500 µg/ml. Thirdly, low cytotoxic were *P. lappacea, J. extensa, S. ellipticum* stembark, *A. sessilis, L. nigritana* stembark and *C. zenkeri* root showing less than 40% activity at 500 µg/ml. Lastly, non-toxic were *C. molle, A. parvoniana* fruit and *L. acida* stem bark with no activity against the HeLa cell line.

4.1.2 Further screening of five selected plant extracts against HT29 and MCF-7 cell lines

S. ellipticum leaves, *C. paniculatum* leaves, *C. trigyna*, *D. cordata* and *C. prostrata* showing over 50% activity at 500 µg/ml against HeLa cancer cells were selected for further screening against HT29 and MCF-7 cancer cell lines at 250 and 500 µg/ml. The plant names, parts used and new extract numbers allocated for further screening are summarised in Table 4.1.

Table 4.1 List of five selected plants used in the cytotoxicity assay against HT29 and MCF-7 cells

Extract number	Plant name (Family) [FRIN No.[a]]	Plant part used
P1	*Sapium ellipticum* (Krauss.) Pax. (Euphorbiaceae) [108265]	Leaves
P2	*Combretum paniculatum* Vent. (Combretaceae) [107980]	Leaves
P3	*Celosia trigyna* L. (Amaranthaceae) [84438]	Whole plant
P4	*Drymaria cordata* (Linn.) Willd. (Caryophyllaceae) [107678]	Whole plant
P5	*Cyathula prostrata* (Linn.) Blume (Amaranthaceae) [107232]	Whole plant

[a] FRIN No.: Herbarium number of collections lodged at the Forestry Research Institute (FRIN) at Ibadan, Nigeria

Figure 4.2 shows the cytotoxicity results with extract numbers corresponding to those in Table 4.1. Cisplatin caused 48.5 ± 2.41, 78.0 ± 0.61 and 72.0 ± 1.47, 82.0 ±1.31 % inhibition (SD, n=4) at 10 and 100 µM inhibition for MCF-7 and HT29 cancer cells, respectively. In the MCF-7 breast cancer cell line, *S. ellipticum* had comparable activity to the positive control

cisplatin for all the concentrations tested. *C. prostrata* showed 50% inhibition at 500 µg/ml while *C. paniculatum*, *C. trigyna* and *D. cordata* showed inhibition lower than 50% for all the concentrations tested. However, in the HT29 colorectal cancer cell line none of the tested plants extracts showed inhibition comparable to the control drug cisplatin. All the plant extracts showed inhibition less than 50% in this cell line. Of all the extracts tested, *Sapium* leaves showed the highest activity and this was also observed in the HeLa cancer cell line.

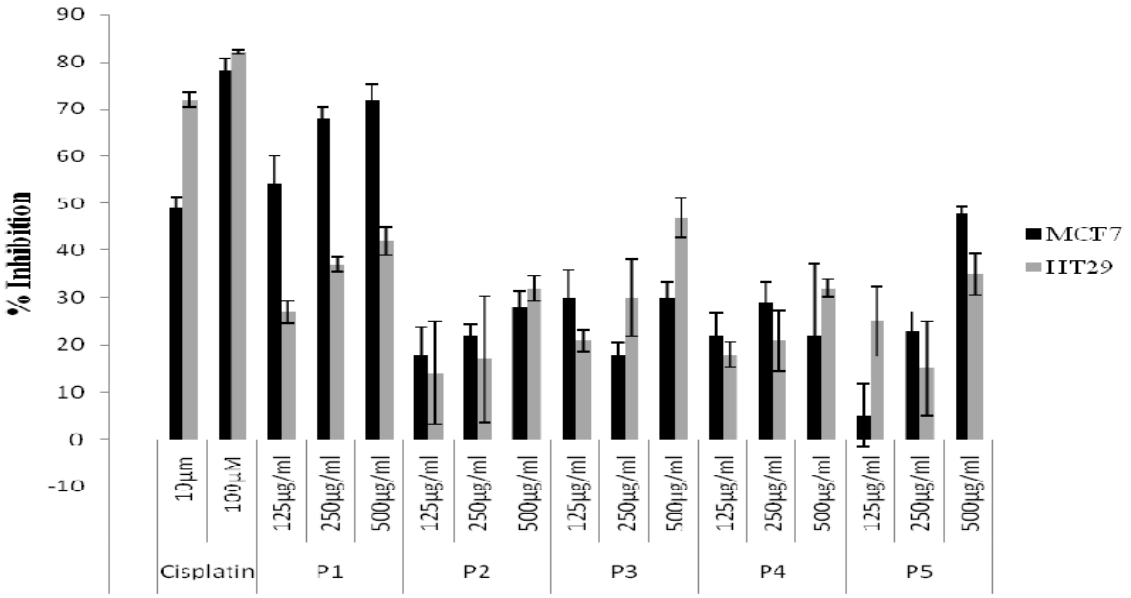


Figure 4.2: Results of cytotoxic activity of extracts P1-P5 on HT29 and MCF-7 cancer cells. Results represent the mean ± SD of quadruplicate determinations. Cisplatin was used as positive control. P1- *Sapium ellipticum*, P2- *Combretum paniculatum;* P3- *Celosia trigyna;* P4- *Drymaria cordata;* P5- *Cyathula prostrata.*

Three extracts were selected for further investigation based on the screening results reported here: *S. ellipticum* and *C. paniculatum* were included in the present study and results are reported in sections 4.1.3, 4.1.4 and 4.3. *C. prostrata* leaf extract was the topic of another MSc study done by Mr Gerald Schnablegger.

4.1.3 Cytotoxic evaluation of *S. ellipticum* leaf crude extract, fractions and compounds

S. ellipticum (Hochst) Pax (Euphorbiaceae) is a tree reaching to about 15 m high. It is widely distributed from Senegal to West Cameroon and across tropical Africa into KwaZulu-Natal in

South Africa [102]. The leaf preparation is used for sore-eyes and abdominal swelling while in Tanzania a preparation of dried leaves is applied to wounds [102].

S. ellipticum (leaf) crude extract is being used in Nigeria as an anti-cancer treatment. The pre-screening of the plant extract against HeLa, HT29 and MCF-7 cancer cells has shown its high cytotoxic activity against HeLa and MCF-7 cancer cell lines with less cytotoxicity against HT29 cancer cell line. Therefore, the crude ethanolic extract, five different fractions prepared from the ethanolic extract and five isolated compounds (Table 2.2) were subjected to further anti-cancer screening.

4.1.3.1 *S. ellipticum* crude extract: IC_{50} determination

4.1.3.1.1 HeLa, HT29 and MCF-7 cancer cell lines

In order to determine IC_{50} values for the crude ethanolic extract of *S. ellipticum* leaves, it was tested against HeLa and MCF-7 cancer cell lines at concentrations ranging from 4 – 500 µg/ml and HT29 at concentrations ranging from 2 – 250 µg/ml. Cisplatin as positive control was tested at concentrations ranging from 1.5 – 100 µM against HeLa cells and at 10 and 100 µM against the other two cell lines.

The results from the MTT assay are shown in Figure 4.3 (A-C). All the treatments showed dose-dependent cytotoxic effects. The results further confirm the observation from the previous section where HT29 cells were the least susceptible to the extract (Figure 4.2 and 4.3 B). In contrast, the three cell lines all seemed to respond with similar sensitivity to cisplatin.

Using GraphPad Prism software, IC_{50} values were calculated for all the dose-response curves where the maximum percentage inhibition was higher than 50%. The IC_{50} for cisplatin against HeLa cells was 7.60 ± 0.39 µM. For the crude extract against HeLa and MCF-7 cells, the IC_{50} values were 88.60 ± 0.03 and 93.09 ± 0.03 µg/ml, respectively (Figure 4.4). An IC_{50} could not be calculated for HT29 cells due to the low percentage inhibition observed.

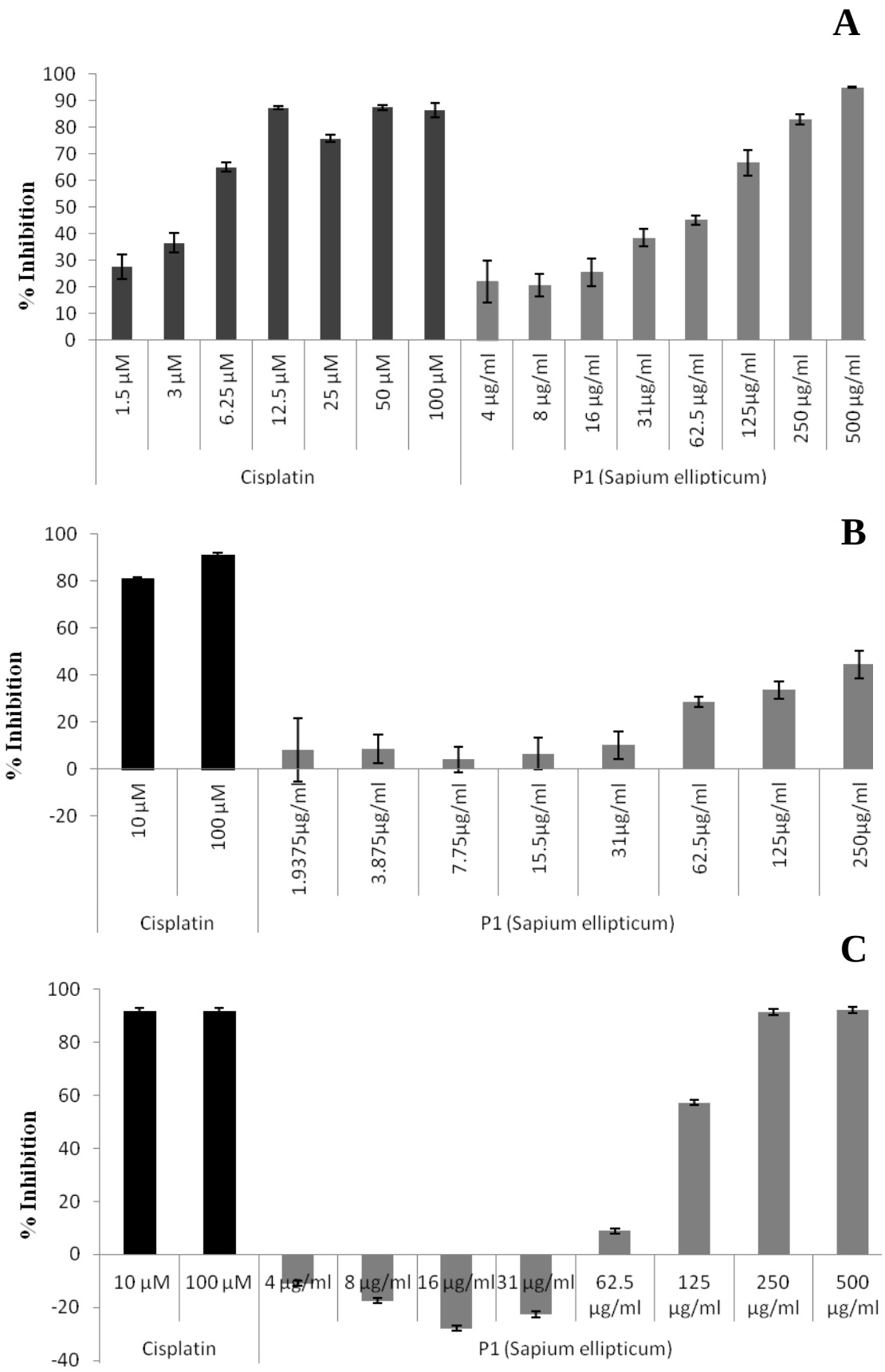


Figure 4.3: Cytotoxicity results of *S. ellipticum* leaf extract against (A) HeLa, (B) HT29 and (C) MCF-7 cancer cells. The positive control, cisplatin was screened at a concentration range of 1.5 – 100 µM against HeLa cancer cells. Results represent the mean ± SD of quadruplicate determinations.

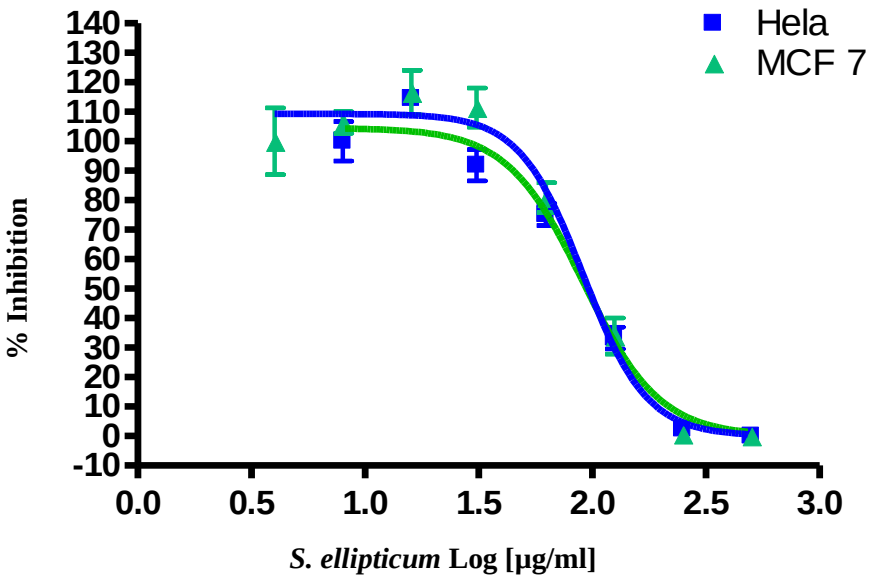


Figure 4.4: Dose response curves of cytotoxic effect of *S. ellipticum* against HeLa and MCF-7 cancer cells. Cells were incubated in quadruplicate in the presence of varying concentrations of *S. ellipticum* crude extract for 48 hours after which an MTT viability assay was performed. The data was analysed using GraphPad Prism software to obtain IC_{50} values. IC_{50} values are reported in Table 4.2. Data points represent the mean ± SD of quadruplicate wells.

4.1.3.1.2 PBMCs and normal human Chang liver cell line

Current anti-cancer drugs do not selectively target cancer cells. Instead, they have the ability to kill rapidly dividing cells whilst being relatively non-toxic to resting (non-proliferating) cells. Although not ideal, this imparts a certain degree of selectivity because cancer cells are constantly dividing whereas the majority of cells in normal tissues are quiescent. In this study *S. ellipticum* leaf crude extract and the positive control cisplatin were screened against PBMCs and Chang liver cells to test their level of cytotoxicity against these normal cells. PBMCs from normal human donors are convenient to use as a normal cell model but they have a limited capacity to proliferate in culture. Chang liver cells were therefore used in their confluent state (non-proliferating) as well as in log phase of their growth curve in order to obtain more information from the results. The Cell Titre Blue assay was used for PBMCs which are cultured in suspension, while the MTT assay was used for the adherent Chang liver cells.

Figure 4.5 (A) shows the cytotoxic activity of cisplatin against PBMCs in concentrations ranging from 1.5 -50 µM. Cisplatin killed more than 50% of the PBMC cells at 12.5, 25 and

50 µM. Cisplatin concentrations ranging from 1.5 – 3 µM showed less than 50 % inhibition. Figure 4.5 (B) shows the results of the cytotoxic evaluation of *S. ellipticum* leaf crude extract against normal, proliferating Chang liver cells and confluent Chang liver cells. The plant showed no inhibitory activity against either the proliferating or the confluent Chang liver cells. It seems to have a growth stimulatory effect on the proliferating cells at 16 and 31 µg/ml. The percentage inhibition seen at 125 µg/ml, which was the highest concentration screened, was 40.68 ± 4.99 (SD, n=4). This was significantly lower than its effect on HeLa, MCF-7 and PBMCs at the same concentration.

To obtain the concentration of cisplatin and *S. ellipticum* required for 50% inhibition in the three cancer cell lines, PBMCs and Chang liver cells the IC_{50} of compounds were determined using the log-dose response curve in the GraphPad Prism 4 program. Ideally, *S. ellipticum* should show no or very low cytotoxic activity against the normal cell models or in other words the IC_{50s} for normal cells should be higher than that of the cancer cells. Table 4.2 shows a summary of the IC_{50} values of cisplatin (positive control) and *S. ellipticum* against HeLa and MCF-7 cancer cell lines and against normal PBMCs and Chang liver cells. The IC_{50} value of cisplatin against HeLa and PBMCs was 7.60 ± 0.39 and 9.91 ± 0.15 µg/ml, respectively. The IC_{50s} of cisplatin on the proliferating cells (HeLa, Chang liver) and PBMCs with a limited capacity to proliferate were all very similar and lower than its IC_{50} on non-proliferating Chang liver cells.

The IC_{50} values of *S. ellipticum* against HeLa, and MCF-7 cancer cells were 88.60 ± 0.03, 93.03 ± 0.03 µg/ml, respectively. The trend seen with cisplatin with respect to lower IC_{50} values on proliferating cells was not observed for the extract. For all further experiments an extract concentration of 125 µg/ml was used against these two cancer cell lines, unless stated otherwise. *S. ellipticum* had no inhibitory activity against HT29 cancer cells at 250 µg/ml and therefore no IC_{50} value could be determined.

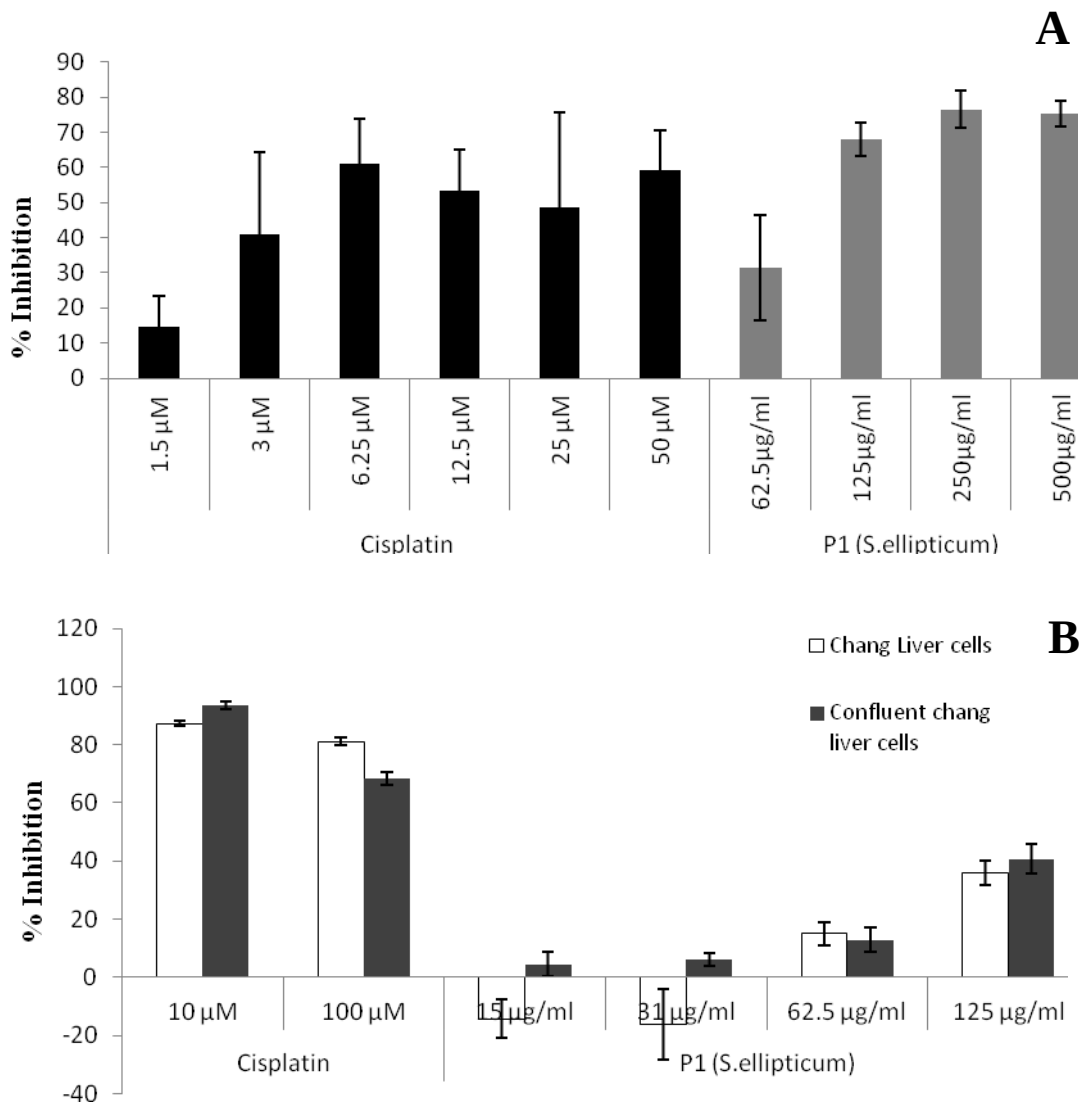


Figure 4.5 Cytotoxic activity of cisplatin and *S. ellipticum* extract against PBMCs (A) and proliferating and confluent Chang liver cells (B). Results represent the mean ± SD of quadruplicate determinations. Cisplatin was used as positive control.

Table 4.2: Summary of IC_{50} values of the cytotoxic effect of cisplatin and *S. ellipticum* crude extract on cancer cells and normal cell models

	Cisplatin (µM)	***S. ellipticum* (µg/ml)**
HeLa	7.60 ± 0.39	88.60 ± 0.03
MCF-7	nd	93.03 ± 0.03
PBMCs	9.91 ± 0.15	77.66 ± 0.14
Proliferating Chang liver cells	9.85 ± 0.09	> 125 µg/ml
Confluent Chang liver cells	25.06 ± 0.12	> 125 µg/ml

nd: not determined

4.1.3.2 *S. ellipticum* fractions and isolated compounds: cytotoxic effects

To determine the active chemical component(s) in the leaf crude extract, responsible for the cytotoxic activity in the three different cancer cell lines (Figure 4.2 and Table 4.2), five fractions (Table 4.3) were prepared from the crude ethanolic extract. These fractions were prepared by a research collaborator at the University of Lagos and kindly supplied for testing. Their growth inhibitory activities were evaluated against MCF-7 cancer cells and confluent Chang liver cells. The IC_{50} value of *S. ellipticum* against MCF-7 was 93.03 µg/ml and therefore 62.5 and 125 µg/ml of the fractions were screened.

Table 4.3: List of prepared fractions and isolated compounds from *S. ellipticum* leaf crude extract.

Plant crude extract	Fractions	Isolated compounds*
Sapium ellipticum (Leaves) Sc	SPE- Petroleum ether	-
	SB - Butanolic	-
	SAQ – Aqueous	-
	SEA – Ethyl acetate	SV 2-2a SV 2-2b SV_2-3-3
	Scf - Chloroform	SCL V2-2 SCL V2-3

*Unidentified compounds, only SV 2-2a and SV 2-2b exhibited cytotoxic activity (discussed later) but insufficient quantities were isolated for structural elucidation.

The cytotoxic screening results of the fractions as listed in Table 4.4 showed that the petroleum ether fraction (SPE) had no cytotoxic activity against MCF-7 cancer cells for both concentrations tested. The aqueous fraction (SAQ) had no growth inhibitory activity at 62.5 µg/ml, but showed more than 50% inhibition at 125 µg/ml. The chloroform (Scf), ethyl acetate (SEA) and butanolic (SB) fractions showed more than 50% inhibition for both concentrations, with the chloroform fraction having the highest growth inhibitory activity for both concentrations. Low to no growth inhibitory activity was seen for all fractions against confluent Chang liver cells, except for the chloroform and ethyl acetate fractions at 125 µg/ml. The butanolic fraction had more than 50% growth inhibitory activity against MCF-7

cancer cells, but less than 50% growth inhibitory activity against both proliferating and confluent Chang liver cells for both concentrations.

Table 4.4: Summary of cytotoxic screening results (% inhibition) of *S. ellipticum* fractions against MCF-7 cancer cell line and confluent Chang liver cells

S. ellipticum leaf crude extract fractions	MCF-7 Cells		Confluent Chang liver cells	
	62.5 µg/ml	125 µg/ml	62.5 µg/ml	125 µg/ml
SPE	-2.32 ± 6.14	11.29 ± 9.58	6.68 ± 5.05*	16.81 ± 2.70***
Scf	79.86 ± 3.20***	90.86 ± 0.81***	17.23 ± 8.99*	76.54 ± 7.19***
SEA	54.23 ± 7.68***	65.73 ± 3.83***	32.00 ± 2.92***	50.591 ± 0.95***
SB	58.87 ± 3.16***	67.84 ± 4.53***	10.30 ± 8.96	29.37 ± 1.66***
SAQ	6.54 ± 22.56	66.633 ± 3.08***	32.668 ± 3.36***	33.496 ± 5.89***

Results represent the mean ± SD of quadruplicate determinations.
Refer to Table 4.3 for description of fraction abbreviations.
* Significantly different from vehicle control; $p < 0.05$
** Significantly different from vehicle control; $p < 0.01$
*** Significantly different from vehicle control; $p < 0.001$

To further determine the active chemical component(s) within the leaf crude extract which exerted more than 50% growth inhibitory activity, five compounds were isolated and purified from *S. ellipticum* crude leaf extract (Table 4.3); three from the ethyl acetate (SEA) and two from the chloroform (Scf) fraction. Isolation was done by Dr AA Sowemimo from the University of Lagos and sent to NMMU for screening. These compounds were screened for cytotoxic activity against HeLa, HT29 and MCF-7 cancer cell lines. Note that the leaf crude extract has shown low growth inhibitory activity against HT29 cancer cells, and thus no or low cytotoxic activity was expected against the cell line. The cytotoxic screening results in Table 4.5 show that compounds $SV_{2\text{-}2a}$ and $SV_{2\text{-}2b}$ induced more than 50% growth inhibitory activity against the MCF-7 cancer cell line.

SV $_{2\text{-}2b}$ showed a growth inhibition of 80.91 ± 2 .86% (SD, n=4) at 31 µg/ml, compared to SV $_{2\text{-}2a}$ which gave a much lower percentage growth inhibition of 7.28 ± 14.77% at the same concentration. The same trend was seen at the higher concentrations tested. SV $_{2\text{-}2b}$ showed 81.38 ± 1.04 and 89.32 ± 0.84% growth inhibition at 62.5 and 125 µg/ml, respectively, while SV $_{2\text{-}2a}$ showed growth inhibition of 58.28 ± 22.69 and 89.92 ± 3.21% at these concentrations. Compound SV $_{2\text{-}2b}$ showed more growth inhibitory activity against MCF-7 cancer cell line than SV $_{2\text{-}2a}$. Due to the small amount of compound that was isolated and purified, the whole amount was dissolved in DMSO when the stock solution was prepared. Unfortunately the compounds were very unstable and lost activity over a short period of time. No further cytotoxic screening could be done due to the instability of the compounds. The structures of these compounds could furthermore not be elucidated due to the limited amounts available and their instability.

Table 4.5: Summary of cytotoxic screening results (% inhibition) of *S. ellipticum* isolated compounds against HeLa, HT29 and MCF-7 cancer cells

	µg/ml	SV $_{2\text{-}2a}$	SV $_{2\text{-}2b}$	SV$_{2\text{-}3\text{-}3}$	SCL V$_{2\text{-}2}$	SCL V$_{2\text{-}3}$
HeLa	16	-	-	-40.38 ± .264	10.66 ± 0.68	1.72 ± 10.09
	31	-	-	-16.99 ± 9.38	31.88 ± 10.90***	9.12 ± 12.10
	62.5	-	-	1.98 ± .980	21.13 ± 10.35**	20.59 ± 6.84***
	125	-	-	19.52 ± 4.38*	7.39 ± 8.25	2.09 ± 6.84
HT 29	16	-	-	17.70 ± 9.31*	15.04 ± 30.33	11.57 ± 9.23
	31	-	-	27.02 ± 4.62***	17.04 ± 14.13	8.44 ± 7.33
	62.5	-	-	39.33 ± 7.21***	-16.26 ± 5.93	5.48 ± 10.47
	125	-	-	36.54 ±4.75***	-10.96 ± 18.04	9.22 ± 15.05
MCF-7	16	-22.12 ± 4.39	17.38±10.91*	-49.64 ± 19.48	0.21 ± 5.35	3.25 ± 10.18
	31	7.28 ± 14.77	80.91± 2.86***	-57.21 ± 19.09	19.23 ± 6.63*	9.53 ± 11.86
	62.5	58.28 ± 22.69*	81.38± 1.04***	-32.91 ± 31.23	-5.78 ± 0.03	-1.65 ± 4.56
	125	89.92 ± 3.21***	89.32± 0.84***	27.41 ± 5.53**	18.52 ± 1.92**	0.42± 0.42

* Significantly different from vehicle control; $p < 0.05$

** Significantly different from vehicle control; $p < 0.01$

*** Significantly different from vehicle control; $p < 0.001$

Very low growth inhibition was seen for SV_{2-3-3}, SCL V_{2-2} and SCL V_{2-3} in HeLa, HT29 and MCF-7 cancer cell lines and in fact at some concentrations these compounds stimulated growth, as represented by the negative percentage growth inhibition in Table 4.5.

4.1.4 Cytotoxic screening of *Combretum paniculatum* against HeLa, HT29 and MCF-7 cancer cells

C. paniculatum leaf crude extract was potentially cytotoxic against HeLa cells showing over 50% activity at 250 µg/ml (Figure 4.1). The activity was much lower against MCF-7 and HT29 cells (Figure 4.2), suggesting possible selectivity for specific cell types, in this case cervical cancer cells. Bioassay guided fractionation was performed with the assistance of Dr AA Sowemimo. Fractions and isolated compounds of *C. paniculatum* (Table 4.6) were prepared in her laboratory and screened at NMMU against HeLa, HT29 and MCF-7 cells using the MTT assay and against PBMCs using the Cell Titre Blue assay. A concentration range of 62.5, 125 and 250 and 500 µg/ml was tested to determine the degree of cytotoxicity.

Table 4.6: List of fractions and isolated compounds prepared from crude ethanolic *C. paniculatum* leaf extract.

Plant crude extract	Fractions	Isolated Compounds	Isolated Compounds Name
Combretum paniculatum	Cpl (Aq) - Aqueous	-	-
	Cpl (PE) - Petroleum ether	-	-
	Cpl (ET) - Ethyl acetate	Cpe 4-4	Pheophorbide
		Cpe 3-4	Pheophorbide a methyl ester
		Cpe 4-9	Not pure

Figure 4.6 (A) Shows the cytotoxic results of the three *C. paniculatum* fractions Cpl (Aq), Cpl (PE) and Cpl (ET) against HeLa, HT 29 and MCF-7 cancer cells. Cisplatin at 10 and 100 µM inhibited HeLa, HT 29 and MCF-7 cell proliferation by >75%. All three fractions exhibited cytotoxicity but only the ethyl acetate fraction (ET) inhibited cell proliferation in all three cell lines by more than 50% at all three concentrations tested. Interestingly, the trend

observed with the crude extract of being more toxic to HeLa cells than MCF-7 and HT29, was not seen with the fractions.

Figure 4.6 (B) shows the dose-dependent cytotoxic effect of the isolated compounds Cpe 4-4, Cpe 3-4 and Cpe 4-9 of *C. paniculatum.* The first two caused significant inhibition of all three cell lines at all the concentrations tested ($p < 0.001$ except Cpe 3-4 against HT29 with $p < 0.01$). Chemical analysis of Cpe 4-9 revealed that it was not pure and contained a number of contaminating compounds. Its cytotoxic activity was lower than that of Cpe 4-4 and comparable to Cpe 3-4 and it was decided not to purify it further.

For Cpe 4-4, it was only at the lowest concentration of 62.5 µg /ml on HT29 cells that it was not able to inhibit proliferation by >50%. A similar trend was observed for Cpe 3-4, except that the % inhibition of HT29 cells was <50% at both 62.5 and 125 µg/ml.

Results from the Cell Titre Blue assay on PBMCs (Figure 4.7) suggest that Cpe 3-4 was less toxic to normal cells than Cpe 4-4. Low toxicity was also seen with Cpe 4-9.

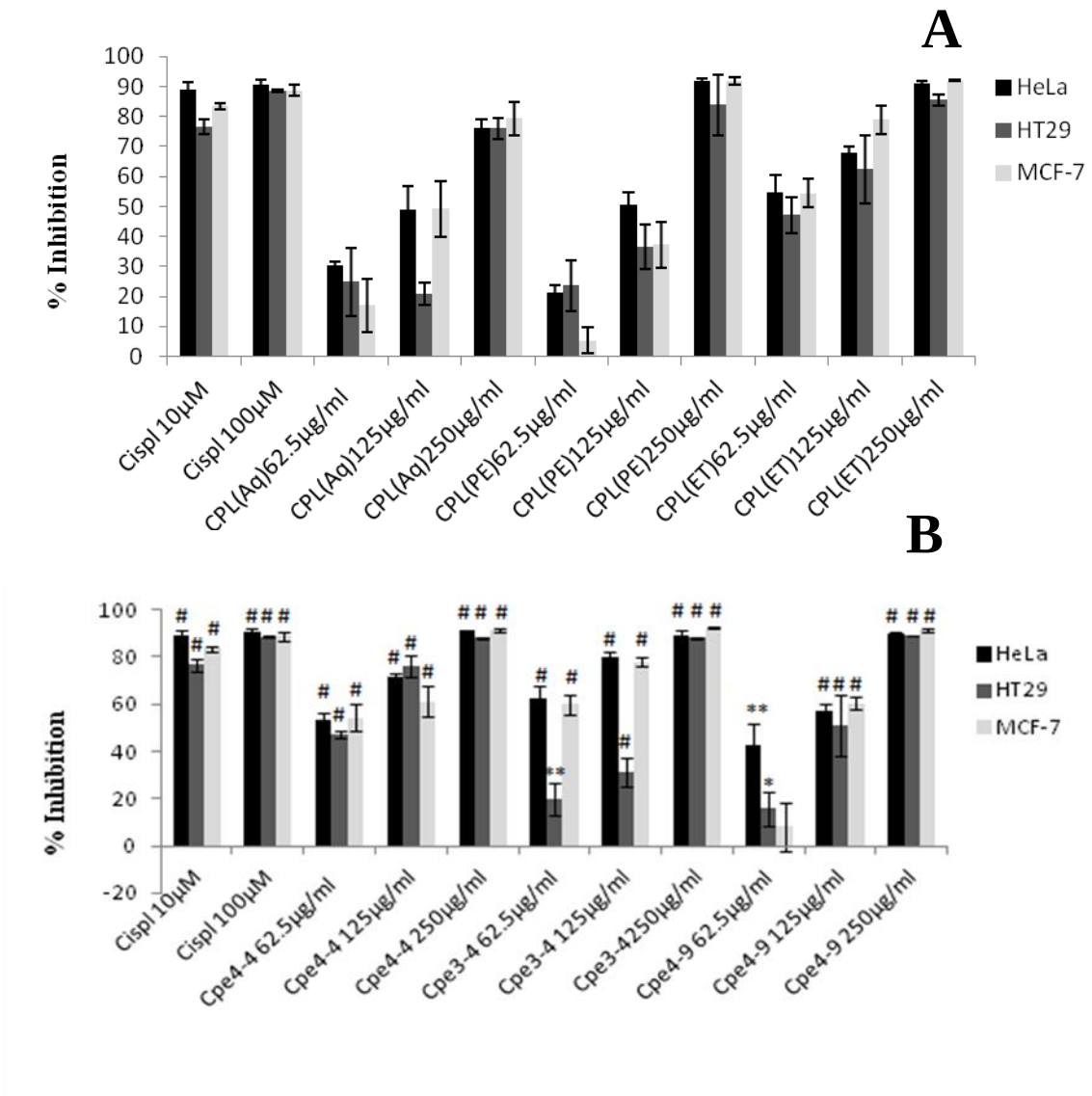


Figure 4.6: Cytotoxicity of *C. paniculatum* fractions (A) and isolated compounds (B) against HeLa, HT29 and MCF-7 cancer cells. Results represent the mean ± SD of quadruplicate determinations. Cisplatin was used as positive control. Cpe4-4, Cpe 3-4 and Cpe 4-9 are as listed in Table 4.2. Significantly different from vehicle control; $p < 0.05$; ** Significantly different from vehicle control; $p < 0.01$; # Significantly different from vehicle control; $p < 0.001$

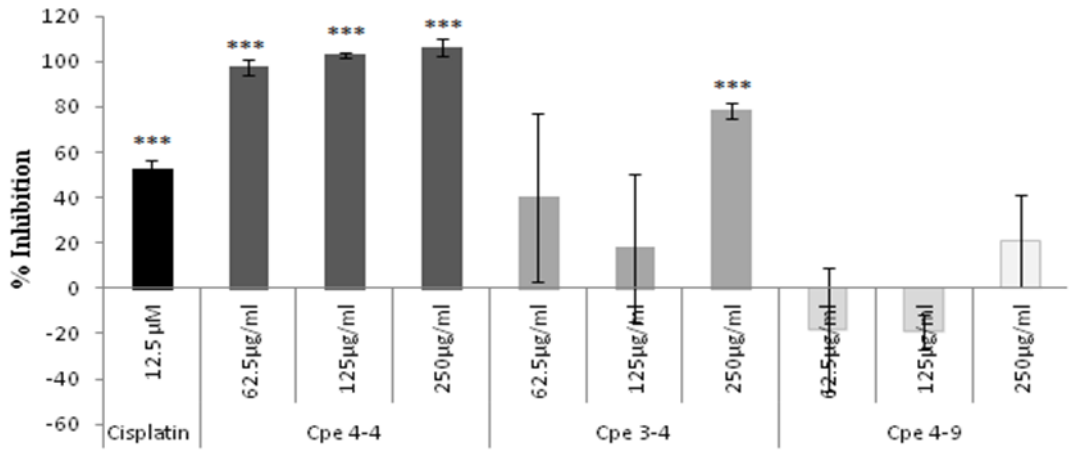


Figure 4.7: Cytotoxic activity of compounds isolated from *C. paniculatum* leaf extract against PBMCs. Results represent the mean ± SD of quadruplicate determinations. Cisplatin was used as positive control. Cpe4-4, Cpe 3-4 and Cpe 4-9 are as listed in Table 4.2. * Significantly different from vehicle control; $p < 0.05$; ** Significantly different from vehicle control; $p < 0.01$; *** Significantly different from vehicle control; $p < 0.001$

4.2 Screening of 17 plant extracts for hTERT inhibition

The nearly universal expression of telomerase reported in almost all human cancer cells but not in most normal cells, provides an attractive target for the treatment of cancer [81]. Inhibition of the enzyme can be achieved through the design of telomerase inhibitors or the suppression of telomerase expression. In this study we used a cellular immunofluorescence - based hTERT flow cytometry assay to measure the amount of the catalytic subunit of the telomerase enzyme present after exposure to the 17 plant extracts at 12.5 and 125 µg/ml for 24 and 48 hours. The levels of the two internal reference proteins GAPDH and β-actin, were measured in the same samples to validate the results found for the measurement of the catalytic subunit of telomerase.

Flow cytometry is a convenient method that allows one to record data for thousands of individual cells in as little as a few seconds. Figure 4.8 (A) shows an example of a 2D dot plot of U937 vehicle control cells (treated with 0.15% DMSO). Each dot represents a single cell passing through the laser beam of the flow cytometer. The position of each dot represents its forward scatter (FS) and side scatter (SS) properties, which relate to size and cell granularity, respectively. Based on the FS and SS, a region can be drawn around a sub

population to isolate events for analysis. The data of all the events within the region can then be displayed in subsequent plots by gating on the region. In this case the fluorescence properties of the cells in region A are displayed in a histogram (Figure 4.8 B).

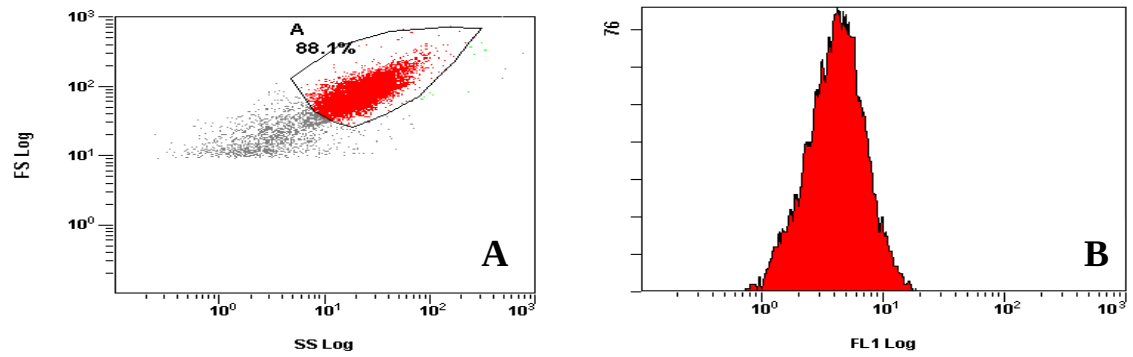


Figure 4.8: Recording of flow cytometry data showing vehicle control cells as example. (A) Detection of cells in region A on 2D dot plot of forward (FS)- vs side (SS)-scatter and (B) a histogram representing the fluorescence properties of the cells in region A. Cells were labelled with anti-hTERT primary and FITC-conjugated secondary antibodies for detection of hTERT, the catalytic subunit of telomerase. A minimum of 10 000 events were recorded per sample on a Beckman Coulter FC500 (Miami FL, USA) flow cytometer.

Histograms of control cells can be compared to that of treated cells by observing their relative positions on a histogram overlay. To illustrate this, Figures 4.9 and 4.10 show the overlay histograms of hTERT and β-actin levels in U937 cancer cells after 24 hours of treatment with 12.5 and 125 µg/ml for the five extracts investigated in Section 4.1.2. The grey shaded area represents the isotype control antibody which distinguishes the non-specific binding. The black histogram represents the control cells (black line), the red histogram cisplatin (50 µM) and P1-P5 at 12.5 µg/ml and 125 µg/ml are represented by the different colours as indicated in the figure captions. A decrease in hTERT levels will cause the histogram to appear to the left on the log scale when compared to the control cells (i.e. lower fluorescence intensity due to less antigen available for the antibody to bind to). Similarly, an increase in the levels of hTERT will cause the histogram to appear to the right, at higher fluorescence intensities than that of the control cells.

The flow cytometry protocol was set up to record 10 000 events for each sample and therefore the histograms were expected to all have the same peak areas. In Figure 4.9 the low concentration exposure for 24 hours for cisplatin showed a smaller peak when compared to the control and plant treatments. This can be explained by cisplatin's known apoptosis inducing properties, through binding to nuclear DNA and subsequently interfering with DNA

replication mechanism. Fewer cells are present in the sample due to elimination via apoptosis.

Figure 4.9 shows the overlay histograms of the effect of P1-P5 on hTERT protein measurement. At the higher concentration, P1, P2 and P3 appeared to have reduced the levels of hTERT relative to the vehicle control cells. In order to quantify these changes, and to allow better interpretation of the results, one can compare the mean fluorescence intensities obtained from these histograms. The results of all 17 plant extracts are summarized in Table 4.7. In order to simplify interpretation, mean fluorescence intensity values for hTERT that were one or more fluorescence unit lower than the control value, were indicated in ***bold italic*** font. Such values suggest a reduction in the levels of hTERT due to the treatment with the plant extract. After 24 hours cisplatin caused an unexpected increase in hTERT expression, however for 48 hours at 12.5 µg/ml and 24 and 48 hours at 125 µg/ml, cisplatin showed a decrease in hTERT protein measurement as was expected. At the low extract concentration at 24 hours no significant changes in hTERT protein measurement were seen when compared to the control cells. At 48 hours a few plant extracts seemed to have caused a decrease in hTERT protein measurement. At 125 µg/ml for both 24 and 48 hours the following plant extracts, 4, 5, 9, 11, 13, 14 and 16 caused a reduction in mean fluorescence intensity of one unit or more when compared to the control cells. The plants from which these extracts originated were *P. lappacea, J. extensa, L. nigritana, C. molle, L. acida, C. achyranthoides* and *C. prostrata.*

The possibility that reductions in hTERT levels were due to non-specific inhibition of gene transcription or protein synthesis and therefore not specifically targeted at hTERT expression or synthesis cannot be excluded. Such a general, non-specific effect would not have any therapeutic use. By quantifying one or more reference proteins in the same cells in which hTERT levels were measured, one would be able to validate the results and observe any possible non-target effects. This approach is similar to that employed in molecular biology where genes widely known as housekeeping genes are used to account for experimental error as opposed to biological differences [132]. GAPDH, an enzyme of glycolysis and β-actin, a cytoskeletal protein, were considered possible reference protein candidates for this study. They were selected on the assumption that their levels of expression would be unaffected by experimental conditions [133]. The internal reference gene GAPDH showed poor inconclusive results (not shown) and further analysis with the enzyme was stopped.

Figure 4.10 shows the overlay histograms of internal reference protein β-actin levels in U937 cancer cells for 24 hours at 12.5 and 125 µg/ml. The grey shaded area represents the isotype control antibody which distinguishes the non-specific binding. The black histogram represent the control cells (black line), cisplatin (50 µM) is represented by the red line and results from the selected five plant extracts at 12.5 and 125 µg/ml are represented by the different coloured lines as indicated in the figure caption. The same negative and positive control cells were used for 12.5 and 125 µg/ml. The reference protein levels should be unaffected by the different treatments, however cisplatin treatment after 24 and 48 hours caused a shift to the right as seen in the overlay histograms with an x-mean value of 6.57 and 26.4 when compared to the x-mean value of 5.0 of the control cells. Table 4.7 summarizes the mean fluorescence intensities of the β-actin protein measurement results of 17 plant extracts after 24 hours of treatment. Only cisplatin was tested at 48 hours. It is clear that the levels of this protein were affected by some of the treatments, especially by cisplatin at 48 hours where a large increase was observed.

Other researchers have reported increases of at least three times in mitochondrial β-actin levels of apoptotic cells [134]. The large increase in x-mean from 3.8 to 26.4 observed in the present study could be explained by β-actin antibody binding the cytosolic, nuclear and accumulated mitochondrial β-actin. Another study revealed that both GAPDH and β-actin mRNA levels vary with cellular proliferation and thus variability in expression emphasises their important physiological roles [133]. It is of great importance that the concentrations of the reference protein should not be influenced by drug treatment and other proteins will have to be considered for validation of the hTERT results in this study.

To further validate the results from this screening experiment, more replicates of each sample would be required. In order to minimise the number of samples for practical reasons, only one sample was analysed for each extract. Within this one sample, a minimum of 10 000 cells were analysed but this result would not reflect experimental variation that may occur between replicate samples.

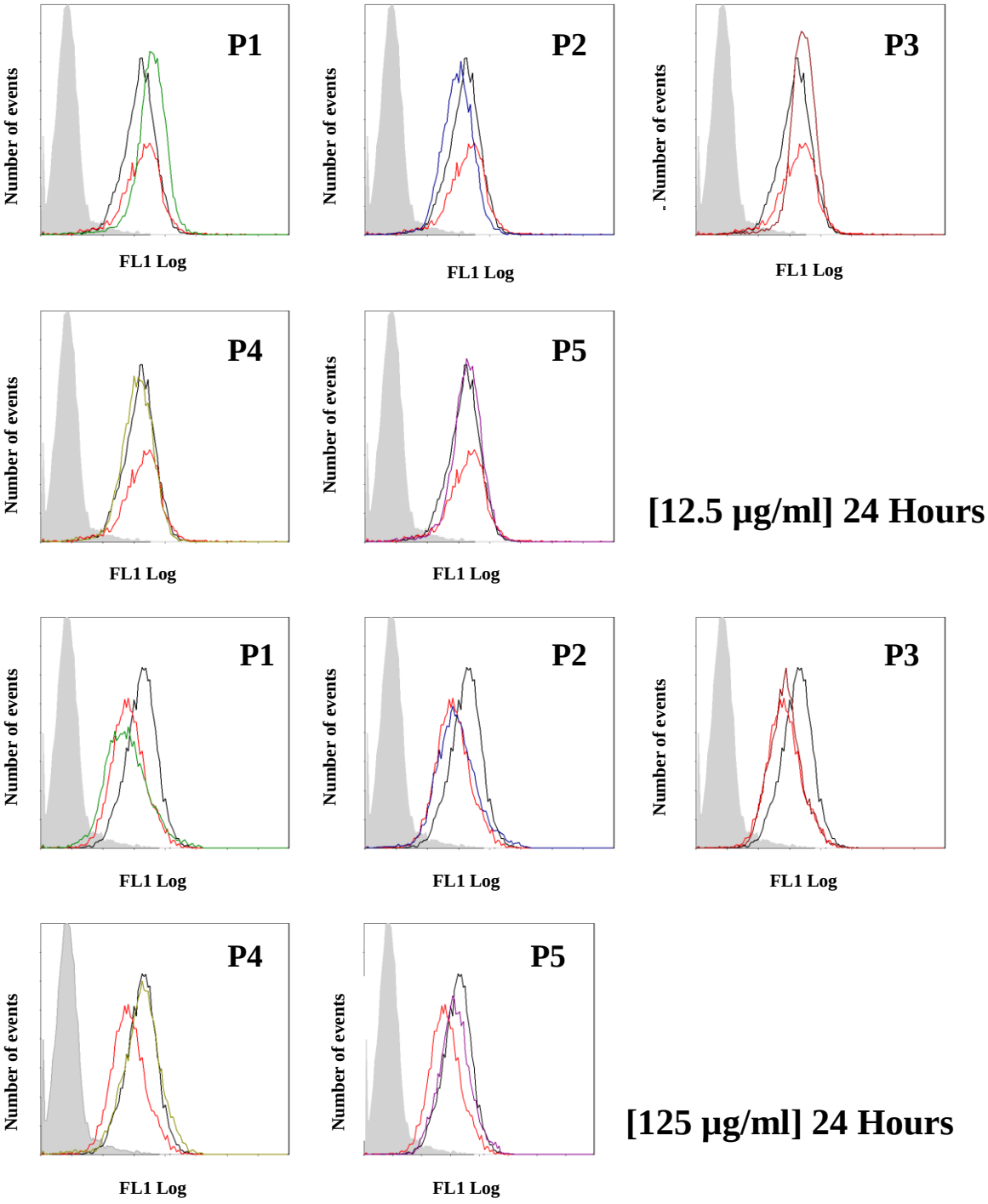


Figure 4.9: Histograms of hTERT protein expression in U937 cancer cells treated with P1- *S. ellipticum*, P2- *C. paniculatum;* P3- *C. trigyna;* P4- *D. cordata;* P5- *C. prostrate* at 12.5 and 125 µg/ml for 24 hours. Cisplatin was used as positive control at 50µM (red histogram). Shaded histogram: isotype control; black: 0.125% DMSO; others colours: plant extracts. After treatment, cells were fixed and permeabilised and hTERT was labelled using a primary anti-hTERT and FITC conjugated secondary antibody. A minimum of 10 000 cells per sample were analysed on a flow cytometer.

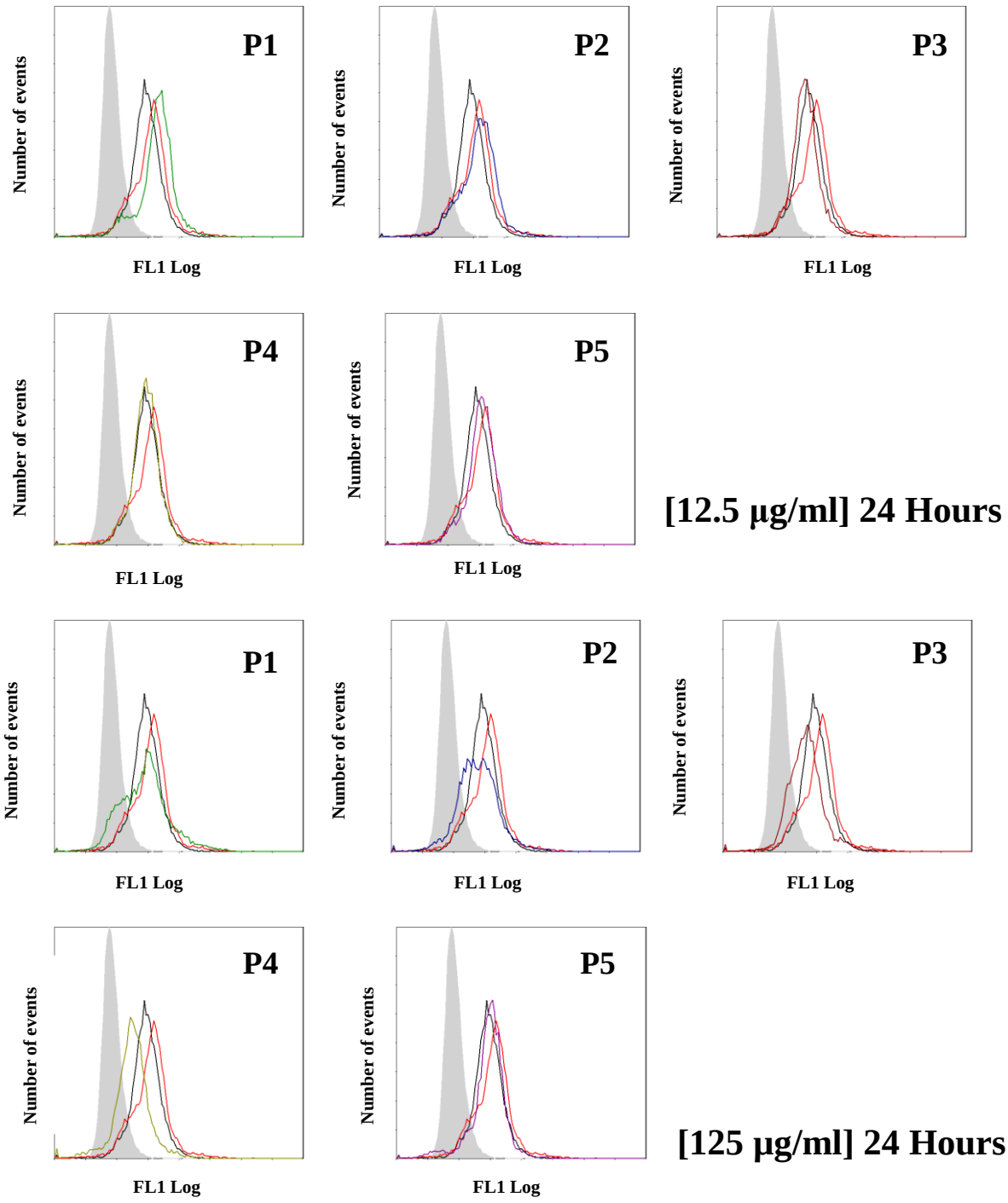


Figure 4.10: Histograms of internal reference protein β-actin expression in U937 cancer cells treated with P1- *S. ellipticum*, P2- *C. paniculatum;* P3- *C. trigyna;* P4- *D. Cordata;* P5- *C.a prostrata*.at at 12.5 and 125 µg/ml for 24 hours. Cisplatin was used as positive control at 50 µM (red histogram). Shaded histogram: isotype control; black: 0.125% DMSO; others colours: plant extracts. After treatment, cells were fixed and permeabilised and β-actin was labelled using a primary anti- β-actin and FITC conjugated secondary antibody. A minimum of 10 000 cells per sample were analysed on a flow cytometer.

Table 4.7: List of hTERT and β-Actin x-mean expression results of the 17 plants screened against U937 cancer cells

Plant No	hTERT X-Mean				β-Actin X-Mean			
	12.5 µg/ml		125 µg/ml		12.5 µg/ml		125 µg/ml	
	24 H	48 H	24 H	48 H	24 H	48 H	24 H	48 H
Control	5.29	6.61	6.3	7.16	5.00	3.8	5.00	3.8
Cisplatin	8.55	***5.21***	***4.05***	***4.57***	6.57	26.4	6.57	26.4
1a – P1	7.76	***5.02***	5.41	***5.88***	6.91	-	5.89	-
1b	6.44	8.94	6.27	***6.1***	6.74	-	6.32	-
2– P2	***4.17***	6.17	***4.97***	6.24	5.96	-	5.68	-
3 – P3	6.83	7.13	***4.59***	6.34	3.72	-	4.85	-
4	5.58	7.03	***1.65***	***5.42***	7.48	-	5.75	-
5	5.55	12.88	***3.38***	***5.94***	7.03	-	5.83	-
6	8.09	12.2	6.73	***4.98***	5.53	-	3.75	-
7	7.36	6.04	***5.32***	6.41	4.7	-	5.66	-
8	9.01	6.18	6.19	***4.51***	4.93	-	2.74	-
9	5.61	6	***5.03***	***4.11***	3.07	-	5.05	-
10	5.87	***5.5***	5.39	***5.35***	5.25	-	-	-
11	5.97	6.35	***4.53***	***5.27***	5.23	-	6.81	-
12	5.34	6.83	5.55	***5.46***	4.99	-	9.34	-
13	7.1	***4.87***	***5.02***	***5.44***	5.46	-	4.33	-
14	5.69	***4.61***	***5.23***	***5.73***	6.67	-	4.46	-
15 – P4	5.41	***4.57***	5.6	***5.43***	4.36	-	3.55	-
16 - P5	5.97	***4.99***	***5.3***	***3.69***	5.59	-	4.48	-

4.3 Mechanism of action of selected extracts and compounds

In view of the promising cytotoxic effects observed for *S. ellipticum* and *C. paniculatum* and the compounds isolated from them (sections 4.1.3 and 4.1.4) as well as the indication that the crude extracts of these two plants might reduce hTERT levels (section 4.2), it was decided to do further investigation into their modes of action.

4.3.1 *S. ellipticum* crude extract

The crude extract of *S. ellipticum* was chosen to investigate the mode of action because of the loss of the two active compounds due to instability (Section 4.1.3.2).

4.3.1.1 Cell cycle analysis

A phenomenon that plays a key role in cancer therapy is cell cycle arrest [135]. The cell cycle is a target for many anticancer drugs, where naturally occurring or synthetic molecules with anticancer activity effects basal cell cycle control mechanisms [135, 136]. To establish whether *S. ellipticum* leaf crude extract inhibited cell growth by blocking cells in a certain phase of the cell cycle or whether it induces apoptosis, HeLa, HT29 and MCF-7 cancer cells were exposed to 125 µg/ml of the crude extract for 6, 16, 24 and 48 hours after which DNA cell cycle analysis was performed.

The acquisition protocol on the flow cytometer was set up to record FS and SS as explained in the previous section (Figure 4.8.A). Once the cell population of interest has been identified, a plot of FL3 peak area vs FL3 peak height was used to eliminate any doublets or cell clumps (Figure 4.11.A). A region was drawn around the cells of interest. The histogram of fluorescence intensity (FL3), plotted on a linear scale as opposed to the log scale in the previous section, was then gated on the region. An example of such a gated histogram is shown in Figure 4.11.B. The peak marked "A" often represents apoptotic cells and is referred to as the sub-G1 peak. Apoptotic cells show up in the sub-G1 peak due to DNA fragmentation occurring during apoptosis and loss of small fragments of DNA from the permeabilized cells. Less PI will therefore bind to these cells [137]. In the G1/G0 phases, cells have one copy of DNA (diploid; 2N) and form a sharp peak labelled "B" in Figure 4.11 B. Cells in G2 or M phase, with two copies of the DNA (tetraploid; 4N) show up in a peak at fluorescence intensity almost double that of G1/G0 as indicated by region "D" while the S-

phase cells having an amount of DNA between 2N and 4N form a "valley" (region "C") between these two peaks. The histogram shown in Figure 4.11 was obtained from the FC500 Beckman Coulter software. The regions indicating the different phases and percentage of cells in each phase are not very accurate and can be calculated more accurately by using software that accounts for the overlap of the different peaks. In this study, MultiCycle software was used for this purpose.

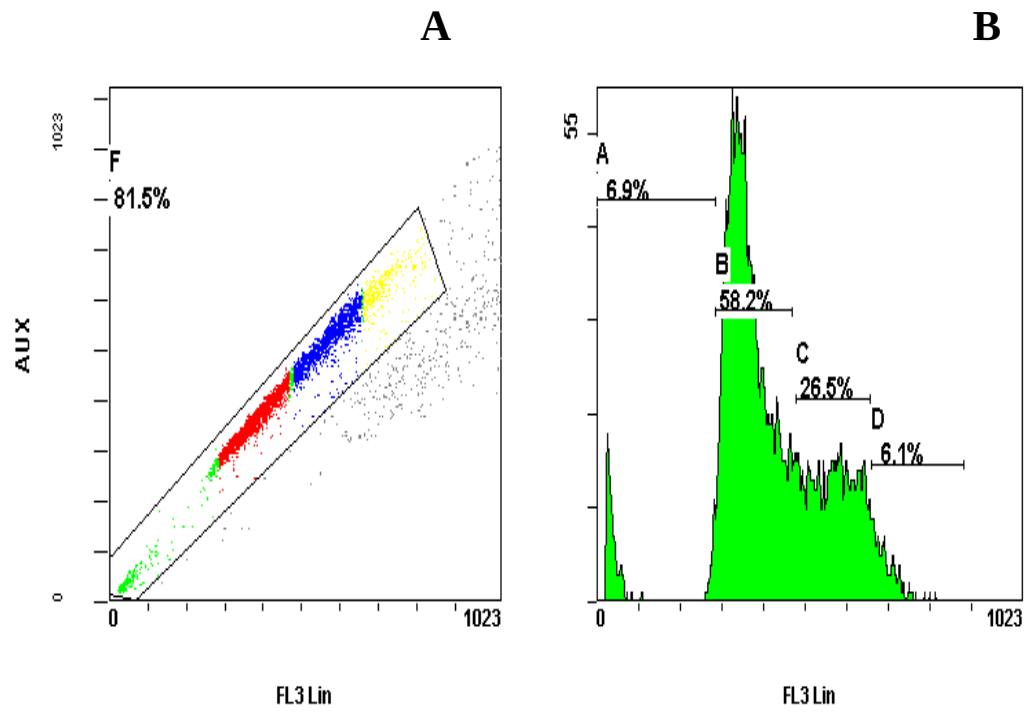


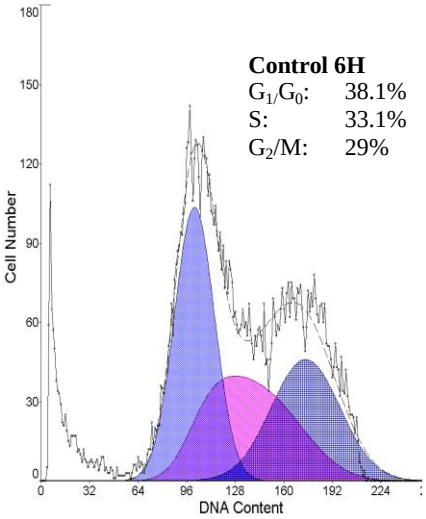
Figure 4.11: Recording of flow cytometry data for cell cycle analysis showing HeLa vehicle control cells as example. (A) Detection of DNA in the linear region on a 2D dot plot of peak area of the electronic signal reaching the photomultiplier tube vs peak height; and (B) histogram representing the fluorescence properties of the cellular DNA in different phases of the cell cycle within the linear region of A. Cells were permeabilized and stained with PI. A minimum of 10 000 events were recorded per sample on a Beckman Coulter FC500 flow cytometer (Miami FL, USA).

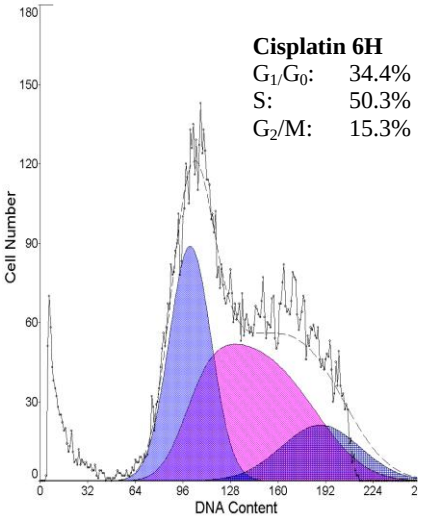
The results for HeLa cells in Figure 4.12 show that cisplatin at 12.5 µM caused an accumulation of cells in the S phase compared to control cells after only 6 hours of exposure. Cells further accumulated in the S phase after 16 hours with a large increase in the size of the sub-G1 peak, corresponding to apoptotic cells. After 24 hours a large sub-G1 peak, corresponding to apoptotic cells, was still observed with an accumulation of cells in the G2/M phase. Further accumulation of cells in the G2/M phase and an even larger peak are

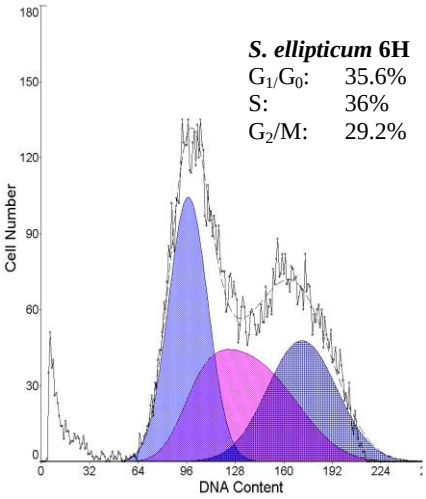
seen in the sub-G1 phase after 48 hours. *S. ellipticum* treatment (125 µg/ml) caused accumulation of HeLa cells in the G2/M phase when compared to control cells after 16 hours. After 48 hours, cells accumulated in the G1/G0 phase by 66.2% compared to 40% of the control cells. *S. ellipticum* slightly increased the cells in the G1/G0 phase over the 6 to 24 hours exposure time, while after 48 hours a 23.45% increase was noted. It seems that the cells were being blocked in the G1/G0 phase of the cell cycle. It would be interesting to see the percentage of cells in the G1/G0 phase after 60 and 72 exposure times.

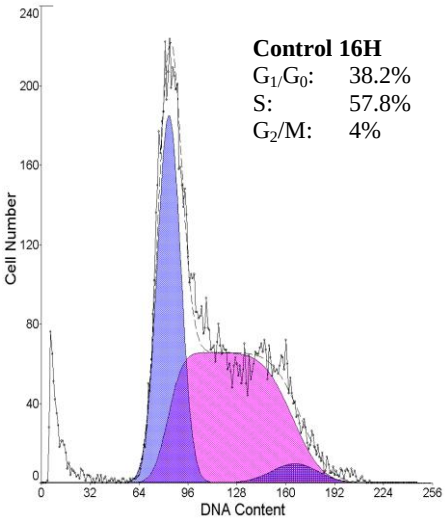
The cisplatin results for HT29 in Figure 4.13 show a similar trend as seen with HeLa cells, with the percentage cells in S phase increasing relative to the control. *S. ellipticum* treatment caused cells to start to accumulate in the G1/G0 phase after 16 hours of treatment. A further accumulation of cells in the G1/G0 phase was seen, with accumulation of cells in the G2/M phase. After 48 hours there was a slight increase in the percentage of cells in the G1/G0 phase, with more cells accumulating in the G2/M phase. The extract appears to have a dual effect.

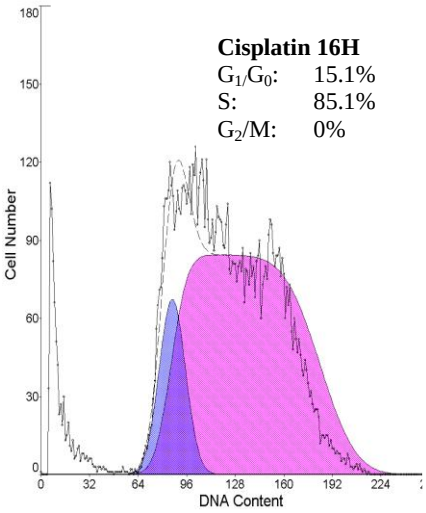
The effect of cisplatin on MCF-7 cells was again induction of S phase arrest with an accompanying increase in the size of the sub-G1 peak (Figure 4.14). *S. ellipticum* treatment caused no significant changes in the cell cycle phases over the exposure time up to 24 hours. Only after 48 hours did cells accumulate in the G2/M phase of the cell cycle. A large peak corresponding to possibly apoptotic cells could be seen as a sub-G1 peak for both 24 and 48 hours when compared to control cells. It seems that *S. ellipticum* caused a G2/M phase arrest in the MCF-7 cancer cells, by blocking the cells in this phase of the cell cycle.

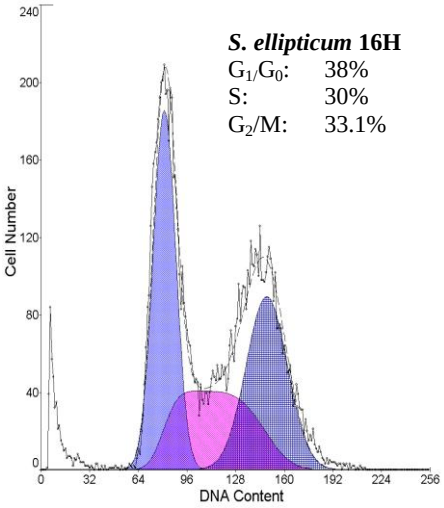

Control 6H
G_1/G_0: 38.1%
S: 33.1%
G_2/M: 29%
Cell Number
DNA Content


Cisplatin 6H
G_1/G_0: 34.4%
S: 50.3%
G_2/M: 15.3%
Cell Number
DNA Content


S. ellipticum 6H
G_1/G_0: 35.6%
S: 36%
G_2/M: 29.2%
Cell Number
DNA Content


Control 16H
G_1/G_0: 38.2%
S: 57.8%
G_2/M: 4%
Cell Number
DNA Content


Cisplatin 16H
G_1/G_0: 15.1%
S: 85.1%
G_2/M: 0%
Cell Number
DNA Content


S. ellipticum 16H
G_1/G_0: 38%
S: 30%
G_2/M: 33.1%
Cell Number
DNA Content

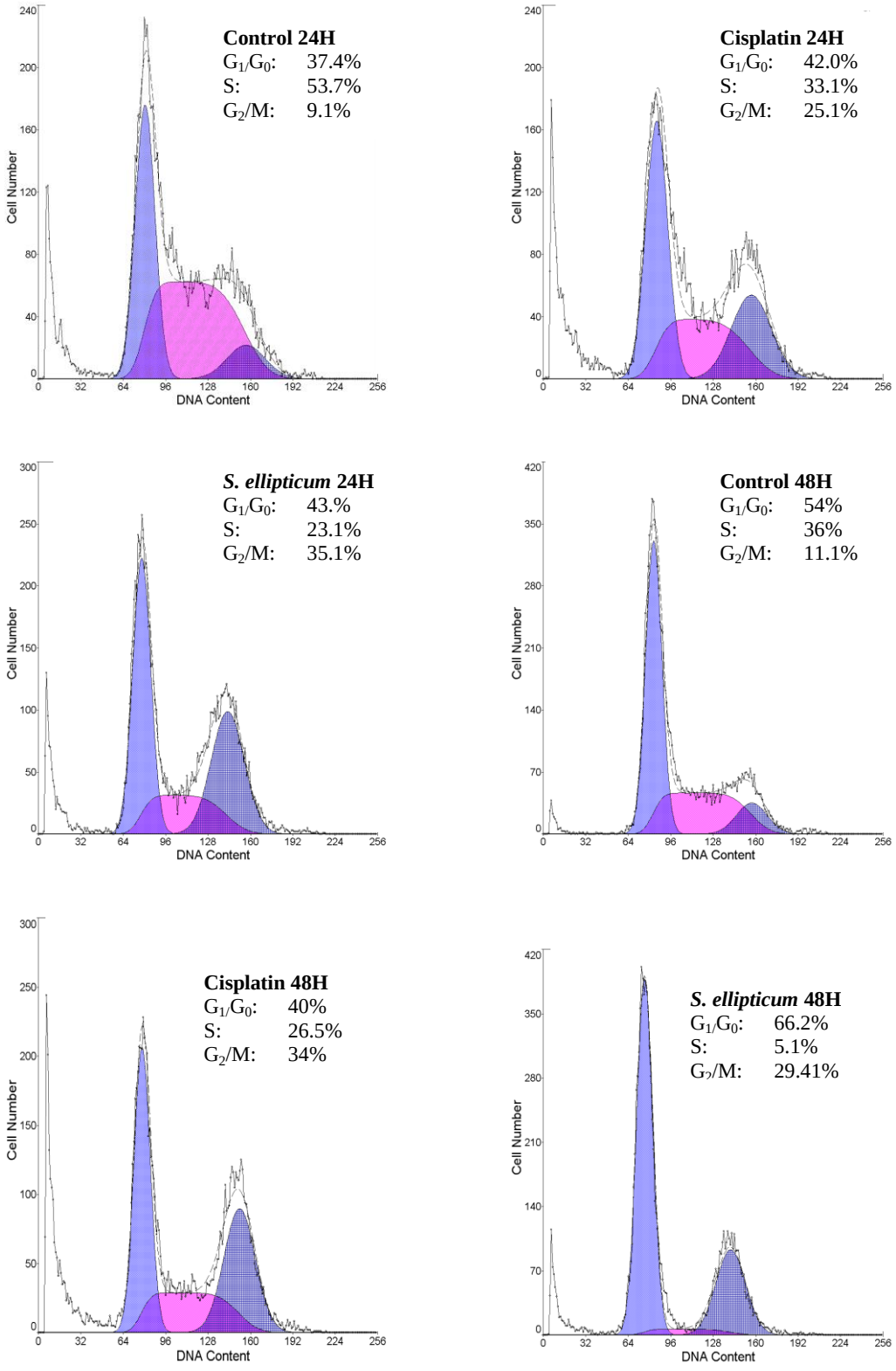
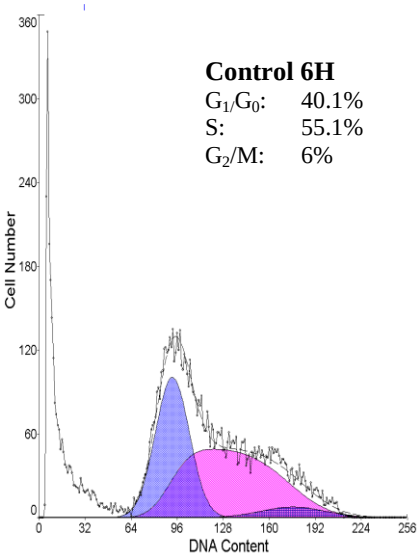
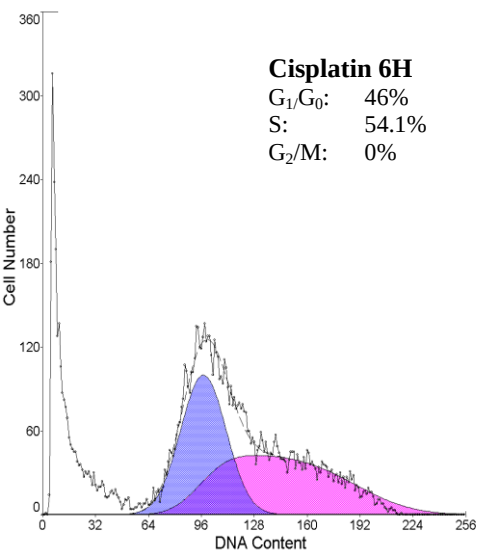


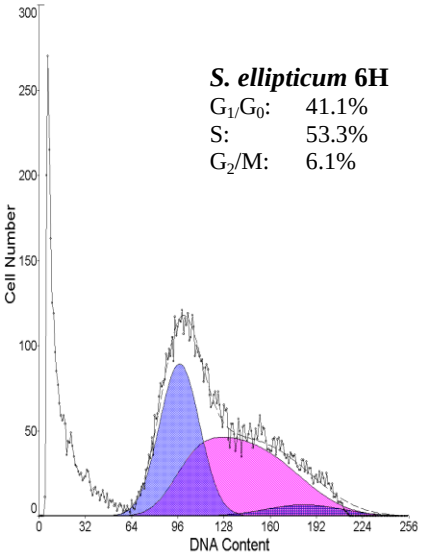
Figure 4.12: Cell cycle analysis of HeLa cells treated with *S. ellipticum* leaf extract for 6, 16, 24 and 48 hours. HeLa cells were treated with 0.125% DMSO (control), 12.5 µM cisplatin as positive control and 90 µg/ml of *S. ellipticum* leaf extract. Data from 10 000 cells in each sample was recorded and analysis performed using MultiCycle software.



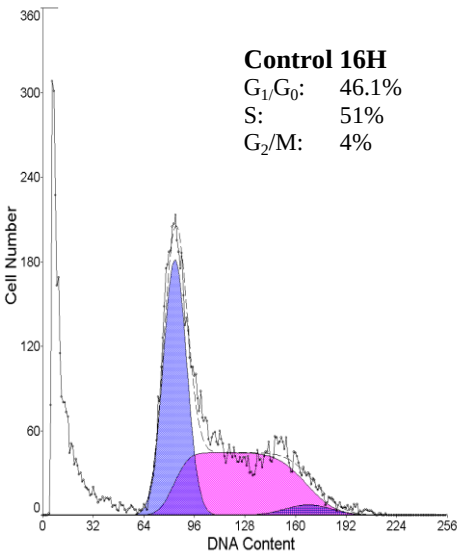
Control 6H
G_1/G_0: 40.1%
S: 55.1%
G_2/M: 6%



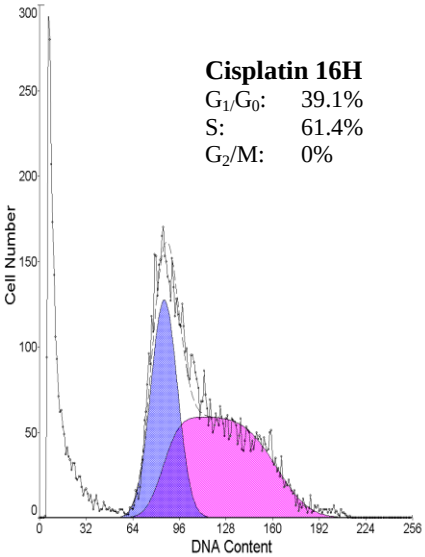
Cisplatin 6H
G_1/G_0: 46%
S: 54.1%
G_2/M: 0%



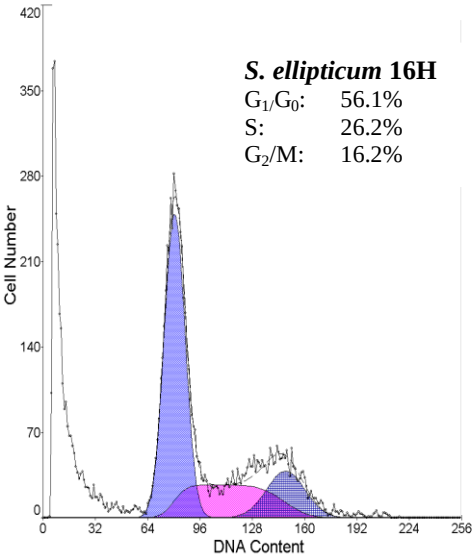
***S. ellipticum* 6H**
G_1/G_0: 41.1%
S: 53.3%
G_2/M: 6.1%



Control 16H
G_1/G_0: 46.1%
S: 51%
G_2/M: 4%



Cisplatin 16H
G_1/G_0: 39.1%
S: 61.4%
G_2/M: 0%



***S. ellipticum* 16H**
G_1/G_0: 56.1%
S: 26.2%
G_2/M: 16.2%

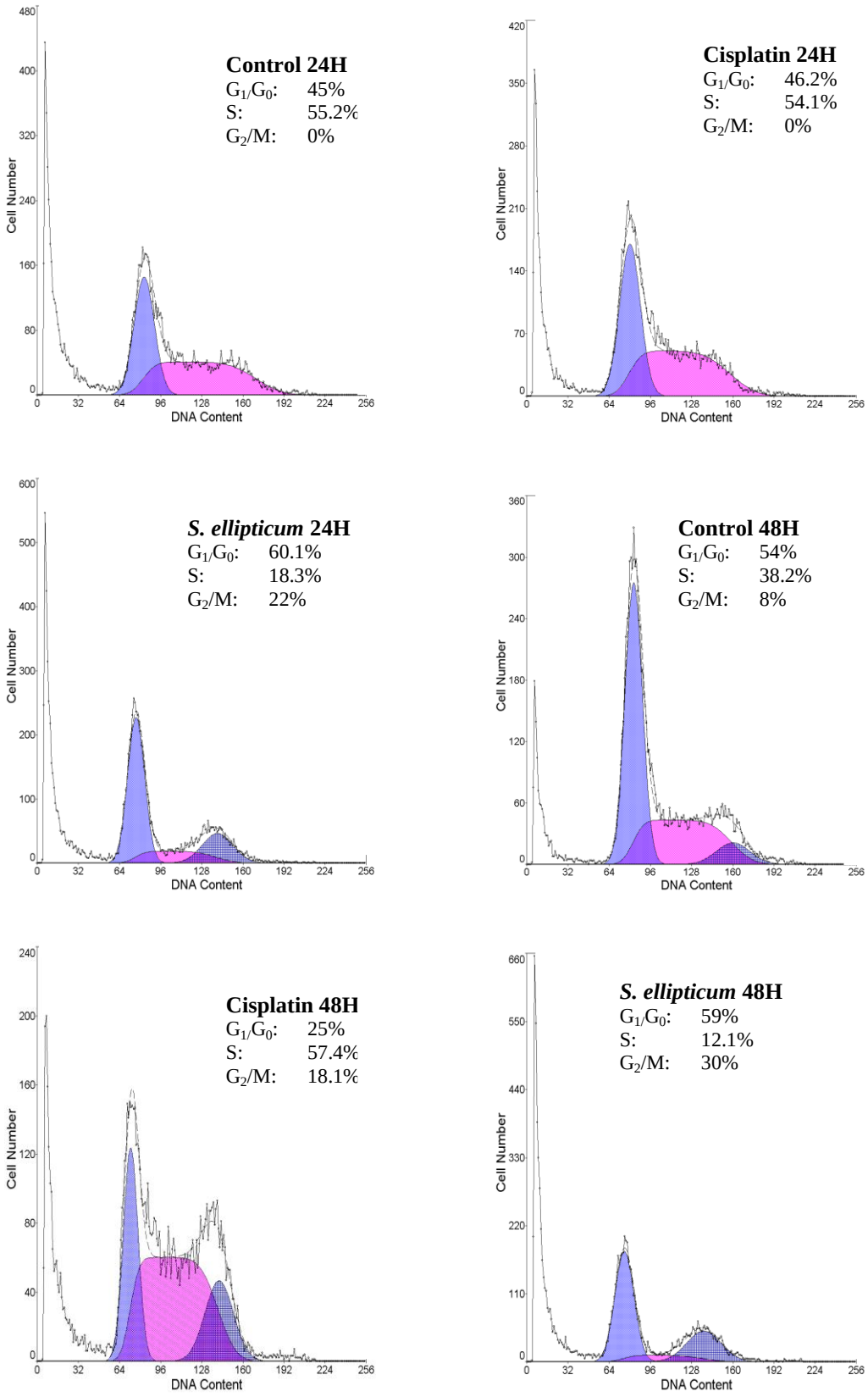
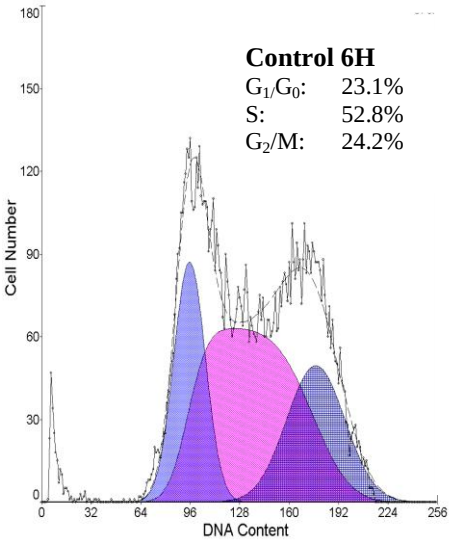
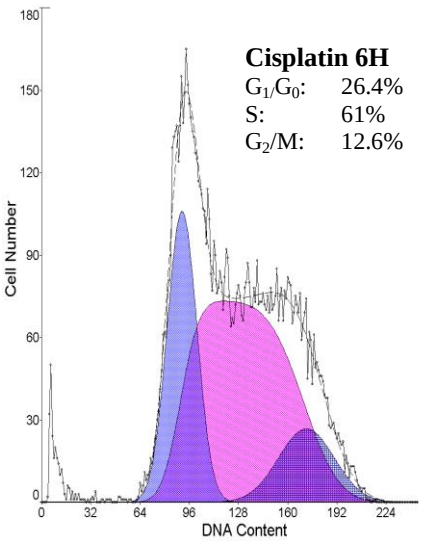
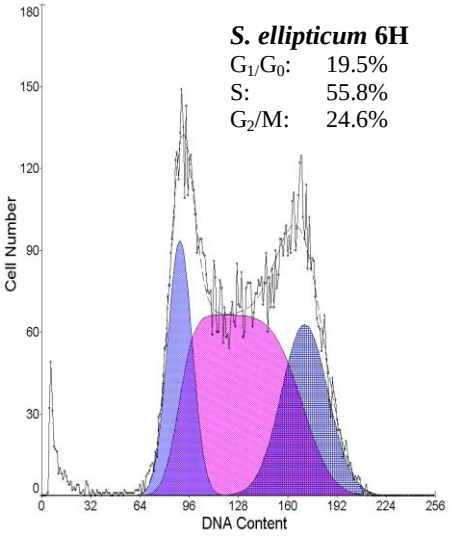
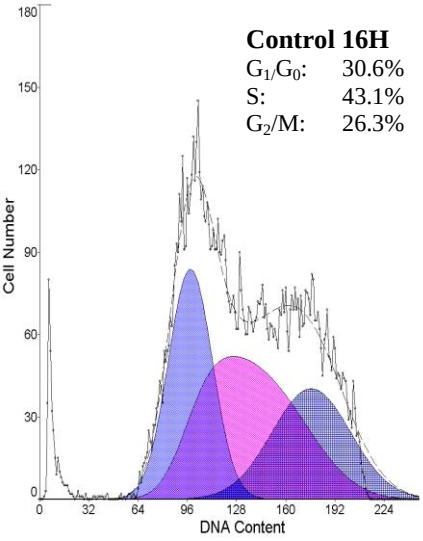


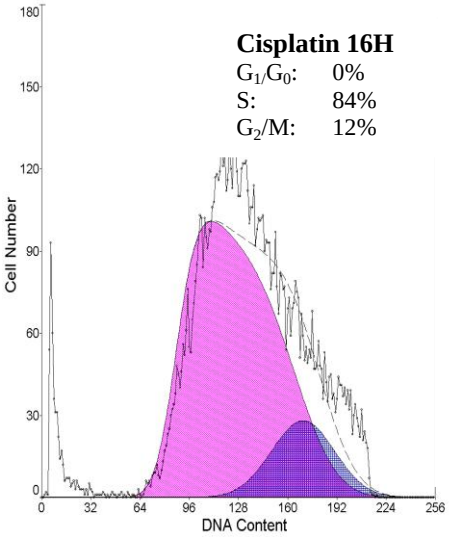
Figure 4.13: Cell cycle analysis of HT29 cells treated with *S. ellipticum* leaf extract for 6, 16, 24 and 48 hours. HT29 cells were treated with 0.125% DMSO (control), 12.5 µM cisplatin as positive control and 90 µg/ml of *S. ellipticum* leaf extract. Data from 10 000 cells in each sample was recorded and analysis performed using MultiCycle software.

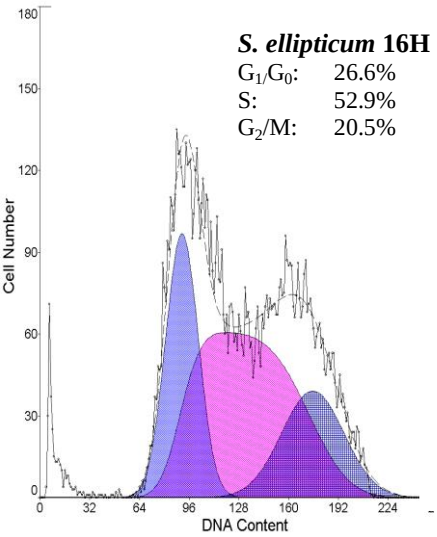

Control 6H
G1/G0: 23.1%
S: 52.8%
G2/M: 24.2%
Cell Number
DNA Content


Cisplatin 6H
G1/G0: 26.4%
S: 61%
G2/M: 12.6%
Cell Number
DNA Content


S. ellipticum 6H
G1/G0: 19.5%
S: 55.8%
G2/M: 24.6%
Cell Number
DNA Content


Control 16H
G1/G0: 30.6%
S: 43.1%
G2/M: 26.3%
Cell Number
DNA Content


Cisplatin 16H
G1/G0: 0%
S: 84%
G2/M: 12%
Cell Number
DNA Content


S. ellipticum 16H
G1/G0: 26.6%
S: 52.9%
G2/M: 20.5%
Cell Number
DNA Content

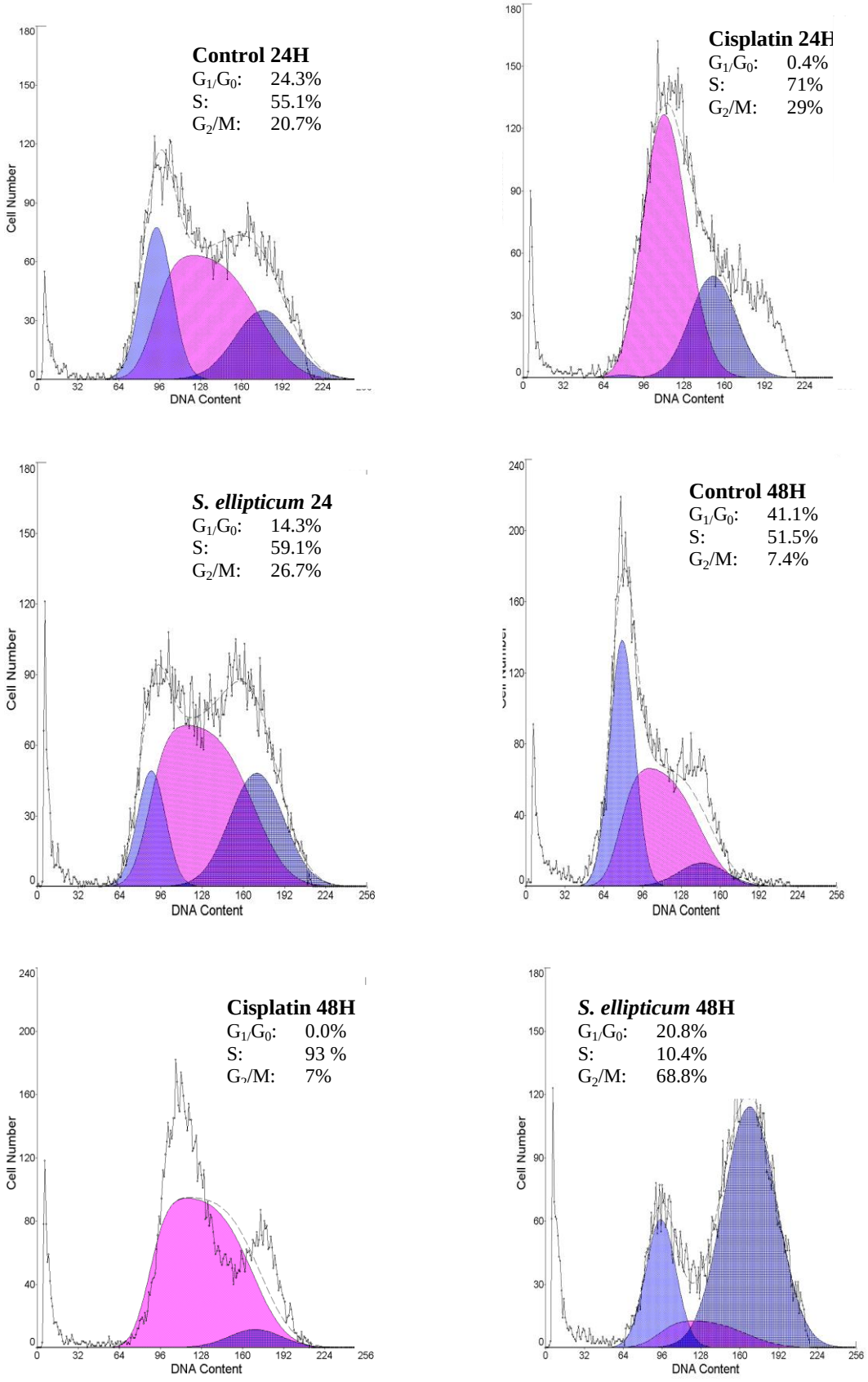


Figure 4.14: Cell cycle analysis of MCF-7 cells treated with *S. ellipticum* leaf extract for 6, 16, 24 and 48 hours. MCF-7 cells were treated with 0.125% DMSO (control), 12.5 µM cisplatin as positive control and 90 µg/ml of *S. ellipticum* leaf extract. Data from 10 000 cells in each sample was recorded and analysis performed using MultiCycle software.

4.3.1.2 Mechanism of Cell Death: apoptosis or necrosis

The increases observed in the sub-G1 peak with cisplatin and *S. ellipticum* in the previous section prompted further investigation into the possibility of apoptosis induction. Although the presence of a sub-G1 peak may indicate the presence of apoptotic cells, it is not a conclusive result and needs to be verified with a more specific apoptosis assay such as the Annexin V assay. The three cell lines used in the previous sections are adherent and not ideal for the Annexin V assay. Early in apoptosis, cells lose their phospholipid membrane asymmetry and expose PS at the cell surface while maintaining their plasma membrane integrity. This results in the exclusion of dyes such as PI. Damage to adherent cells during collection with trypsin and EDTA, either in combination or separately allow access of annexin V-FITC to internally located PS, and, hence giving false positive results [138]. For this reason, a suspension cancer cell line, U937 histiocytic lymphoma, was used to establish whether the mode of cell death induced by the plant extract was apoptotic or necrotic.

The flow cytometry protocol is set up to record and plot FS and SS on a 2D dot plot as described previously (section 4.2). The cells are located on this dot plot and marked by drawing a region around them. Further plots, described below, are gated on this region. Annexin V-FITC binds and stains PS which is translocated from the inner- to the outer layer of the plasma membrane at the early stages of apoptosis. Since the cells are not permeabilised during the staining protocol, PI will only enter cells with compromised membrane integrity, usually necrotic cells. However, as the apoptotic process progresses *in vitro* in the absence of the immune system, cell membrane integrity is lost and both PI and Annexin V-FITC can stain these cells. The results are typically plotted on a 2D dot plot with log of PI fluorescence (FL3) on the y-axis and log of Annexin V-FITC fluorescence (FL1) on the x-axis. Live cells will not stain with either PI or Annexin V-FITC (PI^- and Annexin $V\text{-}FITC^-$); necrotic cells will stain positive for PI only (PI^+ and Annexin $V\text{-}FITC^-$); early apoptotic cells will be PI^- and Annexin $V\text{-}FITC^+$ and finally, late apoptotic cells will be PI^+ and Annexin $V\text{-}FITC^+$. Once the results have been recorded, the flow cytometer software is used to do electronic colour compensation. This is necessary because of the overlapping fluorescence emission spectra of the two dyes.

To investigate the mode of cell death in U937 suspension cancer cells after 24 hours of treatment with cisplatin and *S. ellipticum,* cells were stained with Annexin V-FITC and PI. The results are shown in Figure 4.15. Cisplatin at 5 µM increased the percentage apoptotic

cells (quadrant A4) by 12.2% compared to the vehicle control cells. Figure 4.15 C shows the results of U937 treated with a higher concentration of cisplatin (50 µM). The apoptotic cells increased to 31.7% while an increase in the secondary necrotic (late apoptotic) cells was also observed. As explained previously, this might be due to the fact that no phagocytosis takes place *in vitro* and as the apoptotic process progresses, cell membrane integrity is lost, so that PI can enter cells and stain the DNA. Figure 4.15.D shows the results of U937 cells treated with 125 µg/ml *S. ellipticum.* The plant extract caused only a small increase in apoptosis from 2.3% to 4.1% when compared to the positive control cells. These results conclude that *S. ellipticum* does not cause apoptosis in the U937 cancer cells.

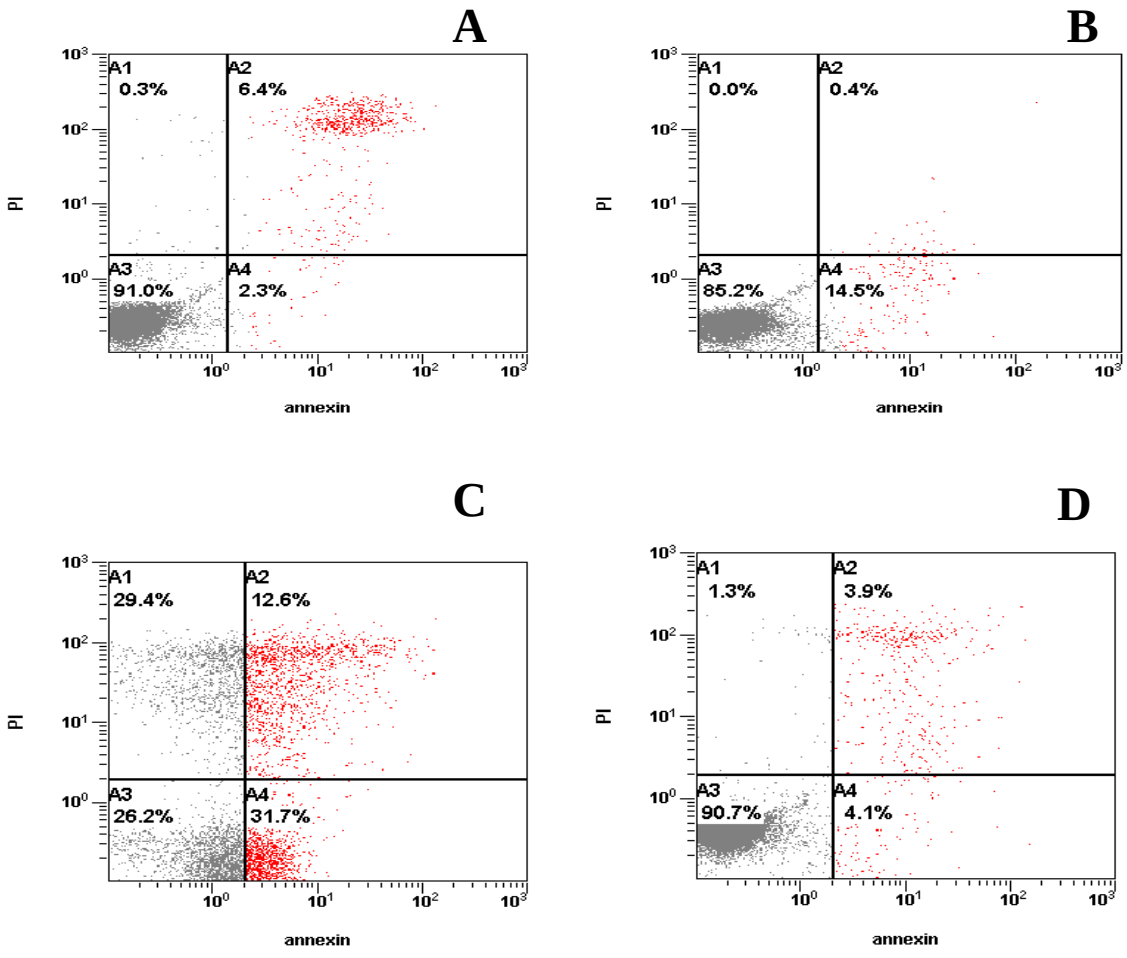


Figure 4.15: Flow cytometry analysis of apoptosis by Annexin V-FITC (*x*-axis) and propidium iodide (*y*-axis) staining. (A) Vehicle control cells, (B) Positive control cisplatin at 5 µM, (C) Positive control cisplatin at 50 µM and (D*) S. ellipticum* at 125 µg/ml. A minimum of 10 000 events were recorded for each sample. The experiment was done in duplicate and a representative plot of each treatment is shown.

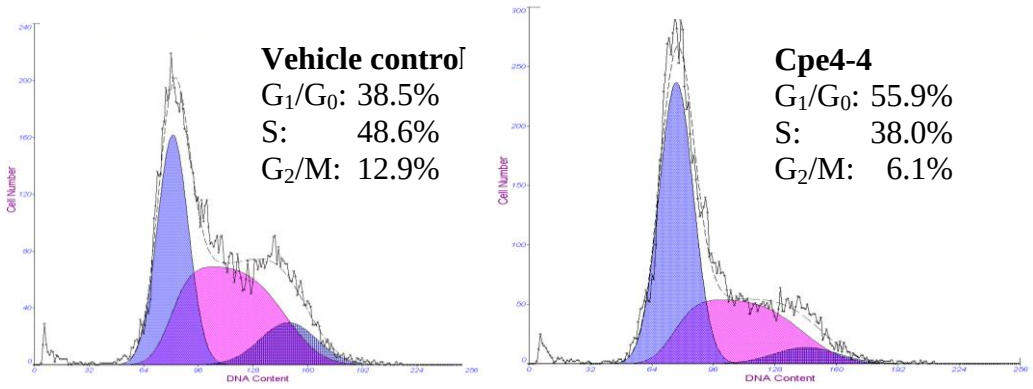
4.3.2 Compounds isolated from *C. paniculatum*

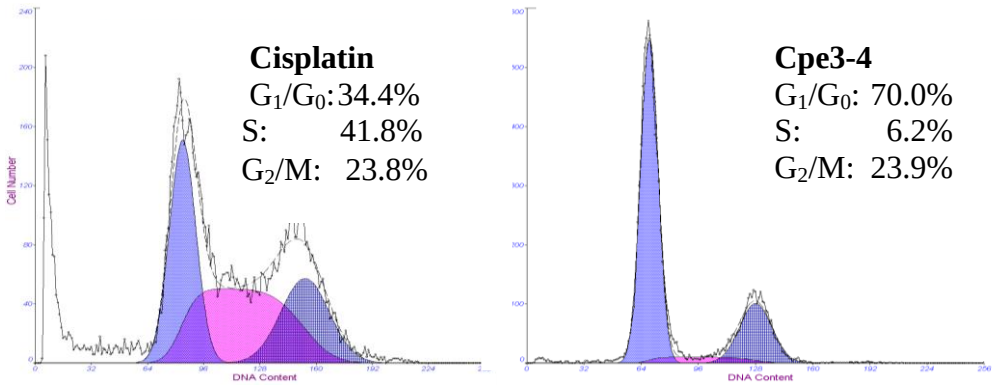
4.3.2.1 Cell cycle analysis

HeLa cells were used to establish whether the purified compounds (Cpe 4-4, Cpe 3-4) induce cell cycle arrest. Cisplatin at 50 µM typically caused an accumulation of the cells in the S phase after 48 hours of treatment (Figure 4.16). A large increase in the size of the sub-G1 peak, corresponding to apoptotic cells, can already be seen after 24 hours of cisplatin treatment and the effect was more pronounced after 48 hours. The two isolated compounds both induced cell cycle arrest in the G1/G0 phase. Although Cpe 4-4 was more toxic to HeLa cells, Cpe 3-4 caused a more enhanced effect on the cell cycle after 24 hours. In comparison with the control cells where 38.5% of the cells were found in the G1/G0 phase, 70% of Cpe 3-4 and 55.9% of Cpe 4-4 treated cells have accumulated in the G1/G0 phase (Figure 4.17) After 48 hours of treatment, around 80% of the cells were arrested in the G1/G0 with both compounds. Interestingly, the sub-G1 peak did not increase with either of the two compounds, even after 48 hours. The cell cycle arrest results revealed that the two compounds act in a very similar way through induction of cell cycle arrest in the G1/G0 phase. No increase was observed in the sub-G1 (apoptotic) peak and therefore it would appear as if the reduction in viable cell numbers compared to control treated cells was due to inhibition of proliferation rather than cytotoxicity.

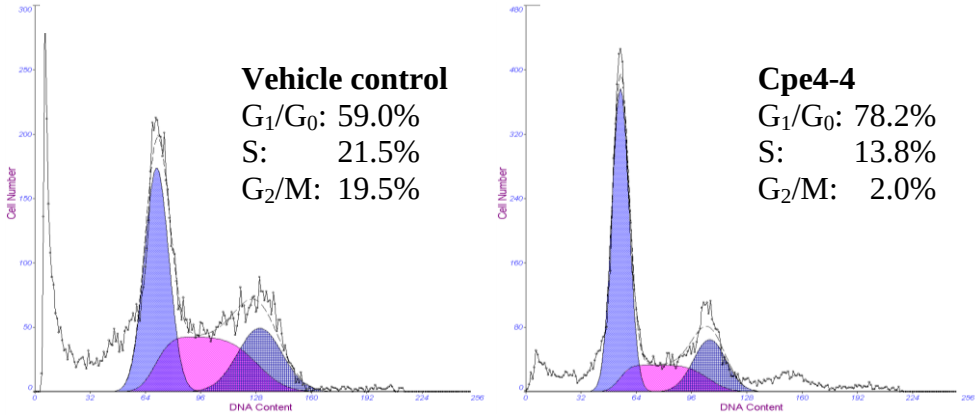
The two compounds were identified by Dr Sowemimo and collaborators from Beijing, China as pheophorbide a (Cpe 3-4) and pheophorbide a methyl ester (Cpe 4-4). This is the first report on these two compounds, from *C. paniculatum* and the induction of cell cycle arrest in HeLa cells in the absence of photoactivation. Our results further suggest that the methyl ester is a more potent inhibitor of proliferation than pheophorbide a in at least two of the three cell lines investigated. It was interesting and encouraging to see that the pheophorbide a was more cytotoxic than its methyl ester to PBMCs isolated from normal human blood cells.

A: 24 HOURS





B: 48 HOURS



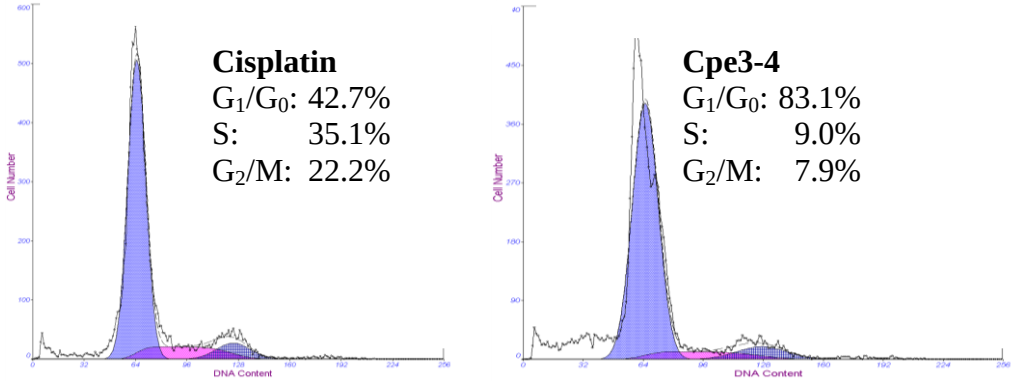


Figure 4.16: Cell cycle analysis of HeLa cells treated with compounds for (A) 24 hours and (B) 48 hours. HeLa cells were treated with 0.125% DMSO (vehicle control), 50 µM cisplatin as positive control, 62.5 µg/ml of pheophorbide a (Cpe4-4) or pheophorbide a methyl ester (Cpe3-4). Data from 10 000 cells in each sample was recorded and analysis performed using MultiCycle software.

4.4 Cytotoxic screening of two more medicinal plant species

The research done for this project formed part of an ongoing African Laser Centre-funded collaboration between researchers from the Nelson Mandela Metropolitan University in Port Elizabeth, South Africa and the University of Lagos in Lagos, Nigeria. After screening of the first 17 extracts was complete, Dr Sowemimo supplied us with another two crude extracts from two plant species for screening. The plants were *U. chamae* and *L. cupanioides.* One of these, *L. cupanioides* was fractionated, the fractions screened and the most active fractions identified for further investigation by another student. The cytotoxicity results obtained for these crude extracts and fractions against HeLa, HT29 and MCF-7 cells are reported in this section.

Figure 4.17 shows the cytotoxic results of *U. chamae* and *L. cupanioides* crude extracts against HeLa, HT29 and MCF-7 cancer cell lines. Cisplatin at 10 µM inhibited growith by 77.14 ± 2.84, 51.81 ± 6.99 and 75.00 ± 2.76 µg/ml (SD, n=4) for HeLa, HT29 and MCF-7 respectively. *U. chamae* gave 50% growth inhibition at 125 µg /ml against HeLa and MCF-7 cancer lines. *U. chamae* showed very little growth inhibition against HT29 cancer cell line compared to HeLa and MCF-7 cells. This seemed to be the trend with most of the plant extracts. *L. cupanioides* root extract (LR) showed no inhibitory activity against the three cell lines for both 62.5 and 125 µg/ml. However it seems at 62.5 µg/ml the root extract stimulated growth in the MCF-7 cancer cell line. *L. cupanioides* leaf extract (LL) showed inhibitory activity against HeLa and MCF-7 cancer cell lines, with the greatest inhibitory effect against HeLa cells, where both 62.5 and 125 µg/ml gave more than 50% inhibition. *U. chamae* and *L. cupanioides* (leaf and root) extracts had no cytotoxic activity against the HT29 cell line.

As mentioned above, *L. cupanioides* leaf crude extract (LL) had no growth inhibitory activity against the HT29 cancer cell line. Results in Table 4.8 show that of the fractions prepared from LL, only the ethyl acetate fraction b (ETb) at 250 µg/ml was able to inhibit HT29 proliferation by more than 50%.

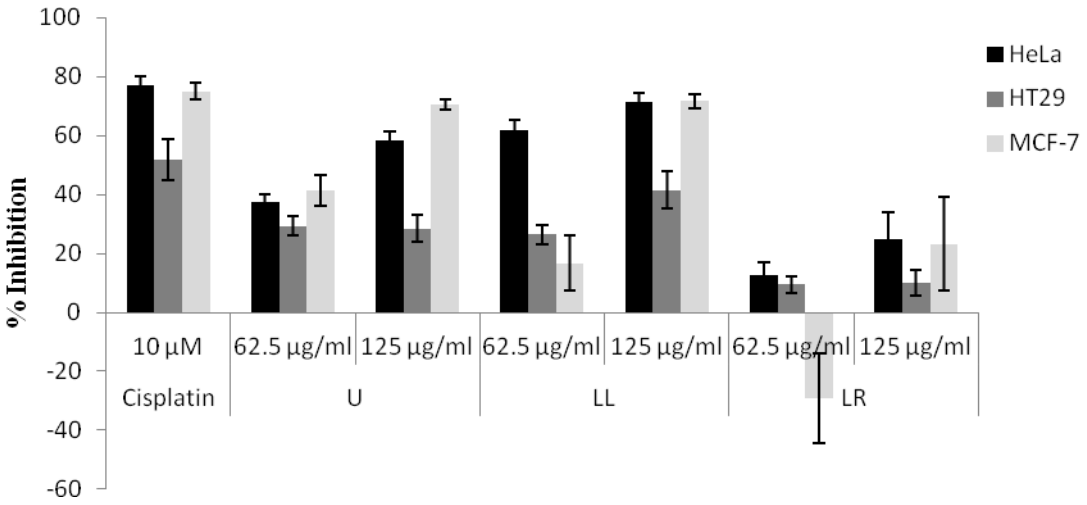


Figure 4.17: Screening results of *U. chamae* and *L. cupanioides* leaf (LL) and root (LR) extract against HeLa, HT29 and MCF-7 cancer cells. Results represent the mean ± SD of quadruplicate determinations. Cisplatin was used as positive control.

The aqueous fraction showed no inhibitory activity at or above 50%. The butanolic fraction inhibited both HeLa and MCF-7 cancer cell lines, showing greater activity against HeLa at 250 µg/ml, but having no activity at 125 µg/ml. Ethyl acetate a and b fractions showed growth inhibitory activity against both cell lines, with both fractions showing greater activity against the Hela cancer cells, however fraction b failed to inhibit the HeLa cells with the lower concentration, but inhibited growth in the MCF-7 cancer cells over all concentrations tested.

These two fractions, LL(Eta) and LL(Etb) were tested further by another student and shown to induce growth arrest in the G1/G0 phase which culminates in apoptosis induction (unpublished results).

Table 4.8: Cytotoxic screening results (% inhibition) of *L.cupanioides* fractions against HeLa, HT29 and MCF-7 cancer cells

	[Extract] (µg/ml)	LL (Aq)	LL (B)	LL (Etb)	LL (Eta)	LL (H)	LL (Cl)	LL (M)
HeLa	62.5	-34.47 ± 15.07	-10.89 ± 6.54	33.83 ± 14.62*	56.79 ± 3.51***	-15.41 ± 6.48	-22.89 ± 15.20	-44.29 ± 8.69
	125	0.95 ± 10.58	33.62 ± 6.35**	54.14 ± 7.82***	56.74 ± 3.71***	9.57 ± 3.06	5.83 ± 4.331	41.0 ± 1.65**
	250	43.44 ± 6.68**	73.20 ± 4.95***	82.55 ± 1.28***	83.43 ± 1.60***	34.66 ± 2.55**	41.93 ± 5.01**	82.34 ± 2.73***
HT 29	62.5	-4.26 ± 9.39	6.10 ± 8.11	40.91 ± 3.40***	9.97 ± 4.32*	31.42 ± 20.89*	3.99 ± 7.28	-16.645 ± 8.03
	125	-2.35 ± 12.11	42.20 ± 3.87***	16.53 ± 8.90*	19.11 ± 4.81**	12.78 ± 7.47*	19.81 ± 5.07**	-11.61 ± 9.85
	250	9.85 ± 4.65	42.56 ± 9.48***	51.11 ± 5.47***	31.65 ± 3.72***	34.58 ± 4.85***	17.82 ± 4.56**	-15.24 ± 9.99
MCF-7	62.5	-3.46 ± 10.82	20.53 ± 12.39	60.38 ± 2.81***	57.49 ± 2.71***	-8.65 ± 12.08	1.37 ± 11.69	-30.93 ± 15.43
	125	37.03 ± 21.30*	62.39 ± 4.48***	65.06 ± 5.09***	50.65 ± 4.14***	7.35 ± 10.76	-14.34 ± 35.49	37.54 ± 7.61**
	250	71.61 ± 3.88***	57.64 ± 5.66***	67.63 ± 3.88***	68.95 ± 4.19***	60.36 ± 9.93***	41.50 ± 7.54*	63.69 ± 3.95***

LL – *L.cupanioides* leaf crude extract was fractionated using the following solvents:, Aq-Aqueous (water), B-butanol, Et- ethyl acetate, H- hexane, Cl- chloroform and M -methanol. Two fractions, a and b, were collected for the ethyl acetate elution.

* Significantly different from vehicle control; $p < 0.05$

** Significantly different from vehicle control; $p < 0.01$

*** Significantly different from vehicle control; $p < 0.001$

Chapter 5: Discussion and conclusion

The 16 plants in this study are being used in South-Western Nigeria for the management of cancer by traditional medical practitioners. However the main problem facing the use of traditional medicines is the proof requirement that the active compounds contained in the medicinal plants are useful, safe and effective [101].

Of 16 plants screened for *in vitro* anticancer activity against HeLa cancer cells, *C. molle* leaves, *A. parvoniana* fruit, and *L. acid*a stembark were non toxic. However, *C. molle* leaves showed a decrease of one or more than one fluorescence unit in the level of hTERT protein at 125 µg/ml for both 24 and 48 hours when compared to the control cells. Research has shown that the alcoholic extracts of leaves and water extracts of *C. molle* twigs have capacity to reduce sarcoma tumours in animals [139]. This effect may be explained by the hTERT inhibition observed in the present study.

Previously, researchers have reported that only the root and stembark of *A. parvoniana* has cytotoxic activity [115] and the absence of toxicity in the fruit extract is therefore not surprising. No published research was found on anticancer activity of *L. acid*a stembark although in the current study the plant extract showed a decrease in hTERT levels at the higher concentration for 48 hours.

P. lappacea, *J. extensa* stembark, *S. ellipticum* stembark, *A. sessilis*, *L. nigritana* stembark and *C. zenkeri* root were moderately toxic, inducing less than 40% inhibition of cell growth in HeLa cells at 500 µg/ml. With the exception of *S. ellipticum* stembark, all these extracts reduced hTERT staining by one or more than one fluorescence unit when compared to the control cells at 125 µg/ml for 24 and 48 hours, suggesting reduced hTERT expression and telomerase activity.

A literature search of previously published work on the plants with moderate cytotoxicity yielded the following: dichloromethane and methanol extracts of *P. lappacea* has shown cytotoxic effects with IC_{50} values of 66.5 and > 100 µg/ml and 68.3 and > 100 µg/ml against J774 and W138, respectively and aqueous extract with IC50 values > 100 µg/ml against both cell lines [140]. Ethanolic extracts of *A. sessilis* were reported to significantly reduce pancreatic adenocarcinoma Panc-1 cell proliferation with an IC_{50} of 27 µg/ml [141]; however another publication showed no cytotoxic activity against HeLa cells [142]. No published

research on anticancer activity of *J. extensa, S. ellipticum, L. nigritana* or *C. zenkeri* root could be found.

E. conyzoides leaves, *H. barteri* leaves *C. achyranthoides* were moderately toxic, inhibiting HeLa cell growth by 40-50% at 500 µg/ml. No publications of any cytotoxicity or telomerase inhibitory activity of these three plants has previously been recorded; however they too showed one or more than one fluorescence unit less hTERT protein expression when compared to the control cells at 125 µg/ml for 24 and 48 hours.

Extracts with the best potential toxicity against HeLa cells were *S. ellipticum* leaves, *C. paniculatum, C. trigyna, D. cordata* and *C. prostrata,* showing over 50% inhibitory activity at 500 µg/ml. Only *S. ellipticum* leaves showed one or more than one fluorescence unit less hTERT protein expression when compared to the control cells at 125 µg/ml for 24 and 48 hours. The other four extracts showed a decrease in one or more than one fluorescence unit for either 24 or 48 hours at one of the tested concentrations. Even though these plants showed over 50% cytotoxic activity against HeLa cells, no publications about possible anticancer activity was found in the literature. Further screening of these five plant extracts against MCF-7 cancer cells showed that *S. ellipticum* leaves showed comparable activity to the positive control cisplatin. *C. prostrata* showed a 50% inhibition at 500 µg/ml while *C. paniculatum, C. trigyna* and *D. cordata* showed inhibition lower than 50% for all the concentrations tested.

IC_{50} values of 88.60 ± 0.03 and 93.03 ± 0.03 µg/ml were obtained for *S. ellipticum* leaf extract against HeLa and MCF-7 cancer cells, respectively. This extract was also tested on PBMCs and Chang liver cells to determine its toxicity on normal cells. The extract had an IC_{50} of greater than 125 µg/ml against both proliferating and confluent Chang liver cells whereas the IC_{50} for PBMCs (77.66 µg/ml) was similar to that of the cancer cell lines. These results point toward possible cell type selectivity of the extract but this needs to be confirmed by testing more cell types.

In an attempt to determine the active compound(s) within the plant leaf extract, five fractions (petroleum ether, butanol, aqueous, ethyl acetate and chloroform) were prepared and subjected to further screening against MCF-7 and confluent Chang liver cells to determine their degree of cytotoxicity. The most active fractions were those prepared in the solvents ethyl acetate (SEA) and chloroform (Scf), with polarity indices of 4.4 and 4.1, respectively. In comparison, the polarity index of water is 10.2 and that of the other two solvents used are

0.1 (petroleum ether) and 3.9 (butanol). This indicates that the active compounds have intermediate polarities. Both fractions were more toxic to MCF-7 cells than confluent Chang liver cells, indicating possible antiproliferative effects. This result was more encouraging than the result for the crude extract which was much more toxic to the Chang liver cells.

Of the five compounds that were isolated from the two active fractions of *S. ellipticum*, two of the ethyl acetate compounds were active against MCF-7 cells, one with an IC_{50} below 62.5 µg/ml ($SV_{2\text{-}2a}$, Table 4.5), and the other below 31 µg/ml ($SV_{2\text{-}2b}$). Unfortunately only a small amount of each compound was isolated for screening and after reconstitution in DMSO they both soon lost activity. Future studies could attempt re-isolation and stability testing under different conditions so that their structures can be elucidated and their activities investigated further. The other three compounds were inactive or less active than $SV_{2\text{-}2a}$ and $SV_{2\text{-}2b}$ and did not warrant further investigation. The chloroform fraction was more active than the ethyl acetate fraction, yet no active compound could be isolated from it in this study. Future work could involve isolation of more compounds from the chloroform fraction for screening.

The crude *S. ellipticum* leaf extract induced cell cycle arrest in G1/G0 as well as G2/M phase in all three cell lines, with the most prominent effect being in HeLa and MCF-7 cells. This effect only became prominent after 24 hours and was more enhanced at 48 hours. These results suggest that the extract has a dual effect, most likely due to the presence of more than one active compound. It would be interesting to establish whether the two unstable isolated compounds, $SV_{2\text{-}2a}$ and $SV_{2\text{-}2b}$, were responsible for this dual effect.

The increase in the sub-G1 peak observed during cell cycle analysis after treatment with *S. ellipticum* leaf extract was not accompanied by PS translocation and thus could not be confirmed as apoptosis. However, a small increase in the percentage secondary necrotic cells (PI^+ and Annexin V-$FITC^+$) was seen, when compared to the control cells. This increase was too small to fully explain the percentage growth inhibition observed with the MTT assay. These results conclude that *S. ellipticum* does not cause apoptosis in the U937 cancer cells. It seems to have an antiproliferative effect through induction of cell cycle arrest rather than a cytotoxic effect.

Possible antiproliferative mechanisms that could be investigated in future are; to establish whether the cell cycle was arrested in the G1 phase or if cells were forced to exit the cell cycle and remain in G0 and whether the G2/M arrest occurred in the G2 or M phase. By measuring the levels of CDK inhibitors (such as p21 or p27) and cyclins and/or measuring

the activities of specific cyclin-CDK complexes, it should be able to resolve these questions [21, 27, 28]. If the cells are found to be in the G0 phase, the possibility exists that the extract induces differentiation or senescence. The indication that the *S. ellipticum* extract reduced hTERT levels may further point towards the possibility of senescence in cells treated with this extract [79].

C. paniculatum showed more than 50% growth inhibitory activity against HeLa cells at 250 µg/ml. During bio-assay guided fractionation, three fractions (aqueous, petroleum ether and ethyl acetate) were screened. All three showed cytotoxic activity but the highest activity was obtained with the ethyl acetate fraction. The observation that three fractions with vastly different polarities were all active may indicate that the extract contains more than one active compound.

Three compounds were isolated from the ethyl acetate fraction and subjected to further screening against HeLa, HT29 and MCF-7 cancer cells. The isolated compounds showed a dose-dependent cytotoxic effect. Two of them were identified by collaborators in China and the third, Cpe 4-9, was found to contain impurities. Cpe 4-9 had a cytotoxic effect lower than that of Cpe 4-4 (Pheophorbide a) and comparable to Cpe 3-4 (Pheophorbide a methyl ester), which then lead to the decision not to purify it further. Results from a cytotoxicity assay performed with PBMCs further suggest that the methyl ester was less toxic to normal cells than pheophorbide a. These two compounds were then used to establish whether they induced cell cycle arrest against HeLa cells. Results revealed that the two compounds act through induction of cell cycle arrest in G1/G0 phase. No increase was observed in the sub-G1 (apoptotic) peak therefore it would appear that reduction in viable cell numbers compared to control cells are due to inhibition of proliferation rather than cytotoxicity.

Numerous *Combretum* species have been illustrated to be cytotoxic to cancer cell lines in the past [145], but this was the first study done on *C. paniculatum.* The most well known and best studied species is *C. caffrum* from which the combretastatins A-1 and A-4 have been isolated. These compounds inhibit tubulin polymerisation and therefore prevent mitotic spindle formation at very low micromolar concentrations [145, 146]. It is possible that these compounds were present in the crude extract used in the present study although they were not isolated from the fractions. Future studies should investigate this possibility by further characterising the fractions obtained from the crude extract. This was the first study to report on the cytotoxic activity of *C. paniculatum.* It was also the first to show that the two

compounds pheophytin a and its methyl ester were able to induce G1/G0 arrest which may contribute to the growth inhibition observed with the crude extract. The potent induction of growth arrest and the absence of toxicity of the methyl ester on PBMCs supports our suggestion that the compound is not cytotoxic but cytostatic. Apart from the two isolated compounds and possibly the combretastatins, many other compounds may contribute to the activity of the crude extract. Examples include tannins and flavonoids [145].

Since this study forms part of an ongoing collaboration two more plant extracts *U. chamae* and *L. cupanioides* leaf (LL) and root (LR) extracts were supplied for cytotoxic screening against HeLa, HT29 and MCF-7 cancer cells. No published work could be found on the anticancer potential of these two species and they were shown here, for the first time, to inhibit HeLa, MCF-7 and to a lesser extent, HT29 cell growth. It has been reported that the extracts of *U. chamae* has mutagenic effects and a cytotoxic tribenzylated flavanone named uvarinol, has been isolated from *U. chamae* [123]. It is possible that this compound was responsible for the activity observed in this study but this needs confirmation.

L. cupanioides root extract (LR) showed very low inhibitory effect against all three cancer cell lines at both concentrations. The leaf extract (LL) showed more potential, especially against HeLa and MCF-7 cells. This extract was fractionated using water, butanol, ethyl acetate, hexane, chloroform and methanol and the ethyl acetate fraction was most active, again with higher activity against HeLa and MCF-7. Further investigation of this fraction was not within the scope of the present study. Polyphenolic compounds of the plant extract have been recognised and are well known to exhibit a number of biological activities of which an anticancer effect is one [144]. This could form the basis of further work on the ethyl acetate fraction in future.

In summary, a total of 20 extracts from 18 plant species were screened for *in vitro* cytotoxic activity against cancer cell lines. Seven of these showed good potential achieving $\geq$ 50% inhibition in one or more cell line at 125 μg/ml. Their IC_{50} values are therefore < 125 μg/ml, which can be considered a realistic and potentially useful concentration when moving on to *in vivo* models [143]. The relatively high percentage of extracts with positive results should not be surprising, considering that all these plants are used in Nigerian traditional medicine for the treatment of various types of cancer. Two of the seven best extracts were followed up by other students. *C. prostrata* was shown to induce apoptosis via the extrinsic pathway, while *L. cupaniodes* leaf extract was found to induce G1/G0 arrest, followed by apoptosis. The

induction of cell cycle arrest was accompanied by an increase in the number of senescent cells. The work from these two studies has not been published yet.

Two extracts were followed up in the present study, namely *S. ellipticum* and *C. paniculatum.* Two active compounds were isolated from each of these extracts but only the *C. paniculatum* compounds could be identified as pheophorbide a and pheophorbide a methyl ester. This study further identified a number of extracts and/or fractions that were investigated by other students in our research group or can still be investigated in future.

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Appendix 1:

Articles published from this study

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Short Communication

Cytotoxicity evaluation of selected Nigerian plants used in traditional cancer treatment

Abimbola Sowemimo[1], Maryna Van de Venter[2]*, Lucinda Baatjies[2] and Trevor Koekemoer[2]

[1]Department of Pharmacognosy, Faculty of Pharmacy, University of Lagos, College of Medicine Campus, Idi-Araba, Lagos, Nigeria,
[2]Department of Biochemistry and Microbiology, Nelson Mandela Metropolitan University, Port Elizabeth 6031, South Africa.

Accepted 27 January, 2011

Herbal medicines have received much attention as a source of new anticancer drugs. However, scientific studies have been conducted to a limited extent with few medicinal plants. This study investigates the cytotoxic activity of some Nigerian medicinal plants used locally in the treatment of cancer. The ethanolic extracts of five plants were evaluated using the MTT assay on the HT29 and MCF-7 cell lines. *Sapium ellipticum* leaves showed a greater cytotoxic activity than *Combretum paniculatum*, *Celosia trigyna*, *Drymaria cordata* and *Cyathula prostata* and it was comparable to the activity of the reference compound Cisplatin in the MCF-7 cell line. In the HT29 cell line, all the plants showed less than 50% activity at 500 µg/ml. The results showed that *Sapium* exhibited a greater cytotoxic activity than all the plants tested and this provides scientific evidence to support the traditional use of the plant.

Key words: *Sapium*, traditional medicine, cytotoxicity, cancer, cisplatin.

INTRODUCTION

The use of plants and their products fpr medicinal benefits has played a significant role in nearly every culture on earth. Historically all traditional remedies were obtained from plants and recent estimates have suggested that several thousands of plants have been known with medicinal applications in various cultures (Richardson, 2001; Wargovich et al., 2001). Local herbalists have been treating various cancers- and cancer-related conditions for ages (Sofowora, 1984). Many plants have been reported to be useful in the management of such conditions. Plants have been the source of well known anticancer drugs such as campthothecin, podophyllotoxin and paclitaxel (Wani et al., 1971; Stahlhut et al., 1999; Lee, 2004). In this paper, we report the cytotoxic activity of the ethanolic extracts of *Sapium ellipticum* (Krauss.) Pax., *Combretum paniculatum* Vent., *Celosia trigyna* L., *Drymaria cordata* (Linn.) Willd. and *Cyathula prostata* (Linn.) Blume against colon and breast cancer cell lines. Selection of these plants was based on their frequency in recipes for the management of cancer from an ethnobotanical survey of traditional medical practitioners in South-Western Nigeria and our earlier investigation on the cytotoxic activity of some anticancer plants using the HeLa cell line (Sowemimo et al., 2009).

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MATERIALS AND METHODS

Plant material

The plants were collected from the Olokemeji Forest Reserve and the Campus of Obafemi Awolowo University, Ile-Ife in Nigeria in October, 2006. They were authenticated by comparison with corresponding herbarium specimens by Mr Daramola at the Forestry Research Institute, Ibadan, Nigeria (FRIN) where voucher specimens were also deposited. The plants were dried in a hot air

Table 1. List of plants used in the cytotoxicity assay against HT29 and MCF-7 cells

Extract number	Plant name (family) [Frin no. [a]]	Plant part used
P1	*Sapium ellipticum* (Krauss.) Pax. (Euphorbiaceae) [108265]	Leaves
P2	*Combretum paniculatum* Vent. (Combretaceae) [107980]	Leaves
P3	*Celosia trigyna* L. (Amaranthaceae) [84438]	Whole plant
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P5	*Cyathula prostata* (Linn.) Blume (Amaranthaceae) [107232]	Whole plant

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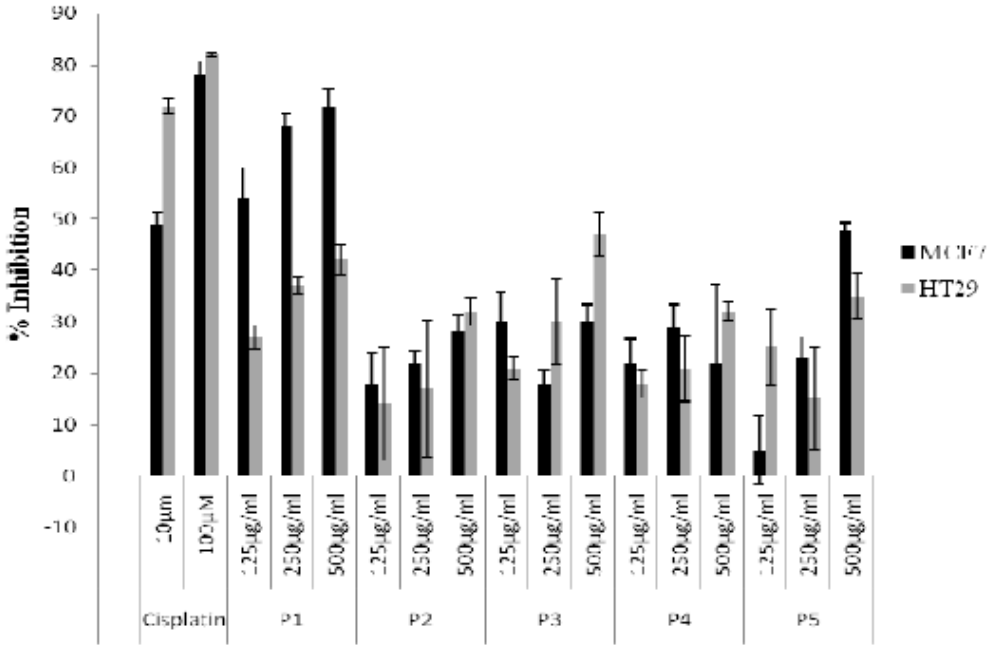

Figure 1. Results of extracts on MCF-7 and HT29 cancer cells. Results represent the mean ± standard deviation of quadruplicate determinations. Cisplatin was used as positive control. P1-*S. ellipticum*, P2 – *Combretum paniculatum*, P3- *Celosia trigyna*; P4-*D. cordata*; P5- *Cyathula prostata*.

oven at 40°C, ground to powder and stored in amber coloured bottles. 100 g each of powdered plant material was macerated with 80% ethanol at room temperature respectively. The resulting extracts were filtered and concentrated to dryness *in vacuo* at room temperature. Table 1 shows the various plant parts used in this work.

Cytotoxicity assay

The method of Koduru et al. (2007b), a modification of the MTT assay (Mossman, 1983) was used to determine the cytotoxic effect of the plant extracts on HT29 (colon cancer) and MCF-7 (breast cancer) cell lines. The cells were seeded into 96-well culture plates (Nunc) at 6000 cells/well in RPMI1640:10% fetal bovine serum (FBS) and left for 24 h. Plant extracts or cisplatin (positive control) were added and the cells incubated for a further 48 h after which the medium was replaced with 200 µl MTT (Sigma) (0.5 mg/ml in RPMI 1640:10% FBS). The MTT was removed after further 4 h incubation at 37°C and the purple formazan product dissolved in DMSO. The absorbance was measured at 540 nm on a multiwall scanning spectrophotometer (Multiscan MS, Labsystems). All incubation steps were carried out in a 37°C humidified incubator with 5% CO_2.

RESULTS AND DISCUSSION

Figure 1 shows the cytotoxicity results with extract numbers corresponding to those in Table 1. Cisplatin caused 48.5 ± 2.41, 78.0 ± 0.61 and 72.0 ± 1.47, 82.0 ±1.31 (SD, n=4) at 10 and 100 µM inhibition for MCF-7 and HT29 respectively. In the MCF-7 cell line, *Sapium* had comparable activity to the positive control Cisplatin for all the concentrations tested. *Cyathula* showed a 50% inhibition t 500 µg/ml while *Combretum*, *Celosia* and

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Of all the extracts tested, *Sapium* leaves showed the highest activity and this was also observed in our earlier work with the HeLa cell line (Sowemimo et al., 2009). The plant has also been reported to have antioxidant activity (Adesegun et al., 2008) and this may have a role to play in the observed activity in the cancer cell lines as antioxidants play a complex role in cancer prevention. Our results justify its inclusion in traditional recipes for cancer treatment and thus indicate its potential for biopharmaceutical use. It is suggested that the plant be taken for further bioassay guided experiments in order to isolate its bioactive principles.

ACKNOWLEDGEMENTS

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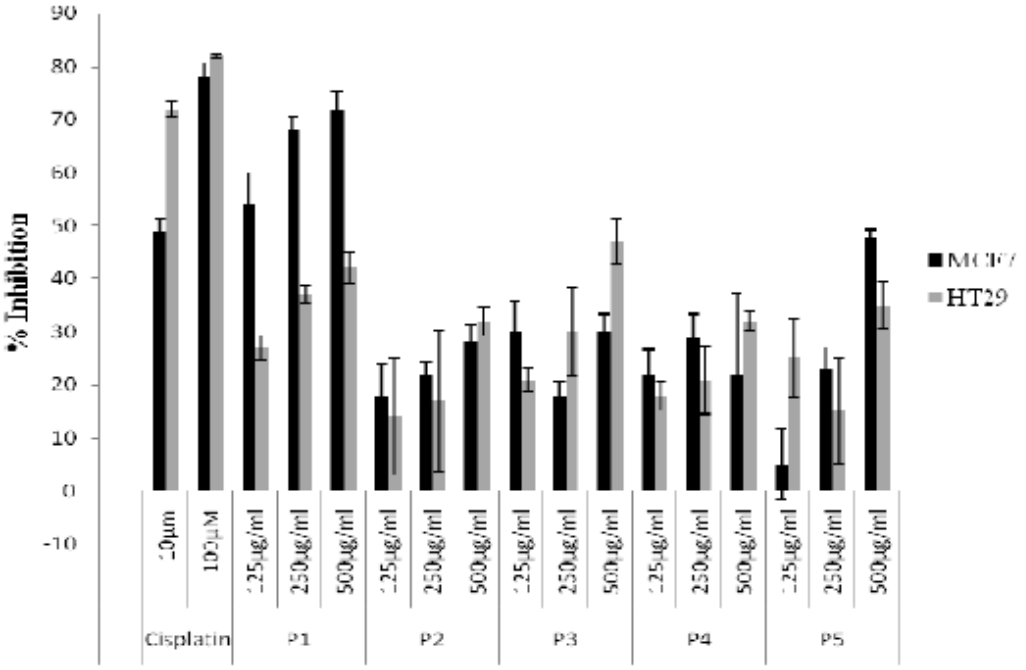

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Appendix 2:

Article accepted for publication

(Corrected proof manuscript)

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Full Length Research Paper

Cytotoxic compounds from the leaves of *Combretum paniculatum* Vent

Abimbola Sowemimo[1]*, Maryna van de Venter[2], Lucinda Baatjies[2], Trevor Koekemoer[2], Saburi Adesanya[3] and Wenhan Lin[4]

[1]Department of Pharmacognosy, Faculty of Pharmacy, University of Lagos, College of Medicine Campus, Idi-Araba, Lagos State, Nigeria.
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Combretum paniculatum* Vent. (Combretaceae) is a shrub that is widespread in tropical Africa. It is used locally in the treatment of carcinomous tumors. The cytotoxic activity of pheophorbide a and pheophorbide a-methyl ester isolated from the leaves of *C. paniculatum* were investigated. *In vitro* cytotoxicity of the compounds were evaluated against HT-29, MCF-7 and HeLa cancer cell lines using the methyl thiazolyl tetrazolium (MTT) assay and peripheral blood mononuclear cells. Cell cycle analysis was performed using propidium iodide staining and flow cytometry. Pheophorbide a was more toxic to MCF-7 and HeLa cells than its methyl ester, while the opposite result was seen in HT-29 cells. The methyl ester killed all the peripheral blood mononuclear cells from healthy donor blood while less inhibition was observed with pheophorbide a. Both compounds induced cell cycle arrest in HeLa cells in the G_0/G_1 phase, with pheophorbide a-methyl ester yielding a more enhanced G_1/G_0 arrest. After 24 h of exposure, the percentage of HeLa cells in the G_0/G_1 phase was 38.5% in vehicle control cells, 55.9% in pheophorbide a and 70.0% in pheophorbide a-methyl ester treated cells. The *in vitro* effect of *C. paniculatum* on cancer cell lines may be ascribed to the presence of pheophorbide a and its methyl ester. They exert this effect through the induction of cell cycle arrest in the G_0/G_1 phase of the cell cycle.

Key words: Cytotoxicity, cancer, *Combretum*, pheophorbide a, pheophorbide a-methyl ester.

INTRODUCTION

Combretum paniculatum Vent. (Combretaceae), a scandent shrub or robust liane with vivid scarlet flowers attaining 15 m length is wide spread in tropical Africa. Traditionally, the leaf sap is used externally for gonorrhea in Tanganyika, while the galled leaves are ground with salt and the paste applied to the tongue and inside the mouth of babies with stomatitis in Ivory Coast. The leaves and the aqueous extract of the inflorescence have been reported to have action against carcinomous tumours (Burkill, 1985). The anti-HIV activity of the plant has also been reported (Asres et al., 2001). Antimicrobial compounds such as cholest-5-en-3-ol, 2-phyten-1-ol, gallocatechin and apigenin have been reported from the plant (Samdumu, 2007). There is however no report on the cytotoxic compounds from this plant.

In our earlier investigation of plants used in the management of cancer by traditional medical practitioners in South Western Nigeria, the ethanolic extract of the leaves of *C. paniculatum* showed significant cytotoxic activity against HeLa (cervical cancer), HT-29 (colon cancer) and MCF-7 (breast cancer) cells (Sowemimo et al., 2009,

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2011).

This present work reports the isolation and cytotoxic activity of two compounds (pheophorbide a and pheophorbide a-methyl ester) isolated from the leaves of *C. paniculatum* against colon (HT-29), breast (MCF-7) and cervical (HeLa) cancer cell lines *in vitro*, peripheral blood mononuclear cells (PBMCs) and their effect on the cell cycle.

MATERIALS AND METHODS

Leaves of *C. paniculatum* Vent. (Combretaceae) were collected from the Olokemeji Forest Reserve, Nigeria in July 2008 and authenticated by comparison with corresponding herbarium specimens at the Forestry Research Institute, Ibadan, Nigeria (FRIN) where a voucher specimen (FHI 107980) was also deposited. The plant was air dried for two days followed by drying in a hot air oven at 40°C and ground to powder.

Extraction and isolation

The powdered plant material (1 kg) was macerated with ethanol (3 L) at room temperature for 72 h. The ethanolic extract was concentrated *in vacuo* to get a dark green residue (24.8 g). This residue was suspended in water and partitioned successively with petroleum ether, EtOAc and n-BuOH. The yield was 9.5, 1.2 and 0.8 g, respectively. The EtOAc fraction was subjected to vacuum liquid chromatography (VLC) gradient elution with petroleum ether and ethyl acetate which gave 6 fractions. Fractions 3 (90 mg) and 4 (96 mg) were further subjected to column chromatography over silica gel eluting with petroleum ether : EtOAc (7:3) and (2:1), respectively, to yield C-1 (5 mg), a light green solid and C-2 (4 mg), a light green solid respectively.

Cytotoxicity assay

Cervical (HeLa), colorectal (HT-29) and breast (MCF-7) cancer cells were seeded at 6,000 cells/well in 96-well plates and left to attach overnight at 37°C in a humidified incubator and 5% CO_2. PBMCs (peripheral blood mononuclear cells) were isolated from venous blood of a healthy donor using heparinized Vacutainer® CPTTM cell preparation tubes (Beckton Dickinson, Plymouth, UK) within 30 min of collection. PBMCs were seeded at 100,000 cells/well in round bottomed 96-well plates. The isolated compounds were resuspended in DMSO (0.25%), sonicated for 15 min, and complete RPMI-1640 medium was added to reach concentrations of 62.5 to 250 µg/ml. Cisplatin (10 and 100 µM) was used as the reference drug. The cells were treated for 48 h after which the medium was replaced with 200 µl MTT (Sigma) (0.5 mg/ml in RPMI 1640:10% FBS). After a further 4 h incubation at 37°C, the MTT was removed and the purple formazan product was dissolved in DMSO and absorbance measured at 540 nm using a BioTek® PowerWave XS spectrophotometer (Winooski, VT, USA). CellTiter-Blue® assay (Promega) was performed according to manufacturer's instructions for the PBMCs and fluorescence was read at $544_{Ex}/590_{Em}$ using a Fluoroskan Ascent FL fluorometer (ThermoLabsystems, Finland). All experiments were performed in quadruplicate.

Cell cycle analysis

HeLa cells were seeded into 10 cm cell culture dishes (Nunc) at 1.15×10^5 cells per ml and incubated in a 37°C incubator supplemented with 5% CO_2 for 24 h. The isolated compounds (62.5 µg/ml) or cisplatin (50 µM) were added and the cells were incubated for a further 24 and 48 h. DNA cell cycle analysis was performed using the Coulter® DNA Prep™ Reagents Kit (Beckman Coulter). The assay was carried out as per kit instructions and the results were analyzed on a Beckman Coulter FC500 flow cytometer. MultiCycle software for cell cycle analysis was used to calculate the percentage of cells in each of the cell cycle phases.

RESULTS AND DISCUSSION

The phytochemical investigation of the leaves of *C. paniculatum* gave two known compounds, pheophorbide a (C-1) and pheophorbide a-methyl ester (C-2) established with the aid of NMR spectroscopic techniques in addition to comparison with data found in literature (Nozawa et al., 1992; Ohsima et al., 1994; Rho et al., 2003).

The comparative growth inhibition results (Figure 1) suggest that at 62.5, 125 and 250 µg/ml, pheophorbide a and pheophorbide a-methyl ester were most effective against MCF-7 breast and HeLa cervical cancer cell lines. In the HT-29 colon cancer cell line, pheophorbide a showed more than 50% inhibition only at 250 µg/ml, while pheophorbide a-methyl ester showed more than 50% inhibition at 125 and 250 µg/ml. Pheophorbide a was however more active than pheophorbide a-methyl ester in the other two cell lines tested. The results from the CellTiter Blue® assay on the PBMCs (Figure 2) suggested that pheophorbide a is less toxic than its methyl ester to normal cells.

HeLa cells were used to establish whether the two purified compounds induced cell cycle arrest. Cisplatin at 50 µM typically caused an accumulation of the cells in the S phase after 48 h of treatment (Table 1). A large increase in the size of the sub-G_1 peak, corresponding to apoptotic cells, was seen after 24 h of cisplatin treatment and the effect was more pronounced after 48 h. The two isolated compounds both induced cell cycle arrest in the G_1/G_0 phase. Although, pheophorbide a was more toxic to HeLa cells, the methyl ester caused a more enhanced G_1/G_0 arrest on the cell cycle after 24 h. The percentage of HeLa cells in the G_1/G_0 phase was 38.5% for the vehicle control, 55.9% for the pheophorbide a and 70.0% for the pheophorbide a-methyl ester treated cells (Table 1). After 48 h of treatment, about 80% of the cells were arrested in G_1/G_0 with both compounds. Interestingly, the sub-G_1 peak did not increase with either of the two compounds, even after 48 h.

Data demonstrating the anti-tumor activity of chlorophyll related compounds are not too common. However, pheophorbide a isolated from *Clerodendrum calamitosum* has been reported to display both strong and broad activity against epidermoid carcinoma of the nasopharynx (KB), ovarian carcinoma (1A9), kidney carcinoma (CA KI-1), malignant melanoma (SK-MEL-2), Ileocecal carcinoma

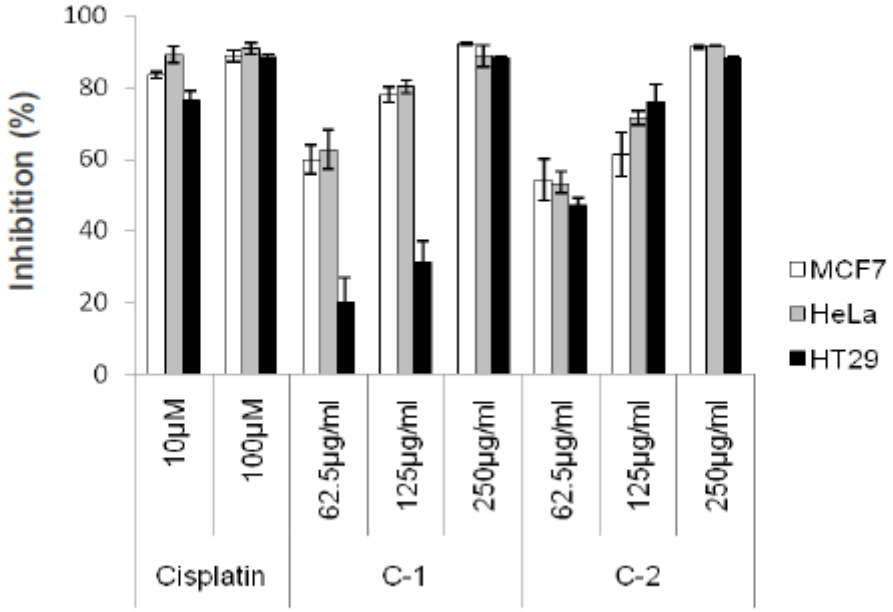

Figure 1. Screening results of compounds in MCF-7, HT-29 and HeLa cancer cells. Results represent the mean ± standard deviation of quadruplicate determinations. Cisplatin is the positive control.

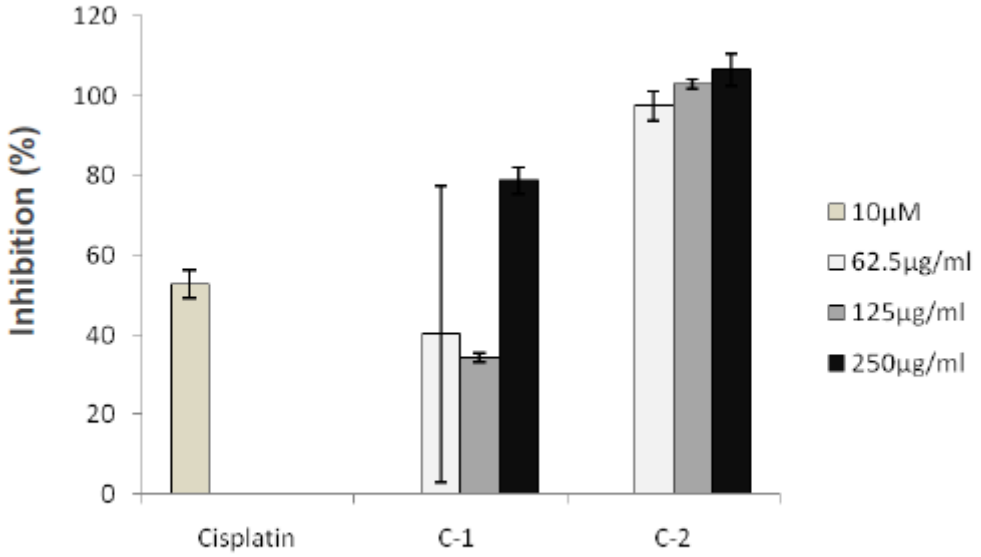

Figure 2. Percentage inhibition of compounds on PBMCs. Results represent the mean ± standard deviation of quadruplicate determinations. Cisplatin is the positive control. Pheophorbide a, C-1; pheophorbide a-methyl ester, C-2.

(HCT-8), human lung cancer (A549) and breast adenocarcinoma (MCF-7) (Cheng et al., 2001), while pheophorbide a-methyl ester from *Garuga pinnata* has been reported to show photo-dependent cytotoxic activity (Kashiwada et al., 1997).

Most previous studies have reported on the photodynamic activities of pheophorbide a and its derivatives (Tang et al., 2006) and not on its cytotoxic activity and mechanism of action in the absence of photo-irradiation. In a recent study by Mohan et al. (2010), fractions of an extract from *Typhonium flagelliforme* were shown to induce G_1/G_0 arrest and apoptosis in CEMss human T4-lymphoblastoid cells. The most active fraction contained, amongst other compounds, four pheophorbide related

Table 1. Effect of pheophorbide a and its methyl ester on the cell cycle distribution of HeLa cells after 24 and 48 h of exposure.

Exposure time (h)	Treatment	G_1/G_0 (%)	S (%)	G_2/M (%)
24	Vehicle control	38.5	48.6	12.9
	Cisplatin	34.4	41.8	23.8
	C-1 (pheophorbide a)	55.9	38.0	6.1
	C-2 (pheophorbide a-methyl ester)	70.0	6.2	23.9
48	Vehicle control	59.0	21.5	19.5
	Cisplatin	42.7	35.1	22.2
	C-1 (pheophorbide a)	78.2	13.8	2.0
	C-2 (pheophorbide a-methyl ester)	83.1	9.0	7.9

Vehicle control, cells treated with 0.125% DMSO; 50 µM cisplatin is the positive control; G_1/G_0, cells in the G_1 and G_0 phase of the cell cycle; S, cells in the synthesis phase of the cell cycle; G_2/M, cells in the G_2 and M phase of the cell cycle.

compounds including pheophorbide a and its methyl ester. The cell cycle arrest corresponds to the results from our study. The observation of apoptosis by Mohan et al. (2010) on CEMss cells and not on HeLa cells as in the present study could indicate that the compounds do not have identical effects in all cell types or the presence of other compounds in their extract were responsible for apoptosis induction. Chan et al. (2006) also reported induction of apoptosis by pheophorbide a in Hep3B hepatoma cells while being non-toxic in the normal human liver cell line WRL-68.

The cell cycle analysis results revealed that the two compounds act in a very similar way through induction of cell cycle arrest in the G_1/G_0 phase. No increase was observed in the sub-G_1 (apoptotic) peak and therefore, it appeared that the reduction in viable cell numbers as compared to vehicle control treated cells was due to inhibition of proliferation rather than cytotoxicity. This is the first report on the isolation of the two compounds, pheophorbide a and its methyl ester from *C. paniculatum* and the induction of cell cycle arrest in HeLa cells in the absence of photoactivation. Our results further suggested that pheophorbide a is a more potent inhibitor of proliferation than its methyl ester in at least two of the three cell lines investigated. Interestingly, the methyl ester derivative was more cytotoxic to PBMCs isolated from normal human blood.

The results obtained in this work further support the traditional use of the plant in the treatment of cancer.

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