



Anti-carcinogenic activity of *Centella asiatica* and *Elytropappus rhinocerotis* on a human colon cancer cell line

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Student's signature

DEDICATION

This work is dedicated to the memory of my late brother and friend-

Ashley Dwarka

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ABSTRACT

Recently our understanding of cancer has advanced in the realization that apoptosis and the genes that control it have a profound effect on the malignant phenotype. It is now clear that some oncogenic mutations disrupt apoptosis, leading to tumor initiation, progression or metastasis. Conversely, compelling evidence indicates that other oncogenic changes promote apoptosis, thereby producing selective pressure to override apoptosis during multistage carcinogenesis. Finally, it is now well documented that most cytotoxic anti-cancer agents induce apoptosis, raising the intriguing possibility that defects in apoptotic programs contribute to treatment failure. Because the same mutations that suppress apoptosis during tumor development also reduce treatment sensitivity, apoptosis provides a conceptual framework to link cancer genetics with cancer therapy. An intense research effort is uncovering the underlying mechanisms of apoptosis, such that, in the next decade, one envisions that this information will produce new strategies to exploit apoptosis for therapeutic benefit. Plants have a long history in cancer treatment. More than 3000 species have been known for their anti-cancer potential. Over 60% of currently used anti-cancer agents are derived in one way or another from higher plants. Indeed, compounds derived from natural sources, including plants, have played, and continue to play, a dominant role in the discovery of leads for the development of conventional drugs for the treatment of most human diseases especially cancer. Thus the aim of this study was to investigate if *Centella asiatica* and *Elytropappus rhinocerotis* possess anti-cancer potential and determine the effect on the modulation of apoptosis.

In South Africa *C. asiatica* is known anecdotally to treat various forms of cancers and *E. rhinocerotis* is known to treat colic and diarrhoea. The anti-cancer activity of *C. asiatica* has been studied in some parts India but *E. rhinocerotis* has not been investigated. This study was conducted using polarity guided fractionation (aqueous, ethanolic, methanolic and hexane), thereafter these extracts were tested for their toxicity on a colon cancer cell line (CaCO-2) and on normal cells

(PBMC). Subsequently, the most active extract was used to isolate the active fraction. The fraction that displayed toxicity on the CaCO-2 cells were further investigated for their ability to induce apoptosis by observing the morphological effects and DNA changes using acridine orange-ethidium bromide staining. Apoptosis was confirmed using Annexin V- PI staining. Nuclear effects were studied by DNA fragmentation and by agarose gel electrophoresis. Nuclear fragmentation was studied by flow cytometry using bromodeoxyuridine (BrDU). Pro-apoptotic changes were determined with Caspase III enzyme levels using flow cytometry. The results were compared to the effect of a known anti-carcinogen - Taxol. The anti-oxidant activity was also evaluated for the different extracts. The ethanolic extracts of both *C. asiatica* and *E. rhinocerotis* showed more than 100% radical scavenging activity. The methanolic extract (125 µg/ml -500 µg/ml) showed cytotoxicity on the CaCO-2 cells and a proliferative effect on the PBMC. Apoptosis was confirmed in the methanolic extract for both plants and was therefore used to carry forth this study. This included early apoptotic changes observed by the morphological study i.e., membrane blebbing, nuclear condensation and the presence of apoptotic bodies, in both *C. asiatica* and *E. rhinocerotis* fractions demonstrated more non-viable apoptotic cells than the methanolic extracts. Late changes of apoptosis were also found as indicated by DNA laddering and a positive outcome with BrDU. Both the active fractions from *C. asiatica* and *E. rhinocerotis* showed more DNA laddering and active caspase III than the methanolic extract. These features indicate that *C. asiatica* and *E. rhinocerotis* cause apoptotic death of colon cancer cells CaCO-2.

In conclusion, there was a significant increase in apoptosis of CaCO-2 cells with little alteration of PBMC in the presence of the methanolic extract of *C. asiatica* and *E. rhinocerotis*. The semipure fractions resulted in changes related to late apoptosis. The results suggest that *C. asiatica* and *E. rhinocerotis* induces apoptosis in CaCO-2 cells which is an important step in elucidating the underlying molecular mechanism for anti-tumour activity.

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LIST OF ABBREVIATIONS

7-AAD	:	7-amino-actinomycin D
AIF	:	Apoptosis-inducing factor
AO	:	Acridine orange
BrDU	:	Bromodeoxyuridine
CCM	:	Complete culture medium
COX	:	Cyclooxygenase
CPT	:	Camptothecin
DMSO	:	Dimethyl sulphoxide
DNA	:	Deoxyribonucleic acid
DPPH	:	2,2-Diphenyl-1-Picrylhydrazyl
ELISA	:	Enzyme-Linked ImmunoSorbent Assay
EtBr	:	Ethidium bromide
FCS	:	Foetal Calf Serum
MEM	:	Minimum essential media
MeOH	:	Methanol
MRC	:	Medical Research Council
NCI	:	National Cancer Institute
PBMC	:	Peripheral Blood Mononuclear Cell
PBS	:	Phosphate buffered saline
PI	:	Propidium iodide
POH	:	Perillyl alcohol
PS	:	Phosphatidylserine
RBC	:	Red blood cell
RNS	:	Reactive nitrogen species

ROS	:	Reactive oxygen species
R _t	:	Retention time
RT	:	Room temperature
TLC	:	Thin-layer chromatography
TNF	:	Tumor necrosis factor
UV	:	ultraviolet
WHO	:	World Health Organization
XTT	:	2,3-bis[2-Methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5 carboxyanilide inner salt

CHAPTER 1: RATIONALE OF STUDY

1.1 The need to find natural anticancer compounds as an alternative to conventional drugs

According to the WHO, poverty and lack of modern medicine forces 65-80% of the world's population to depend exclusively on plants for principle health care. Many chemotherapeutic regimens do exist but are compounded with extreme toxicity. During the 1960s the National Cancer Institute (USA) began to screen plant extracts with antitumor activity (Monks *et al.*, 2002). Natural compounds isolated from medicinal plants, as rich sources of novel anticancer drugs, have been of increasing interest since then.

While many conventional drugs or their precursors are derived from plants, there are fundamental differences between administering a pure chemical and the same chemical in a plant matrix. Synergy is an important concept in herbal pharmacology. In the context of chemical complexity, it applies if the action of a chemical mixture is greater than the arithmetical sum of the actions of the mixture's components: the whole is greater than the sum of the individual parts (Mills and Bone, 2000). In other words, components of plants which are not active themselves can act to improve its stability, solubility, bioactivity or half-life of the active components. Hence a particular chemical might in pure form have only a fraction of the pharmacological activity that it has in its plant matrix.

It is a widely recognized fact that numerous synthetic chemotherapeutic drugs exert along with a positive effect also harmful side effects such as cardiotoxicity, central neurotoxicity, pericarditis and hepatotoxicity. To the contrary, in the plant world, one very often encounters strongly active substances coexisting with the other compounds that mitigate their negative side effects. Because of

this, in recent years a return to phytotherapy has been observed. Therefore there has been a renewed call by the World Health Organization (WHO) to screen plant material for the presence of biologically active compounds. It is firmly believed that a great, yet still not fully revealed, therapeutic potential exists in plants, because so far only a few percent out of 250,000 plant species have been investigated with regard to their usefulness in medicine (Bhattacharjee, 2008).

We stand at a turning point in cancer chemotherapy. The last 50 years have been dominated by drugs that are not highly specific to cancer cells. Being non-specific, these drugs also destroy normal cells, and in the process can cause significant and sometimes deadly adverse effects. However, the future does look brighter, for an eventual success in the fight against cancer. As a science, the field of plant compound research can contribute to a greater understanding of plants and their effect on tumors which can lead to the development of successful therapies.

A successful anticancer drug should kill or debilitate cancer cells without causing unnecessary damage to normal cells. This idyllic situation is achievable by inducing apoptosis in cancer cells. The life span of both normal and cancer cells is extensively affected by the rate of apoptosis. Thus, modulating apoptosis may be useful in the management and therapy or prevention of cancer. Significantly, natural products are providing such templates. It is thus considered imperative to screen apoptotic inducers from plants, either in the form of crude extracts or as compounds isolated from them. Confirmation has emerged from various studies that put forward products derived from plants are helpful in the treatment as well as in the prevention of cancer.

1.2 Why *Centella asiatica* and *Elytropappus rhinocerotis*?

Ethnobotanic knowledge has indicated that *Centella asiatica* and *Elytropappus rhinocerotis* are used for their anti-carcinogenic properties. *C. asiatica* is found as a weed and belong to the family

Umbelliferae. The aqueous extract of *C. asiatica* possesses antioxidant (Gupta and Flora, 2006), anti-inflammatory, immunomodulating, antitumour (Babu *et al.*, 1995), antiproliferative (Yoshida *et al.*, 2005) and radioprotective properties. *E. rhinocerotis* is an aromatic shrub belonging to the family *Asteraceae*. It is used for the treatment of colic, wind, diarrhea and acidity in young children. It is also used for the treatment of cancer and ulcers (Dekker *et al.*, 1988).

Although there is an extensive number of research publications available on the anticancer and biological activity of *C. asiatica* in India and other parts of the world, the development of therapeutic agents and research into the medicinal properties of the South African species has remained a somewhat neglected subject. *E. rhinocerotis* is indigenous to South Africa. Scientifically *E. rhinocerotis* has not been studied for its medicinal or anticancer properties. The study of these two South African traditional plants also has not been taken seriously or documented as fully as in other traditional societies, such as Indian. Our knowledge of these two South African medicinal plants are rather limited.

As there is sufficient ethno botanical knowledge that *Centella asiatica* species found in India contains potential anti-cancer properties, and to uncover if *Elytropappus rhinocerotis* contains potential anti-cancer compounds, therefore in this study we investigated the anticancer and apoptotic activity of *C. asiatica* and *E. rhinocerotis* on human colon cancer (CaCO-2) cell line.

To achieve this, our objectives were:

1. To determine the cytotoxic and the anti-oxidant activity of the non-polar based extract (hexane) and polar (aqueous, ethanolic and methanolic) of *C. asiatica* and *E. rhinocerotis* on colon cancer cell line (CaCO-2).
2. To identify the active fraction by the bioassay guided fractionation

3. To determine the effect of the active extract and selected fractions on the morphology, cell membrane integrity, nuclear changes and cell cycle changes on CaCO-2 cell organelles.

This thesis is designed in six phases. The first stage gives the overall literature review regarding plants and their use in cancer. The second phase emphasizes the problem encountered and elaborates on the rationale for this research. The methodology carried out for this study is outlined in the third phase. It describes the cytotoxic and proliferative effect of the aqueous, ethanolic, methanolic and hexane extract of *Centella asiatica* and *Elytropappus rhinocerotis* on CaCO-2 cells and PBMC, thereafter the isolation of the chemical compound from *Centella asiatica* and *Elytropappus rhinocerotis* responsible for anti-tumour activity. Subsequently, the investigation of the apoptotic potential of the active crude plant extract and the active fractions on the CaCO-2 cell line. The final phase includes an overview of the study and the conclusion thereof.

CHAPTER 2: LITERATURE REVIEW

2.1 Plants and cancer

Plants have been an indispensable source of natural products for medicine. Hundreds if not thousands of indigenous plants have been used by people on all continents as poultices and infusions dating back to the prehistoric era. The chemical constituents of the plant cells that exert biological activities on human and animal cells fall into two distinct groups depending on their relative concentration in the plant body, as well as their major function: primary metabolites, the accumulation of which satisfies nutrition and structural needs and secondary metabolites, which act as hormones, pharmaceuticals and toxins (Kintzios and Barderaki, 2004).






A significant portion of the products derived from secondary pathways serve either as protective agents against various pathogens (e.g. insects, fungi or bacteria) or growth regulatory molecules (e.g. hormone-like substances that stimulate or inhibit cell division and morphogenesis). Due to these physiological functions, secondary metabolites can be a source of anti-cancer drugs, since their direct cytotoxicity has an effect on the course of tumor development. Administration of these compounds at low concentrations may be lethal for microorganisms and small animals but in larger organisms, including humans, they may specifically affect the fastest growing tissues such as tumors (Boik, 2001).

Africa is a continent endowed with an enormous wealth of plant resources. Over 5 000 distinct species are known to occur in the forest regions alone, and most of them have been used for several centuries in traditional medicine for the prevention and treatment of diseases. The continent is believed to have the oldest known human habitation, and is generally considered the cradle human civilization. The ancient kingdoms and empires of Africa had extensively codified healing recipes.

The history of healing arts in Africa can be traced back to 3200 B. C. E. (Danziel, 1937). In South Africa, many people still use plants as medicines as an alternative or supplement to visiting a western health care practitioner (Van Wyk *et al.*, 2000). This is not surprising due to South Africa's cultural diversity as well as its large floral biodiversity. South Africa is home to over 30,000 species of higher plants and 3000 of these species have been found to be used in traditional medicine across the country (Van Wyk *et al.*, 2000). There are over 27 million users of indigenous medicine (Mander, 1998) and an estimated 200,000 indigenous traditional healers, which up to 60% of the population consult with (Van Wyk *et al.*, 2000). Currently, Combretastatin a small organic molecule found in the bark of the African bush willow tree *Combretum caffrum*, has been identified and clinical trials have begun in cancer therapy. Combretastatins are believed to restrict blood flow to tumours and therefore starve them of oxygen.

Traditional utilization of medicines is acknowledged as a way to discover potential future medicines. In traditional practices, numerous plants have been used to treat malignant diseases including cancer. The expansion of resistance by cancerous cells to chemotherapeutic agents causes major problems in the control of this disease. An ethnopharmacological approach has provided leads to identifying potential new drugs from plant sources, including those for many types of cancers (Table 1). There are numerous drugs available in Western medicine that have been directly isolated from plants, or are derived from templates of compounds from plant sources used in the treatment of cancer. However the challenge lies in finding an effective anti-cancer drug that would not have dreadful side effects. Because cancer cells may grow and divide more rapidly than normal cells, many anticancer drugs are made to kill growing cells. But certain normal, healthy cells also multiply quickly, and cancer drugs can affect these cells, too. This damage to normal cells causes side effects. Some anticancer drugs may affect cells of vital organs, such as the heart, kidney, bladder, lungs, and nervous system. Therefore researchers are turning to plant compounds in the optimism to find a successful anti-cancer drug as a template for chemical synthesis.

Table 1: South African indigenous plants used anecdotally for cancer treatment

Plant name (family)	Active Ingredients	Medicinal uses	Bioactivity tested	Reference
<i>Sutherlandia frutescens</i> 	Triterpenoids, amino acids, sugars	TB , AIDS, treatment for cancer	anti-inflammatory , antidiabetic, anti-tumor	Steenkamp and Gouws, 2006.
<i>Salvia Africana-lutea</i> 	Monoterpene-Hydrocarbons	Colds, tuberculosis, cancer, influenza	Antimicrobial, antioxidant, anti-inflammatory, Cytotoxicity, antipyretic	Kamatoua <i>et al.</i> , 2008
<i>Withania somnifera</i> 	Flavanoids, Triterpenoids, Amino acids	Cancer, Anti-aging	Antioxidant, Anti-cancer	Steenkamp and Gouws, 2006.
<i>Tulbaghia violacea</i> 	Diterpenoids, flavanoids	stomach ailments, rheumatism, high fevers and cancer	Antipyretic, anti-tumor and anti-inflammatory	Low <i>et. al.</i> , 2008
<i>Prunus Africana</i> 	Amygalin, hydrocyanic acid	Prostate cancer, HIV	Anti-cancer, Anti-inflammatory	Low <i>et. al.</i> , 2008

Indigenous knowledge of *Centella asiatica* and *Elytrophapus rhinocerotis* have been recorded since 1940 and *C. asiatica* has been documented to be used for the treatment of leprosy, respiratory infections, ulcers, colds and fatigue (Inamdar *et al.*, 1996). *E. rhinocerotis* is used for the treatment of stomach complaints including indigestion, dyspepsia and a lack of appetite (Kamatoua *et al.*, 2008). Scientific information regarding anticancer activity from *C. asiatica* in India was

documented by Babu *et al.* (1995). However there is no affirmative records that show that *E. rhinocerotis* has been studied for its anti-cancer activity.

2.2 Characteristics of plants studied

2.2.1 *Centella asiatica*



Figure 1: *Centella asiatica*

2.2.1.1 Classification and general description

C. asiatica (Fig. 1) is a small herbaceous annual plant of the subfamily Mackinlayoideae of family *Apiaceae*. The stems of the plant are slender with creeping stolons, green to reddish green in color, interconnecting one plant to another. The flowers are pinkish to red in color, born in small, rounded bunches (umbels) near the surface of the soil. *Centella* grows along ditches and in low wet areas. Because the plant is aquatic, it is especially sensitive to pollutants in the water, which are easily incorporated into the plant (Hausen, 1993).

2.2.1.2 Medicinal uses and studies

A decoction of juice from the leaves is thought to relieve hypertension. This juice is used as a general tonic for good health. Several scientific reports have documented *C. asiatica* ability to aid wound healing, which is responsible for its traditional use in leprosy. Upon treatment with *C. asiatica*, maturation of the scar is stimulated by the production of type 1 collagen. The treatment

also results in a marked decrease in inflammatory reaction and myofibroblast production (Inamdar *et al.*, 1996). This could be attributed to the asiatic acid or madecassic acid and their respective glycosides, asiaticoside and madecassoside which are the bioactive terpene acids found in *C. asiatica* (Inamdar *et al.*, 1996). Park *et al.* (2005) found that asiatic acid decreased the viability and induced apoptosis in human melanoma SK-MEL-2 cells. A study by Shukla *et al.* (1999) show that, asiaticoside, has wound healing activity, promotes fibroblast proliferation and increases the level of enzymatic and non-enzymatic anti-oxidants.

2.2.1.3 Phytochemicals in *Centella* genus

Yoshida *et al.* (2005), isolated 9 compounds from the methanol (MeOH) and chloroform extracts: (1) 1, 12-dehydroursolic acid lactone, (2) ursolic acid, (3) pomolic acid, (4) α -dihydroxyurs-12-en-28-oic acid, (5) Asiatic acid, (6) corosolic acid, (7) 8-acetoxy-1,9-pentadecadiene-4,6-diyn-3-ol, (8) β -sitosterol 3-o- β -pyranosid and rosemarinic acid which they tested for antiproliferative effect on human gastric adenocarcinoma (MK1) and murine melanoma (B16F10). They found that these compounds had an antiproliferative effect that ranged from 8-20 μ M.

It was also reported by Babu *et al.* (1995) that a methanolic extract of *C. asiatica* inhibited the proliferation of transformed cell lines. The extract had an IC_{50} of 62 μ g/ml for mouse Ehrlich ascites carcinoma (EAC) cells and 75 μ g/ml for Daltons lymphoma ascetic (DLA) cells. Yoshida *et al.* (2005) found that the methanolic extract from the aerial parts of *C. asiatica* inhibited *in vitro* the growth of MK 1, HeLa, and B16F10 cells which could be due to ursolic acid.

2.2.2 *Elytropappus rhinocerotis*



Figure 2: *Elytropappus rhinocerotis*

2.2.2.1 Classification and general description

E. rhinocerotis (Fig. 2) is a much-branched grey to grey-green aromatic shrub which is 0,6 - 2,5m in height. It comes from the family *Asteraceae*. The leaves are minute, numerous, adpressed to the stem, usually woolly on both surfaces. The flowers inconspicuous, yellow, tubular, born in capitula of mostly 3 florets and well developed. The fruit (an achene) has prominent longitudinal ribs (Dekker *et al.*, 1988). It is common on dry clay flats and slopes throughout the Western and Eastern Cape Provinces, up to Namaqualand (Dekker *et al.*, 1988).

2.2.2.2 Medicinal uses and studies

E. rhinocerotis has traditionally been used for the treatment of colic, wind, diarrhea and acidity in young children; adult use is mainly for digestive disorders and as a bitter tonic to stimulate appetite (Dekker *et al.*, 1988). According to the South African Medical Research Council (Levyns, 1935), no *in vitro* antimicrobial activity against *Pseudomonas aeruginos* (highest concentration of 100 µg/ml tested), *Candida albicans* or *Mycobacterium smegmatis* was observed. Some activity was recorded against *Staphylococcus aureus*. Some preliminary studies on the use of this herb as an

anti-hypoglycaemic agent were carried out during the period 1975-1980 by the late Professor W. Jackson, at the Department of Endocrinology at Groote Schuur Hospital.

2.2.2.3 Phytochemicals in *Elytropappus rhinocerotis*

Microchemical tests indicated the presence of cardiac glycosides, saponins, tannins and reducing sugars. Rhinocerotoic acid, a labdane diterpene, has been isolated from the overground parts of this species (Dekker *et al.*, 1988). Not much is known about this plant as it was not studied for its biological activities and phytochemical properties.

2.3 Cancer and apoptosis

2.3.1 Incidence of Cancer

In South Africa cancer is one the leading causes of deaths. The Cancer Association of South Africa predicts that one in four South Africans will be affected by cancer in his/her lifetime. Cancer incidence rates in South Africa are among the highest rates in Africa (Garcia *et al.*, 2007). It is estimated that there were more than 12 million new cancer cases in 2007 worldwide, of which 5.4 million will occur in economically developed countries and 6.7 million in economically developing countries (Fig. 3). The corresponding estimates for total cancer deaths in 2007 were 7.6 million (about 20,000 cancer deaths a day), 2.9 million in economically developed countries and 4.7 million in economically developing countries (Garcia *et al.*, 2007). The most commonly diagnosed cancers worldwide are lung, breast and colorectal cancers. The most common causes of cancer death are lung, stomach and liver cancers. By 2050, the global burden is expected to grow to 27 million new cancer cases and 17.5 million cancer deaths.

Cancer Incidence Worldwide

Breakdown of the estimated 12.7 million new cases, World-age standardised incidence rates and the most commonly diagnosed cancers by the different regions of the world, 2008.

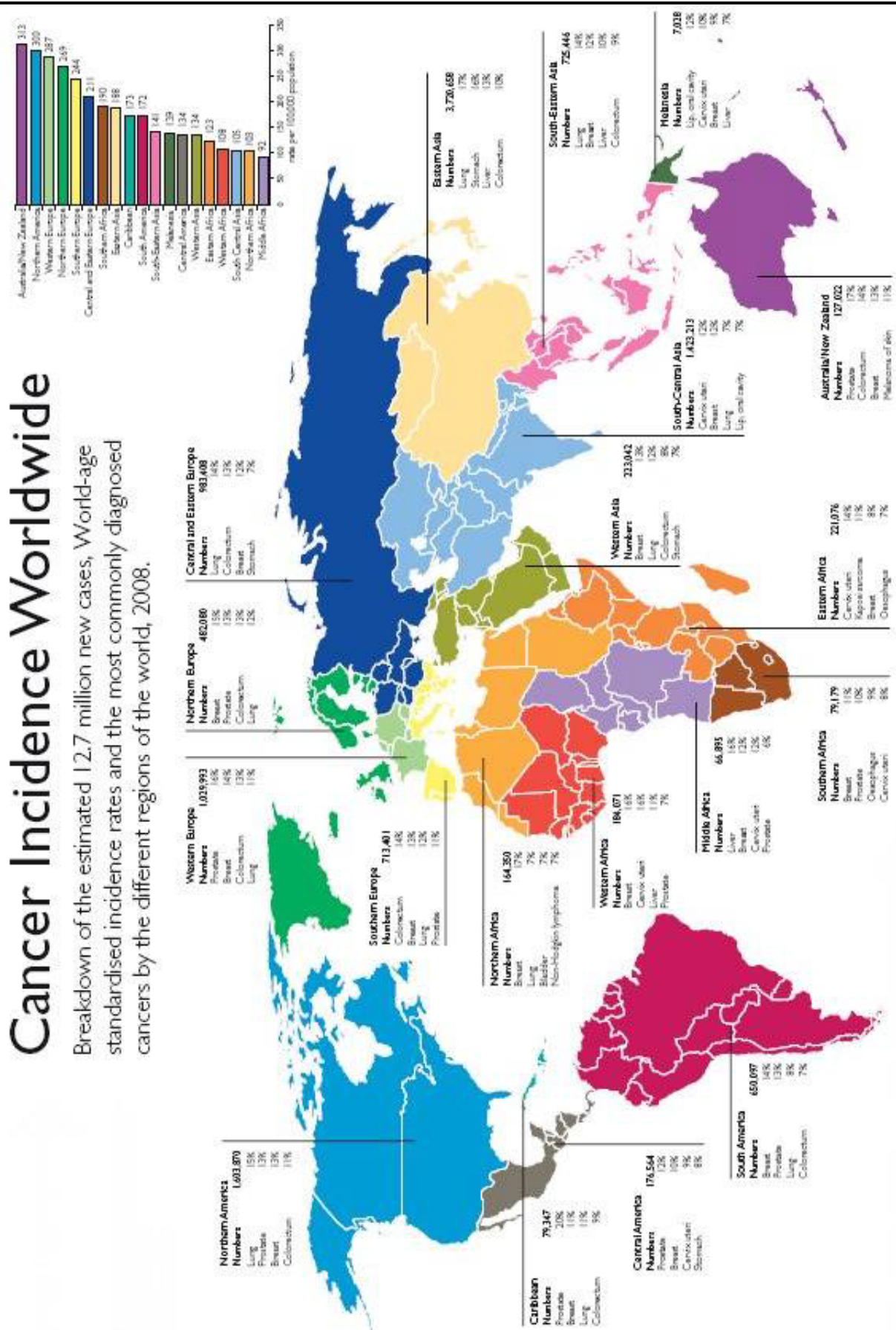


Figure 3: Cancer incidence and mortality worldwide in 2008 (WHO, 2008).

2.3.2 General features of cancer

Cancer is a term used for diseases in which abnormal cells divide without control and are able to invade other tissues. It is not just one disease but many diseases. Cancer cells can spread to other parts of the body through the blood and lymph systems (National Cancer Institute). There are more than 100 different types of cancer. Most cancers are named for the organ or type of cell in which they start.

Cancer arises when a cell acquires sufficient mutations to enable it to survive and multiply free from its normal regulation by soluble extracellular factors and by interaction with its neighbours. The genes that become deregulated are the key proteins that manage these processes. These proteins can be placed into three overlapping categories: (i) those that control signal transduction of extracellular signals regulating cell division; (ii) those that control processes associated with cell invasion; and (iii) those that affect processes associated with the cell cycle and apoptosis (Fig. 4).

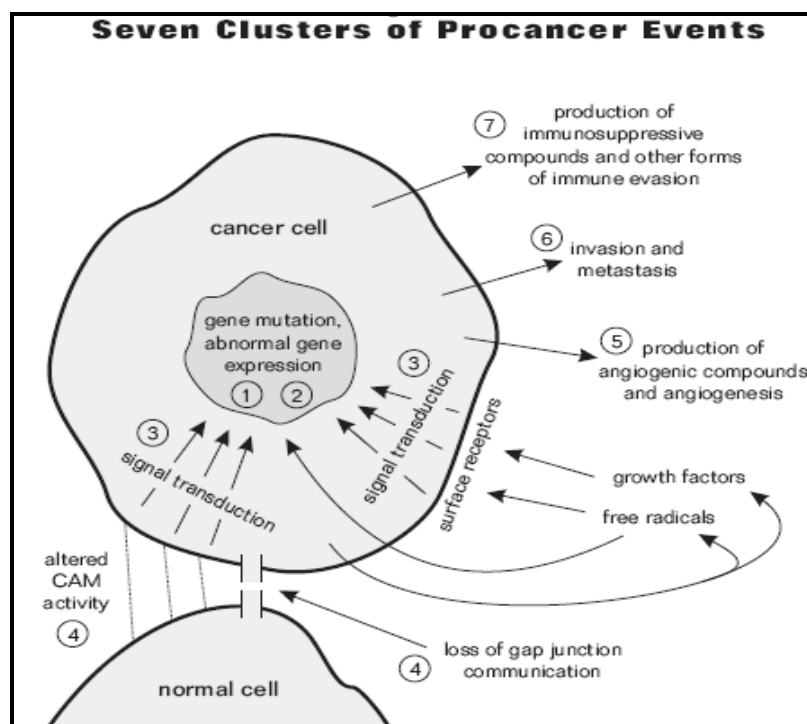


Figure 4: Clusters of procancer events (Boik, 2001).

In some cases the proteins that control these processes become mutated and they function inappropriately (Boik, 2001). The carcinogenic process that arises by accumulation of mutations in these crucial genes follows a stepwise sequence (Peerra and Weinstein, 2002). Initially, tumor is initiated when mutations free the cell from growth restraints, and build up of small cells occurs. As enhanced cell division facilitates the accumulation of further mutations, tumor promotion leads to cells that are more dysfunctional and that have lost further growth controls leading to uncontrolled growth (Fig. 5).

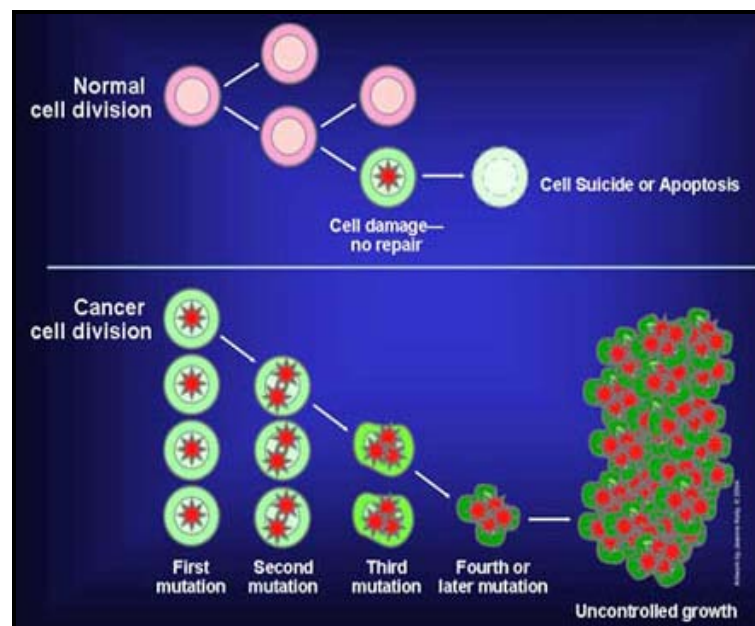


Figure 5: Loss of normal growth control (National Cancer Institute, 2000).

As the colony of these cells grows it has to acquire its own blood supply by organizing growth of blood vessels into the tumor, this process is called angiogenesis. The colonies become invasive when it acquires the ability to break down local structures. During metastasis cells slough off from the tumor mass and travel round the body to establish cell colonies at other sites. The last two processes are elements of progression and are shown in Figure 6. The extent of this determines the tumor type (Peerra and Weinstein, 2002).

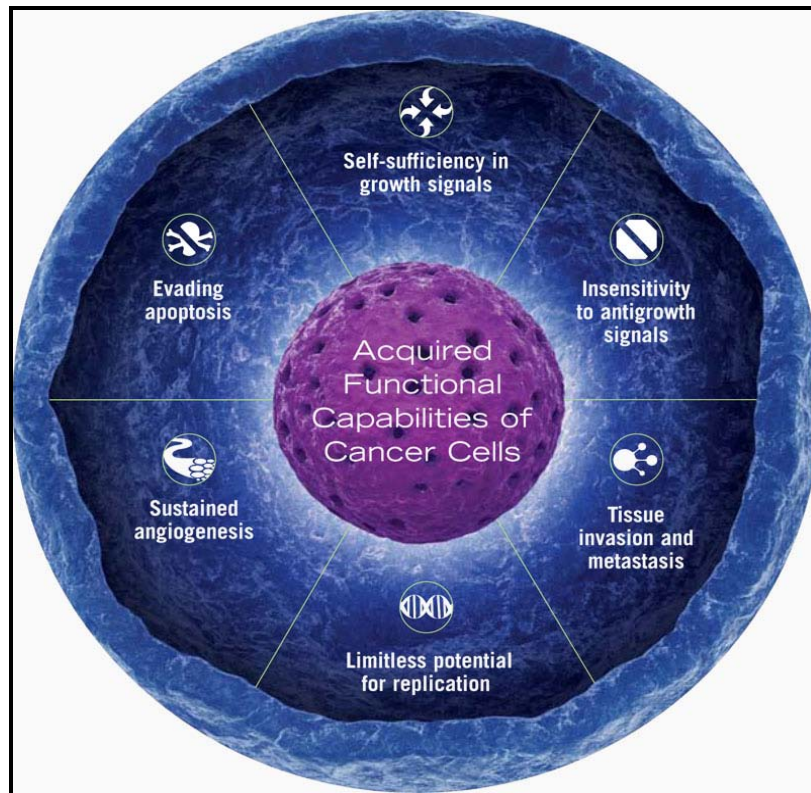


Figure 6: Well-designed capability of a cancer cell (Hanahan and Weinberg, 2000).

2.3.2.1 Free radicals and anti-oxidants in relation to cancer inflammation

Millions of processes occur all the time in the human body. All these processes require oxygen. However that same life giving oxygen can create harmful side effects, or oxidant molecules called free radicals which cause cell damage and lead to cell death. A free radical is an atom or molecule with an unpaired electron. These unpaired electrons are very hazardous and unstable. These unstable electrons collide with other molecules so it can obtain an electron from them. This causes a change in structure of the other molecule and also causes them to become free radicals. Therefore a chain reaction is commenced, where the structure and composition of billions of molecules are affected, changing the DNA, protein structure, enzymes and cells. It has been established that oxidative stress is among the major causative factors in the induction of many chronic and degenerative diseases including atherosclerosis, ischemic heart disease, ageing, diabetes mellitus, cancer, immunosuppression, neurodegenerative diseases and much more (Kamuhabwa *et al.*, 2000). In carcinogenesis, reactive oxygen species are responsible for initiating the multistage

carcinogenesis process starting with DNA damage and accumulation of genetic events in one or few cell lines which leads to progressively dysplastic cellular appearance, deregulated cell growth, and finally carcinoma (Van Cruchten and Van Den Broeck, 2002).

2.3.3 General features of apoptosis

All cells have a finite life span and cell death occurs mainly as a result of passive necrotic processes or due to an active process of programmed cell death, or, “apoptosis” (Park *et al.*, 2005). Apoptosis plays an important role in both human embryonic development and adult tissue homeostasis. Apoptosis is the most common mechanism by which the body eliminates damaged or unneeded cells without local inflammation from leakage of cell contents. Cells that are undergoing apoptosis exhibit a characteristic pattern of morphologic changes, including cell shrinkage, condensation, fragmentation of the nucleus, bubbling of the plasma membrane, known as “blebbing,” chromatin condensation and nucleosomal fragmentation (Valenzuela *et al.*, 2000). The resulting membrane-bound apoptotic bodies are consumed by neighboring cells or by macrophages. In contrast, the necrotic mode of cell death represents a passive consequence of mechanical damage or exposure of the cells to toxins. The morphologic differences between and the physiologic consequences of apoptosis and passive necrosis are shown Figure 7 .

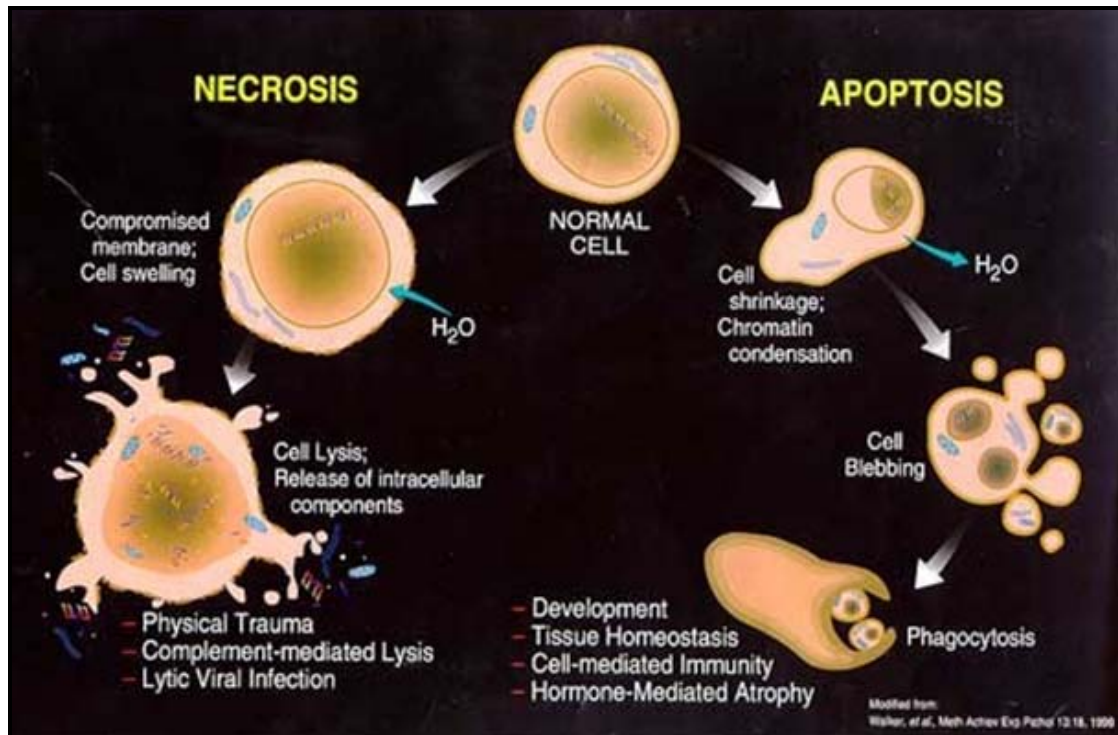


Figure 7: Hallmarks of apoptotic and necrotic cell Death (Van Cruchten and Van Den Broeck, 2002).

Apoptosis is a much slower series of events than necrosis, requiring from a few hours to several days, depending on the initiator (Willingham, 1999). There are different methods to detect whether an extract induces cell death (specifically apoptosis) and by which mechanism these extracts induce cell death. The induction of apoptosis of cancer cells is recognized as a valuable tool for cancer treatment.

2.3.3.1 The apoptotic pathway

Efficient apoptotic pathways are vital for tissue homeostasis and the dysregulation of apoptosis is implicated in a multitude of disease states including cancer. Genetic changes resulting in loss of apoptosis or derangement of apoptosis-signaling pathways in the transformed cells are likely to be decisive components of carcinogenesis (Kaufmann and Vaux, 2003). There are at least two broad pathways that lead to apoptosis, an "Extrinsic" and an "Intrinsic" Pathway as shown in Figure 8.

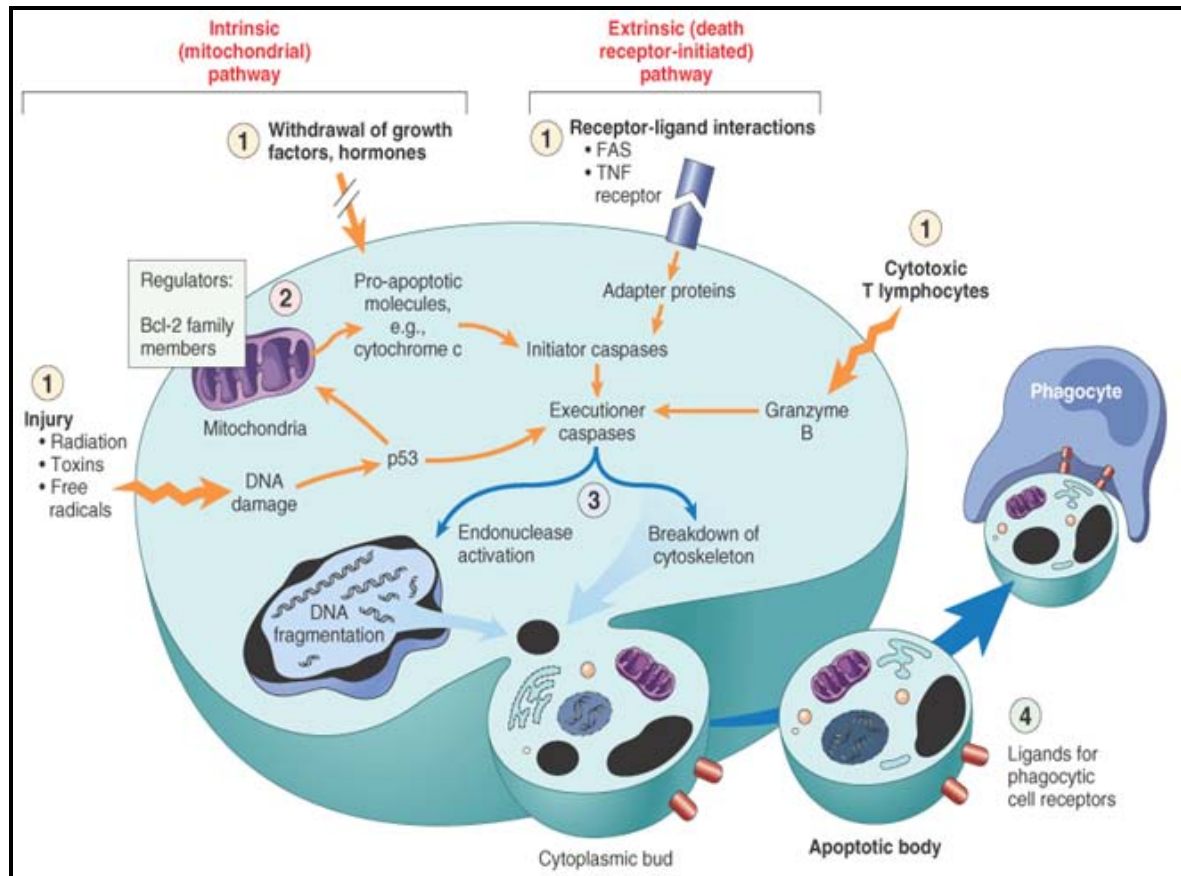


Figure 8: The two major pathways of apoptosis (Vogelstein and Kinzler, 2004).

In both pathways, signaling results in the activation of a family of cys (cysteine) Proteases, named caspases that act in a proteolytic cascade to take apart and eliminate the dying cell. Activation of caspase 3 is tightly regulated by the formation of apoptosome (Fig. 9) which is dependent on the cytochrome c release from the mitochondria into cytosol, which is thought to be regulated by *Bax* and *Bcl-2* (Vogelstein and Kinzler, 2004).

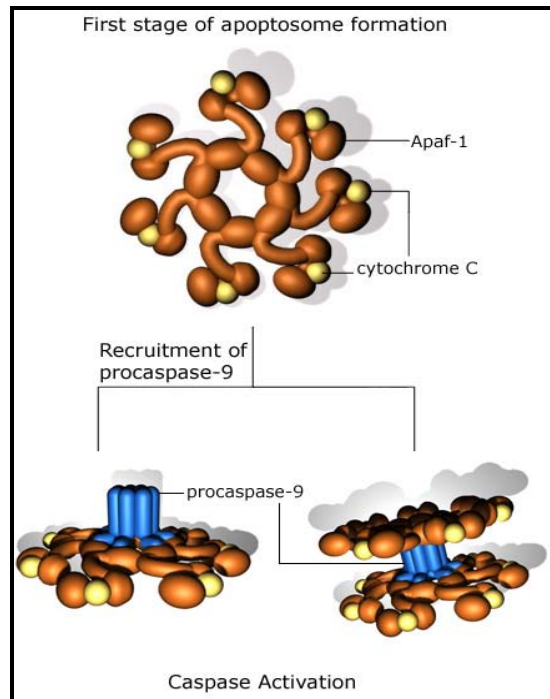


Figure 9: Aposomal formation (Horobin and Kiernan, 2002).

Pro-apoptotic *Bax* forms pores in the outer mitochondrial membrane, releasing cytochrome c while anti-apoptotic *Bcl-2* prevents the opening of mitochondrial transition pore by binding with *Bax*. Damaged mitochondria release cytochrome C and "apoptosis activating factor," which can directly activate caspase 9, resulting in propagation of a direct signal to other downstream caspases through protease activation. Apoptosis-inducing factor (AIF1) is also released from the mitochondrion and then can translocate to the nucleus, bind to DNA, and generate free radicals to further damage DNA. An additional pro-apoptotic stimulus is the bad protein, which can heterodimerize with *bcl2* gene family members to antagonize apoptosis. Importantly, though, bad protein function can be retarded by its sequestration as phospho-bad through the 14-3-3 adapter proteins. The phosphorylation of bad is mediated by the action of the AKT kinase in a way that defines how growth factors that activate this kinase can retard apoptosis and promote cell survival (Kasper *et al.*, 2004). Reactive oxygen species (ROS) have been suggested to act as an upstream signal for caspase-3 activation (Park *et al.*, 2005).

2.3.3.2 Key elements of apoptosis

2.3.3.2.1 Morphological changes

The usual sequence of morphological changes in apoptosis depicted in Figure 10 and are usually, (a) a loss of adhesion to substratum, resulting in cell rounding; (b) a flurry of surface blebbing, (c) shrinkage of the cell and (d) after a long delay of several hours, the lysis of cell membrane. The dynamic nature of the initial rounding and blebbing events makes them easy to spot, and these events are a convenient point at which to designate the beginning of apoptosis. Morphological changes due to apoptosis is studied using acridine orange and ethidium bromide staining. Acridine orange is a nucleic acid selective fluorescent cationic dye useful for cell cycle determination. It is cell-permeable, and interacts with DNA and RNA by intercalation or electrostatic attractions respectively. When bound to DNA, it is very similar spectrally to fluorescein, with an excitation maximum at 502 nm and an emission maximum at 525 nm (green). Ethidium bromide intercalates and stains DNA, providing a fluorescent red-orange stain. Although it will not stain healthy cells, it can be used to identify cells that are in the final stages of apoptosis, such cells have much more permeable membranes (Horobin and Kiernan, 2002). Consequently, ethidium bromide is often used as a marker for apoptosis in cells populations and to locate bands of DNA in gel electrophoresis. The stain may also be used in conjunction with acridine orange (AO) in viable cell counting.

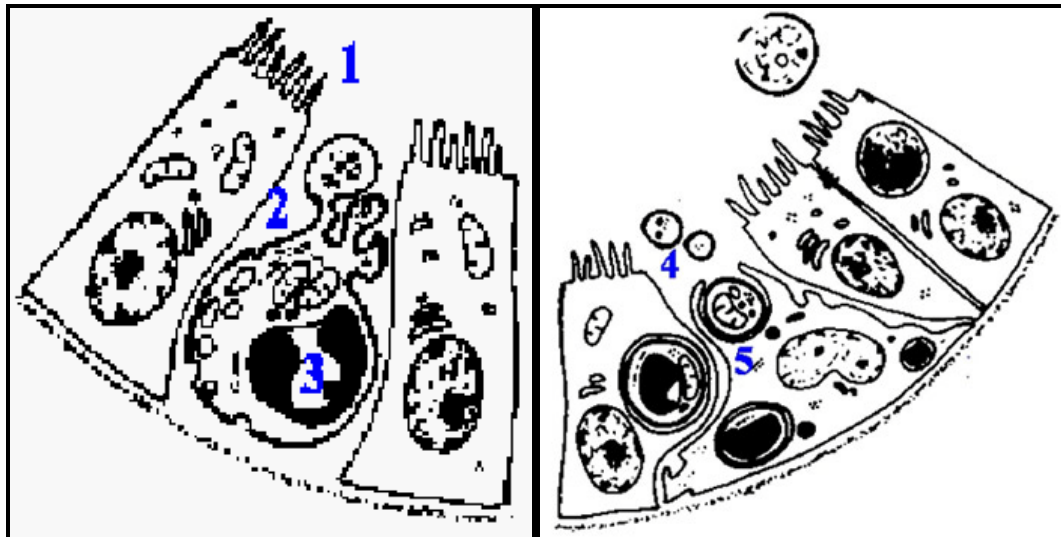


Figure 10: Cell dying by apoptosis. Once the apoptotic programme has been initiated within a cell, several morphological characteristics can be seen (1) cell shrinkage, (2) loss of surface contact with neighbours, (3) chromatin condensation, (4) fragmentation of the apoptotic cell into small apoptotic bodies and (5) phagocytosis by neighbours or macrophages (Berezney *et al.*, 1991).

2.3.3.2.2 Membrane changes

The loss of plasma membrane is one of the earliest features of a cell undergoing apoptosis. Externalization of phosphatidylserine (PS) and phosphatidylethanolamine is a hallmark of the changes in the cell surface during apoptosis. In apoptotic cells, the membrane phospholipid phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing the PS to the external environment (Fig. 11).

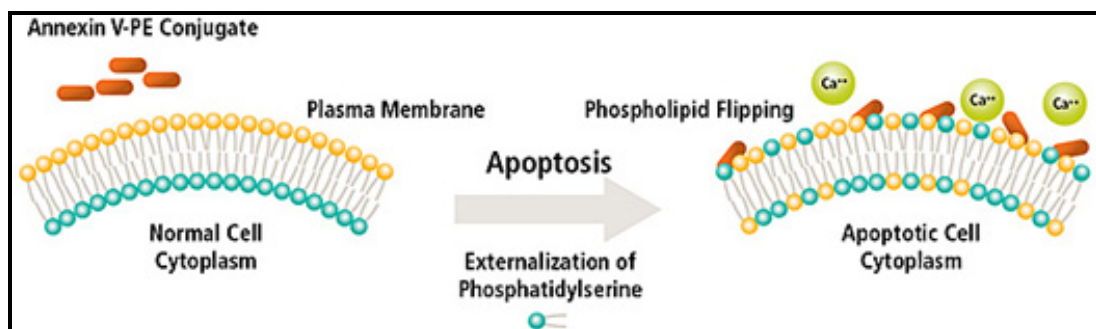


Figure 11: The loss of plasma membrane (Berezney *et al.*, 1991).

Annexin V-FITC/PI is a convenient method used to study these changes. Annexins are a family of structurally related proteins that can bind specifically to cellular membranes. Annexin V is a 35 kDa

Ca²⁺ dependent phospholipid-binding protein that has high affinity for PS and binds to the cells with exposed PS. Staining with Annexin V is typically used in conjunction with a vital dye such as propidium iodide (PI) to allow for the investigation to identify early apoptotic cells. Propidium iodide (PI) is a fluorescent vital dye that stains DNA. It does not cross the plasma membrane of cells that are viable or in the early stages of apoptosis because they maintain plasma membrane integrity. Viable cells with intact membrane exclude PI, whereas membranes of dead and damaged cells are permeable to PI (Berezney *et al.*, 1991).

The Annexin-V FITC/PI kit was designed to detect specific biochemical changes in the cells surface membrane which are the signature events of early apoptosis and analysis can be done by flow cytometry (BD Biosciences). Staining cells with a combination of Annexin-V FITC and PI allow flow cytometry to detect nonapoptotic live cells (Annexin-V positive/PI negative), late apoptotic cells (Annexin-V positive/PI positive) and necrotic cells (Annexin-V negative/PI positive).

2.3.3.2.3 Nuclear changes

Apoptosis is cell-autonomous, and is accompanied by the shrinkage and fragmentation of both cells and their nuclei, loss of microvilli, and extensive degradation of chromosomal DNA. Cleavage of chromosomal DNA into oligonucleosomal size fragments (Fig. 12) is a molecular hallmark of apoptosis. It occurs in response to various apoptotic stimuli in a wide variety of cell types. Molecular characterization of this process identified a specific DNase (CAD, caspase-activated DNase) that cleaves chromosomal DNA in a caspase-dependent manner (Nagata, 2000). The major biochemical marker for apoptotic cell death is the activation of nuclear Ca/mg dependant endonuclease. This enzyme is constitutively present in cell nuclei and catalyzes intranucleosomal DNA fragmentation. Apoptosis has become increasingly important to many areas of biomedical

research and internucleosomal DNA fragmentation is frequently used to show the existence of apoptosis.

The degradation of DNA starts with the generation of high molecular weight fragments of about 300 kb, which are then fragmented to around 50 kb, thereafter more fragmentation occurs and in the end giving rise to about 10-40 kb fragments which in the end give rise to the oligosomal DNA fragments (Babykutty *et al.*, 1995).

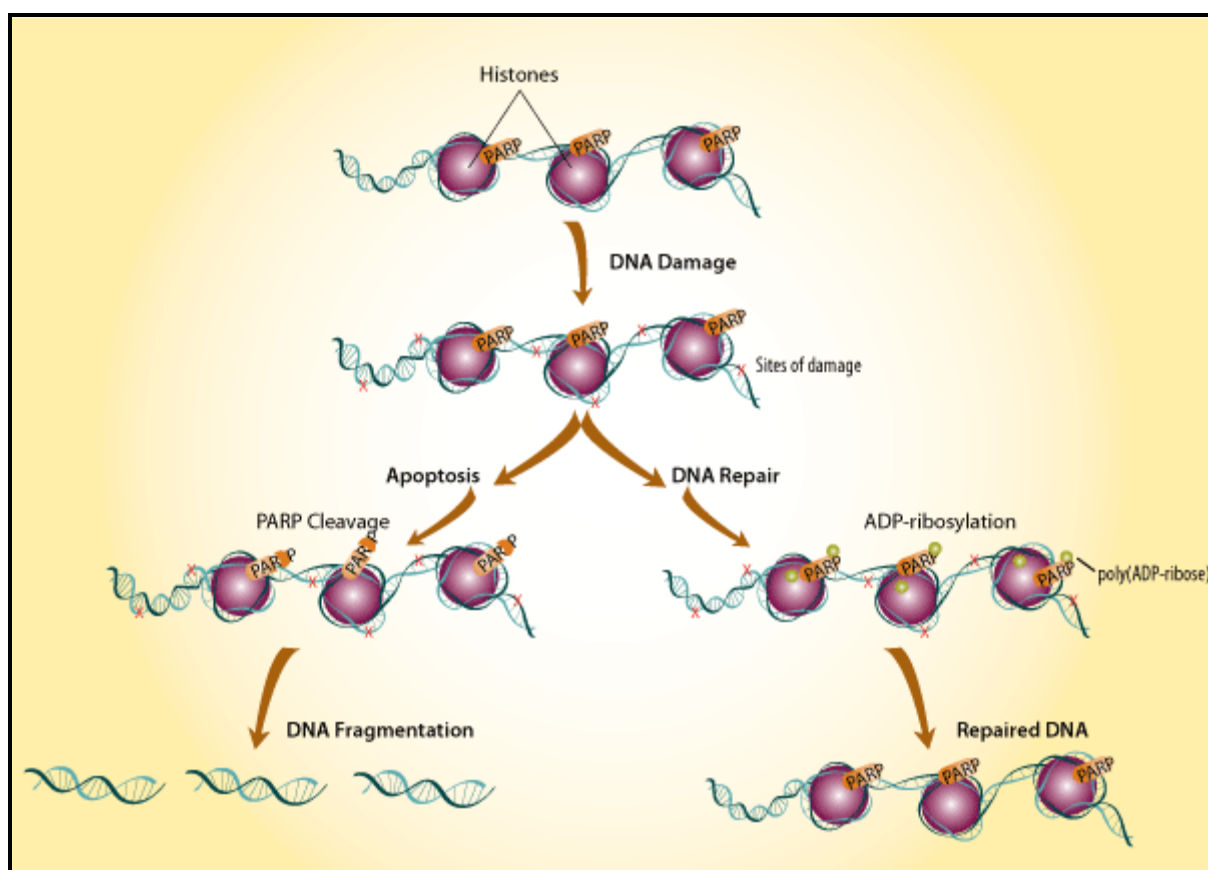


Figure 12: Schematic diagram of DNA organization and fragmentation (Narula *et al.*, 1996).

These oligosomal DNA fragments are double stranded DNA cleavage observed at linker regions between 180-200 base pair fragments. Alternatively DNA extracted from necrotic cells develop as a diffuse smear on the electrophoresis gel. Two frequently used techniques to study nuclear changes are by agarose gel electrophoresis of DNA bands and by flow cytometric analysis using the FITC

BrDU Flow kit. The immunofluorescent staining of incorporated bromodeoxyuridine (BrDU) and flow cytometric analysis provide a high resolution technique to determine the frequency and nature of individual cells that have degraded DNA. In this method, BrDU is incorporated into newly fragmented DNA by cells entering and progressing through the cell cycle. The incorporated BrDU is stained with specific anti-BrDU fluorescent antibodies. The levels of cell-associated BrDU are measured by flow cytometry. Often staining with a dye that binds to total DNA such as 7-amino-actinomycin D (7-AAD) is coupled with immunofluorescent BrDU staining. With this combination, two-coloured flow cytometric analysis permits the enumeration and characterization of cells that are actively fragmented DNA (BrDU incorporation) In terms of DNA fragmentation, when the DNA breaks (fragments) the dye (BrDU) is then allowed to enter the DNA and the total fragmented DNA can be visualized using the flow cytometer (BD Biosciences).

2.3.3.2.4 Cell cycle changes: Caspase 3 activation

Once the apoptotic signals are received by the cell, the final stages that lead to dismantling of the cell are executed by a subfamily of proteases known as caspases. Caspases are highly selective cysteine proteases that have a preference for cleaving proteins after aspartate residues. This specifically ensures that apoptosis is primarily a set of limited proteolytic cleavages rather than a generalized degradative process, resulting in caspase dependant cleavage of hundreds of cellular proteins and ultimately leading to a series of changes characteristic of apoptotic cell death (Chaube and Murphy, 1995).

Caspases can be grouped into two categories: initiator caspases and effector caspases (Fig. 13). Initiator caspases (caspase 2, 8 and 9) are activated as they bind to their appropriate adaptor molecules after which they cleave and thereby activate downstream procaspase and various

proteins. The downstream “effector” caspases (caspase 3, 6 and 7) go on to degrade many cellular substrates that result in characteristic features of apoptotic cell death.

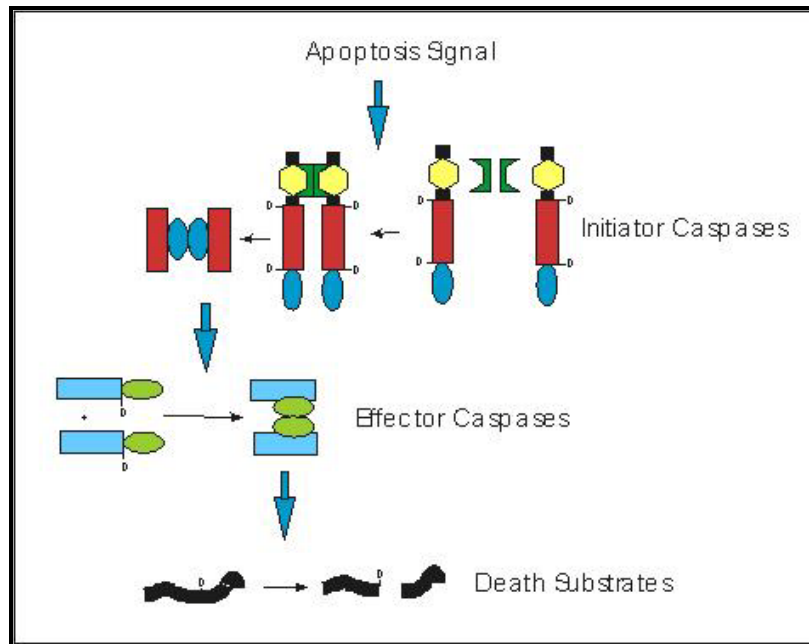


Figure 13: Schematic diagram of the Activation of the Caspase Cascade. Apoptotic signals cause oligomerization of death adaptor proteins, which in turn oligomerize initiator pro-caspases. Oligomerized pro-caspases autoproteolytic activity result in active initiator caspase enzymes. Active initiator caspases then process and activate effector pro-caspases. Active effector caspases cleave various substrates necessary for apoptosis to proceed (Stennicke and Salvesen, 2000).

Caspase 3 is a key protease that is activated during the early stages of apoptosis. Active caspase 3, a marker for cells undergoing apoptosis consist of a heterodimer of 17 and 12 kDa subunits which is derived from the 32 kDa pro-enzyme (Chaube and Murphy, 1995). Active caspase 3 proteolytically cleaves and activates other caspases as well as relevant targets in cytoplasm (Bcl-2) and in the nucleus.

2.4 Plant based cancer chemotherapeutics

In most developed countries conventional cancer treatments include surgery (used for the excision of a tumor), radiation (X rays and gamma rays of a cancerous tumor, causes cancer cell death or apoptosis, preserves the anatomical structures surrounding the tumor and also destroy nonvisible

cancer cells) and chemotherapy (systemic administration of anticancer drugs that travel throughout the body via the blood circulatory system). However, due to poverty and lack of modern medicine 65-80% of the world's population depend exclusively on plants for principle health care (Cordell *et al.*, 1991). The primary objective of any treatment is to destroy the cancer cells and leave the normal tissue intact. Some of the current plant derived anti-cancer compounds are described below.

2.4.1 Camptothecin and topotecan



Figure 14: *Camptotheca acuminata* (Bhattacharjee, 2008).

During a screening program conducted by the National Cancer Institute in late 50s, it was confirmed that a compound from *Camptotheca acuminata* (Fig. 14) had anticancer properties (Pettit *et al.*, 1994). Later, in 1966, a quinoline alkaloid *camptothecin* (CPT) was isolated from bark (and wood), by Wall and other researchers of the Research Triangle Institute (Duke and Ayensu, 1985). After that CPTs became the second most important source of anti-cancer drugs (Kintzios and Barderaki, 2004). Camptothecin (Fig. 15) and its close chemical relatives aminocamptothecin, CPT-11 [irinotecan], DX-8951f, and topotecan are S phase specific anticancer agents that inhibit the activity of the enzyme DNA topoisomerase- I (Cragg, 1998).

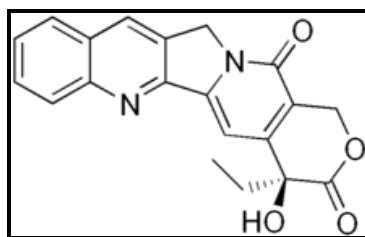


Figure 15: Structure of Camptothecin (Bhattacharjee, 2008).

2.4.2 Paclitaxel and Docetaxel

Paclitaxel (Taxol) illustrated in Figure 16 and docetaxel (Taxotere) are diterpenoids originally extracted from the bark of the *Taxus brevifolius* (Fig. 17). Paclitaxel binds to the microtubules and inhibits their depolymerization (molecular disassembly) into tubulin (Schiff and Horwitz, 1981). This inhibits the cells ability to break down the mitotic spindle during mitosis and consequently cells cannot divide into two daughter cells causing G2-M arrest (Schiff *et al.*, 1979; Schiff and Horwitz, 1981). Paclitaxel is an intravenous drug that is most effective against ovarian carcinomas and advanced breast carcinomas.

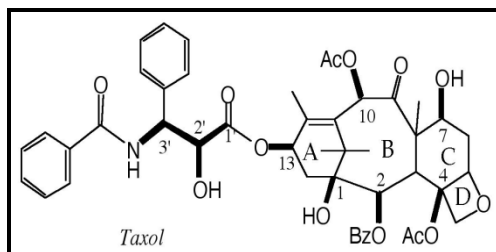


Figure 16: Structure of Taxol (Kintzios and Barderaki, 2004).

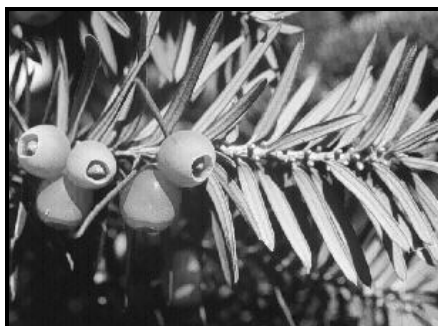


Figure 17: *Taxus brevifolius* (Kintzios and Barderaki, 2004).

Docetaxel also prevents the mitotic spindle from being broken down by stabilizing the microtubule bundles, but clinical trials indicate it is two times more effective than paclitaxel in this process (Vacca *et al.*, 2002). Docetaxel is also an intravenous drug that is being tested on carcinomas of the bladder, cervix, lung, and ovaries.

2.4.3 Colchicine

Colchicine (Fig. 18), a water-soluble alkaloid, was isolated from the *Colchicum autumnale* (Fig 19). It suppresses cell division by inhibiting mitosis by binding to the tubulin molecule, thereby inhibiting its assembly into microtubules and consequently it inhibits the development of spindles as the nuclei are dividing. It is used in veterinary medicine to treat animal cancers. It is also used as an anti-mitotic agent in cancer research involving cell cultures (Vacca *et al.*, 2002).

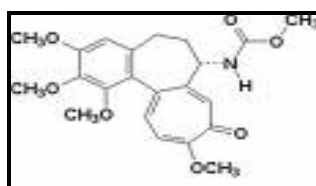


Figure 18: Structure of colchicine (Vacca *et al.*, 2002).



Figure 19: *Colchicum autumnale* (Vacca *et al.*, 2002).

2.4.4 Other plant-based compounds

There are many new plant compound-based drug candidates in active preclinical trials. Inputs from traditional medical knowledge and using modern techniques to speed up the plant compound-based drug discovery have now made us to consider beyond the only 10–15% of plant diversity that have been explored for their pharmaceutical purpose so far (Saklani and Kutty, 2007). Over 60 compounds are in the pipeline, as anticancer drugs alone, from plant sources.

2.4.4.1 Phenolic compounds

Studies have shown that cytotoxic effect of phenols against different tumours is mediated through apoptosis. Tannic acid and caffeic acid induced DNA fragmentation only in HL-60 cells (Hutchings *et al.*, 1996). Gallic acid induced apoptosis in HL-60 RG cells through reactive oxygen species generation, Ca²⁺ influx and activation of calmodulin. Curcumin, a phenolic compound that has been identified as the major pigment in turmeric, induces apoptosis in transformed rodent and human cells in culture. Curcumin mediated the chemopreventive action through inhibition of formation of cyclooxygenase (COX) metabolites, which could provide a mechanism for induction of apoptosis (Kiuchi *et al.*, 1982).

2.4.4.2 Flavonoids

In past etiological studies, intake of certain kinds of polyhydroxy phenols such as flavonoids or lignans in the diet has been correlated with low incidence of colon cancer. A flow cytometric analysis suggested that genistein (a hydroxyisoflavone) induced apoptosis in human promyelocytic HL-60 leukaemic cells. Genistein is also reported to inhibit tyrosine kinase, angiogenesis and cell-cycle progression (Adlercreutz *et al.*, 1982).

2.4.4.3 Alkaloids

Solamargine, an alkaloid purified from the Chinese herb, *Solanum incanum*, has been observed to induce apoptosis in human hepatocyte (Hep-3B) and normal skin fibroblast cells in culture. In addition, the gene expression of TNF receptor I was up-regulated within 30 min of solamargine treatment (Hsu *et al.*, 1996). Since TNF receptor I has been involved in apoptosis, its over-expression may be related to the mechanism of cytotoxicity of solamargine.

2.4.4.4 Polysaccharides

Some polysaccharides found in plants have shown potent activity against various tumours when tested in animals. The mechanism proposed has been the blockage of metastasis by covering galactose-specific binding sites. These activities may have possible therapeutic implications in cancer treatment, from the approach of modulating the immunological functions or by blocking metastasis (Hagmar *et al.*, 1991).

2.4.4.5 Tannins and lignins

Tannins and lignins are present in the natural kingdom in large amounts. Some tannin-related compounds, e.g. tellimagrandin II, induced cytotoxicity and inter-nucleosomal DNA cleavage in HL-60 cells. Their activity increased with polymerization when their concentration was expressed on a molar basis (Su *et al.*, 1995).

2.4.4.6 Terpenoids

Monoterpenes such as perillyl alcohol (POH) derived from lavender, respectively, have been shown to possess chemopreventive properties against mammary, liver and lung carcinogenesis. Reddy *et*

al., (1997), reported that the colon tumours of animals fed with POH exhibited increased apoptosis compared to those fed with control diet. Gross and ultrastructural morphology of POH mediated tumour regression indicated that apoptosis accounted for the marked reduction in the epithelial component.

2.4.4.7 Quinones

Shikonin, a quinone ingredient of *Lithospermum erythrorhizon* induced apoptosis in HL-60 leukaemia cell line. The increase of apoptotic cells by shikonin treatment was preceded by the activation of caspase-3, which plays a central role in apoptotic process (Yoon *et al.*, 1999).

Higher plants continue to retain their historical significance as important sources of novel compounds useful directly as anti-cancer agents, as model compounds for synthetic or semi synthetic structure modifications and optimization, as biochemical or pharmacological probes and as sources of inspiration for generations of synthetic organic medicines. The plant derived compounds described above and many other examples serve to illustrate the continuing value of plant derived secondary metabolites as viable compounds of modern anti-cancer drug development.

CHAPTER 3: METHODOLOGY

Leaves of *E. rhinocerotis* and *C. asiatica* were collected from the Western Cape and Durban areas. The plant material was dried and the compounds from the plant material were extracted with non-polar and polar solvents (hexane, methanol, ethanol and water). These four extracts were used to treat a colon cancer cell line (CaCO-2). Polymorphonuclear leucocytes were used as a control. The effect on the viability of these cells was measured using the XTT assay and the dose and time of exposure was determined. All statistical analysis was done using graphpad prism. The extracts that exhibit toxicity to the CaCO-2 cells and showed minimum damage to the PBMC were used to isolate the bioactive components by bioassay guided fractionation. This procedure is commonly used in the routine isolation of bioactive components from medicinal plants. It involves the separation of the compounds by either preparative TLC or the use of column chromatography to isolate the components of the plant extract by using the appropriate mobile phase. The fractions were collected and together with the most active crude extract, were tested for features of anti-cancer compounds.

Morphological changes induced by the most active crude extract and the separated components was evaluated by observing changes such as membrane blebbing, chromatin condensation and enzymatic cleavage of DNA into oligonucleosomal fragments, and the formation of apoptotic bodies (Hurtwitz *et al.*, 2000). The Annexin V (Bio Vision) and FITC Brdu (BD Sciences) were used to detect and measure possible apoptosis (cell death) as a result of extract treatment. Further features of apoptosis ie. loss of plasma membrane, DNA fragmentation and caspase 3 activity were evaluated by flow cytometric analysis.

Table 2: List of Media and reagents (HPLC grade)

REAGENT	SUPPLIER
Acridine orange	Sigma-Aldrich
Agarose	Merck
DMSO (Dimethyl sulphoxide)	Sigma
DNA ladder detection kit	Biovision
DPPH	Sigma-Aldrich
Ethanol	Merck
Ethidium Bromide	Sigma-Aldrich
Ethyl acetate	Merck
Fetal calf serum (FCS)	Highveld Biological
Ficoll Histopaque	Sigma-Aldrich
FITC BrDU Flow kit	BD BioSciences
FITC Annexin V	BD BioSciences
Fungizone	Sigma-Aldrich
Hexane	Merck
L-glutamine	Highveld Biological
Methanol	Merck
Minimum essential media (MEM)	Highveld Biological
PE caspase 3 apoptosis kit	BD BioSciences
Penicillin/streptomycin	Highveld Biological
Phosphate buffered saline (PBS)	Highveld Biological
Rutin	Sigma-Aldrich
Silica gel 60 F254	Merck
Trypan Blue	Biowhittaker
Trypsin	Highveld Biological
XTT	Sigma-Aldrich

3.1 Extraction and preparation of plant material

Fresh specimens (± 1 kg fresh weight) of *E. rhinocerotis* and *C. asiatica* (Permit no. 9041) were collected from the Western Cape, South Africa in 2009 by Prof Sewram. The taxonomic identity of the plants were authenticated by Prof H. Baijnath (University of KwaZulu Natal). The voucher specimens (1056) were deposited as Baijnath Sn. Ward Herbarium, University of KZN, South Africa.

E. rhinocerotis and *C. asiatica* were de-leafed and washed repeatedly with distilled water. The damaged leaves were removed and dried (25°C; 48 h) in an oven (Memmert B.Owen Jones limited, South Africa). The dried samples were milled in an industrial grinder (Retsch GmbH, West Germany), then stored in labelled schott bottles in cool dark place until further use.

Thirty grams of the dried plant material was continuously stirred in either double distilled water (200 ml); 80% methanol (v/v); 70% ethanol (v/v); or hexane. This was stirred for 24 h on a rotary shaker then the samples were centrifuged (Centrifuge 5810R V5.8) (8000 rpm; 10 min). The supernatants were filtered using Whatman No. 1 filter paper. The aqueous extract was stored frozen (-70°C; 24 h) and then freeze dried (48 h) in a Virtis Benchtop Freeze Dryer. The methanol and ethanol was evaporated from the samples with a Rotary evaporator (Buchi) in a vacuum, which was connected to a Buchi 461 water bath set at a temperature of 50°C and the remaining slurry was freeze dried. Hexane was removed from the plant material by evaporation under room temperature over night. The freeze dried plant material was diluted in Dimethyl sulphoxide (DMSO). The yield obtained from the crude dried leaves of *E. rhinocerotis* and *C. asiatica* was calculated by measuring the weight of the freeze dried powder and using the equation:

$$\% \text{ Yield} = \text{weight of freeze dried powder} / \text{weight of dried crude plant material}$$

3.2 Anti-oxidant activity

The anti-oxidative properties of the crude extracts was tested using the DPPH (1,1-diphenyl-2-picrylhydrazyl radical) photometric assay as described by Choi *et al.*, 2002.

Preparation of plant material: The freeze dried material obtained from the aqueous, ethanol, methanol and hexane extracts (1mg/ml) was diluted to final concentrations of 500 µg/ml, 250 µg/ml, 100 µg/ml, 50 µg/ml, 10 µg/ml and 1 µg/ml in ethanol.

Preparation of Standard: Rutin found in the buckwheat plant *Fagopyrum esculentum*, was used as a comparative standard. DPPH (1 ml; 0.3 mM) in ethanol, was added to the plant sample (2.5 ml) and was allowed to react (30 min; RT). Ethanol (1.0 ml) plus was used as a blank, while DPPH solution in ethanol (2.5 ml) was used as a negative control. The positive control was DPPH solution

(1 ml) plus Rutin (2.5 ml; 1mM). Each test was carried out in triplicate and results are expressed as the mean. The absorbance values were measured in a Varian Cary 1E UV-visible spectrophotometer at 518 nm and the average absorbance values was converted into the percentage antioxidant activity, using the following equation:

$$\text{Scavenging capacity \%} = 100 - \frac{(\text{Abs of sample} - \text{Abs of blank}) \times 100}{\text{Abs of negative}}$$

3.3 Cytotoxic activity

CaCO-2 (Colon cancer cells) were contributed by Prof Sewram from the Oncology Research Unit, MRC. The cells were cultured as described by Freshney (1987). All cell culture procedures were carried out in a laminar flow cabinet containing a UV light, (Scientific Engineering INC). The unit was sterilized with ethanol (70%) before each use.

CaCO-2: The human colonic CaCO-2 cell line has been extensively used over the last twenty years as a model of the intestinal barrier. The parental cell originally obtained from a human colon adenocarcinoma, undergoes in culture a process of spontaneous differentiation that leads to the formation of a monolayer of cells, expressing several morphological and functional characteristics of the mature enterocyte. These cells grow in monolayers, show a cylindrical polarized morphology, with microvilli on the apical side, tight junctions between adjacent cells and express small intestinal hydrolase enzyme activity (Pinto *et al.*, 1983).

PBMC: A peripheral blood mononuclear cell (PBMC) is any blood cell having a round nucleus. For example: a lymphocyte, a monocyte or a macrophage. These blood cells are a critical component in the immune system to fight infection and adapt to intruders. These cells are often extracted from whole blood using ficoll, a hydrophilic polysaccharide that separates layers of blood, with monocytes and lymphocytes forming a buffy coat under a layer of plasma. This buffy coat contains

the PBMCs. PBMCs were used as control cells for this particular section of this study because it had to be assured that the plant extracts were not toxic to normal cells.

3.3.1 Culturing of CaCO-2 cells

3.3.1.1 Thawing of cells

Cell maintenance was performed according to protocols obtained from Freshney (1987). The vials of frozen cells were removed from storage (-70°C) and immediately thawed in a water bath (37°C). The contents of the vial were then transferred into DMEM (15 ml). The tube was then centrifuged (2min; 5000 rpm). The supernatant was discarded and the pellet was resuspended in complete culture medium (5 ml) (CCM) and transferred to a culture flask (25cm²) for incubation.

After two days the cells were transferred to tissue culture flasks (75cm²) (Greiner, Germany) and 10 % CCM (15ml) which comprised of Dulbecco's modified Eagle's medium (DMEM), containing foetal calf serum (10 %) and supplemented with antibiotics (penicillin: 10 000 U/ml, streptomycin sulphate: 10 000 U/ml and 1 mM sodium pyruvate) was added to each flask. Cells were incubated in a humidified incubator under CO₂ (5 %; 37°C). The culture flasks were examined for colour changes and turbidity of the media on a daily basis. This determined the frequency of media changes. The culture was examined under an inverted microscope (Nikon) for cell growth.

3.3.1.2 Trypsinisation of cells

The cells were trypsinized when the culture was confluent (80 %). The medium was decanted and the cells were washed with pre-warmed phosphate buffered saline (PBS) (pH 7.2). Trypsin (1 ml) was added to the flask and this was left to incubate (37°C; 2-3 min). The sides of the flasks were

tapped gently to detach the cells from the surface of the flasks. The cells were resuspended in the medium (20 ml) and was incubated for two days.

3.3.1.3 Freezing of cells.

The cells were pelleted and washed twice with pre-warmed PBS (pH 7.2), resuspended in FCS (0.5 ml) and cooled on ice. Dimethylsulphoxide (DMSO) (20 %) in MEM (V/V 1:4) was prepared as the cryoprotecting agent and also placed on ice. Equal aliquots (0.5 ml) of the cell suspension and the cryoprotective agent was added to a cryotube (Corning, South Africa). The tubes were transferred to the thermos flask and kept overnight (-20°C). The cells were subsequently transferred to a bio-freezer (-70°C) and stored until required.

3.3.1.4 Seeding of cells

Once cells were thawed and incubated in a tissue culture flask (75cm^2) cells were incubated (37°C ; 48 h) to grow to confluency, cells were then trypsinized, centrifuged to remove supernatant and resuspended in of CCM media (20 ml). The cells were enumerated using a haemocytometer and seeded in microtitre plates (24, 48 or 96 well).

3.3.1.5 Cell Enumeration

The cells were enumerated using a haemocytometer. The cell suspension was mixed with equal aliquot of trypan blue (0.2 %) [Biowhittaker, Wakersville USA] (V/V 1:1). This mixture was drawn across the grid by capillary action. The volume of cell suspension that occupied one primary square is 0.1 mm^3 ($1.0\text{ mm}^2 \times 0.1\text{ mm} / 1.0 \times 10^4\text{ ml}$). Only the viable (translucent) cells that lay within or that touched, the left or top boundary were counted. The number of viable cells per ml in the original sample was calculated as follows:

$$\text{Cells/ml} = \text{Average number of cells per primary square} \times 10^4 \text{ dilution factor}$$

3.3.2 Isolation of PBMC

Reagent Buffy coats were donated by the South African Blood Bank (East Coast Region, Pinetown). The isolation of the PBMC was conducted according to the SOP/HPP/01 with minor modifications. This isolation was undertaken under sterile conditions within a class II biological safety cabinet (Lab Aire, South Africa) using standard procedures as per SOP/HPP/01. All reagents were swabbed with ethanol (70 %). An autoclavable plastic bucket was filled with Virkon (2 %) (The Scientific Group, South Africa) solution, and was placed in the hood for bio-hazardous waste.

Buffy coats were carefully mixed to equal parts of pre-warmed PBS with Pen/Strep (1 %) and was carefully layered onto equal volume of Ficoll Histopaque (Sigma-Aldrich, South Africa). Blood + PBS + Ficoll were added in approximately a 1: 1: 1 ratio. Care was taken not to disturb the tubes as mixing would of resulted in a delay of sedimentation of the different cell types.

The tubes were thereafter centrifuged using a bench top centrifuge (1500 rpm; 30 min; RT). After centrifugation, four distinct layers were obtained and the upper plasma layer aspirated and discarded. The mononuclear cell layer, PBMC, which contained the lymphocytes, was carefully removed using a sterile pasteur pipette (3 ml) and transferred into new sterile tube. In the event of red blood cells being present, several drops of double distilled water was added to lyse the red blood cells. Then PBS (pH 7.2) was added dropwise immediately to prevent all the cells from lysing. These tubes were thereafter washed thrice in PBS (45 ml) with pen/strep (5 ml). by centrifugation (1500 rpm; 10 min; RT). After the third wash, all the PBS was removed and the pellet resuspended in RPMI- 1640.

3.3.2.1 Culture and maintenance of PBMC (Freshney, 1987)

The PBMC's were grown aseptically in tissue culture flasks (T 75 cm²) using filter sterilized (0.22 µm), medium (R 10) which comprised of RPMI (435 ml), heat inactivated filtered FCS (50 ml), of L-glutamine (5 ml) and of pen/strep (5 ml). The cultures were incubated (37°C) in a humidified incubator (5 % CO₂). The culture flasks were then examined for colour changes and turbidity of the media on a daily basis. This determined the frequency of media changes. The culture was examined under a microscope (Nikon) for cell growth. The cells were then enumerated using a haemocytometer. Refer to section 3.3.1.5.

3.3.3 Effect of the plants extracts

The XTT cytotoxicity assay was conducted according to Mosmann, (1983) with minor modifications. The assay was carried out in flat-bottomed microtitre plates (96 well) (Cellstar, Greiner, Germany). Cells (50 µl; $\pm 1.2 \times 10^3$) were added into each well, plant extract (50 µl) in a twofold dilution factor (500 µg/ml - 3,17 µg/ml) and media (50 µl) were added to the respective wells. In the control wells cells (50 µl) and media (50 µl) were added respectively. The plates were incubated (37°C) in a humidified incubator (5 % CO₂ atmosphere) for 1 day, 3 days and 5 days. Thereafter XTT (20 µl) reagent were added, the plates were then further incubated (4 h; 37°C) in a humidified incubator (5 % CO₂ atmosphere). The absorbance was read (578 nm) on an ELISA plate reader (Digital Analogue Systems, Italy). All error bars shown represents the standard deviation. Taxol in the same concentration range as the plant extracts, was used as a positive control. The percentage viability was determined using the formula below:

$$\% \text{ Cell Viability} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of untreated cells}} \times 100$$

3.4 Fractionation of compounds

The extracts obtained from four different extraction solvents (aqueous, ethanol, methanol and hexane) were subjected to cell proliferation assay according to the procedure described in Section 3.3. The extract which showed significant inhibitory effect on the CaCO-2 cells and a proliferative effect on PBMC was further subjected to fractionation using TLC.

3.4.1 Determination of optimal solvent system

Before performing TLC, the solvent system required to separate the compounds was determined. TLC aluminium sheets (20 x 20 cm; silica gel 60 F254) were cut into rectangles (10 cm x 1.5 cm). A light pencil line was drawn 1 cm from the bottom and top edge of the chromatographic plate. The sample was spotted on this line (<0.5 mm in diameter) and positioned in a developing chamber. The solvent was allowed to travel up the plate via capillary action until the pencil line sketched across the top border was reached. The plates were then examined under ultraviolet light (312 nm, Camag Universal UV lamp TL-600). Using the above method, a number of different solvent systems comprising; hexane, methanol, ethyl acetate and water in different ratios were evaluated for optimal separation of compounds.

3.4.2 TLC fractionation

The mobile phase used for *C. asiatica* was ethyl acetate: methanol: water (100 : 25 : 15) and for *E. rhinocerotis* was hexane: ethyl acetate (6 : 4). The crude extract of each plant was applied as a line rather 1 cm from the bottom of the plate. The plate was immersed into the development chamber containing the mobile phase and allowed to develop in the saturated chromatography chamber. Thereafter, the plate was removed from the chamber and allowed to air dry. Each band was marked with pencil. A scalpel blade was used to scrape off the bands from the TLC plate. The silica of

each band was suspended in the mobile phase (10 ml) of each of the plants in centrifuge tubes (50 ml). The tubes were shaken to dissolve the isolated compounds and then left to stand for 2 days for the silica to settle to the bottom. A pipette was used to draw out the compounds which were filtered into glass bottles covered in foil. The bottles were placed under the laminar flow to evaporate the mobile phases. The resultant powdered form of the fractions were stored at room temperature until. The fractions obtained through chromatography were subjected to cytotoxic assay on CaCO-2 cells and PBMC's, following that, the most active sample was selected for evaluation of apoptosis. Subsequently, the fraction which showed specific activity towards CaCO-2 was evaluated for its apoptotic effect towards the cell line.

3.5 Apoptotic activity of *C. asiatica* and *E. rhinocerotis*

The active extract and active fractions of *C. asiatica* and *E. rhinocerotis* were evaluated for their ability to induce apoptosis in CaCO-2 cell lines. In this regard, the morphological changes (using acridine orange and ethidium bromide), membrane changes (using Annexin V), nuclear changes (using apoptotic extraction kit and confirmed with BrDU) and cell cycle changes (Caspase 3 activity), were assessed as described below.

3.5.1 Morphological changes

A stock solution (100 X) was made up by adding ethidium bromide (50 mg) and acridine orange (15 mg). This was dissolved in ethanol (1 ml; 95 %) and distilled water (49 ml). Thereafter, this was mixed well and divided into aliquots (1 ml). The working stock solution (1 X) was made up of aliquot (1 ml) of the 100 X stock solution and PBS (99 ml) was diluted into it.

The cells were seeded in plates (24 well) and left to adhere overnight. They were then treated with the methanolic extracts of *C. asiatica* (125 µg/ml, 250 µg/ml and 500 µg/ml) and *E. rhinocerotis* (125 µg/ml, 250 µg/ml and 500 µg/ml). They were also treated with the methanolic extracts of *C. asiatica*-band 2 (250 µg/ml) and *E. rhinocerotis*-band 1 (125 µg/ml).

At day three, the cells were trypsinized and the cell suspension was adjusted to an estimate 100 000 cells/ml in Minimal Essential Medium. Equal volumes (25 µl) of cell suspension and acridine orange-ethidium bromide solution were mixed gently in a tube. Small aliquots were placed underneath the cover slip on a slide. The cells were then observed under the fluorescence microscope set up to excite for fluorescein (i.e., with a 495 nm primary filter and a 515 nm secondary filter). Cells were viewed under the objective (10 X) with viable normal cells staining bright green nuclei and intact structure where the viable apoptotic cells were stained green with highly condensed or fragmented nuclei. Non-viable normal cells were identified as those with chromatin stained bright orange and with an organized structure whilst non-viable with apoptotic nuclei were observed to have highly condensed and fragmented chromatin.

3.5.2 Membrane changes

The Annexin V-PE Apoptosis detection kit (BD Biosciences) was used as per manufacturers protocol. The CaCO-2 cells were seeded in plates (24 well) and left to adhere overnight. They were then treated with the methanolic extracts of *C. asiatica* (125 µg/ml, 250 µg/ml and 500 µg/ml) and *E. rhinocerotis* (125 µg/ml, 250 µg/ml and 500 µg/ml). They were also treated with the methanolic extracts of *C. asiatica*-band 2 (250 µg/ml) and *E. rhinocerotis*-band 1 (125 µg/ml). At day three, the cells were trypsinized, washed twice with PBS and then resuspended in binding buffer (1 X at 50 000 cells/ml). FITC Annexin V (5 µl) and propidium iodide (PI) (5 µl) were added, vortexed and

incubated (15 min) in the absence of light. Thereafter, binding buffer (400 µl) was added to each tube and the results analysed by flow cytometry (BD FACS-flow cytometer).

3.5.3 Nuclear changes

The cells were seeded in 24 well plates and left to adhere overnight. They were then treated with the methanolic extracts of *C. asiatica* (125 µg/ml, 250 µg/ml and 500 µg/ml) and *E. rhinocerotis* (125 µg/ml, 250 µg/ml and 500 µg/ml). They were also treated with the methanolic extracts of *C. asiatica*-band 2 (250 µg/ml) and *E. rhinocerotis*-band 1 (125 µg/ml). At day three, the cells were trypsinized, and pelleted. The Biovisions quick apoptosis ladder detection kit was used as per manufacturer's protocol. The cells were then washed with PBS and the supernatant was removed. Thereafter, the cells were lysed with lysis buffer (35 µl) and enzyme A solution (5 µl) was added to each sample and was incubated (37 °C; 10 min). Thereafter, enzyme B solution (5 µl) was added into each sample and incubated (30 min; 50 °C). Ammonium acetate (5 µl) solution and isopropanol (50 µl) was added to the samples and was further incubated (10 min; -20°C). The DNA was then precipitated, washed with ethanol (70 %) and then dissolved in suspension buffer.

The BioVision's Quick Apoptotic DNA Ladder Detection Kit was used to extract the DNA from the treated cells and gel electrophoresis was used to see these changes. To the DNA sample (2µl) that was prepared, loading buffer (2 µl) was added. Samples were then loaded alongside the molecular weight marker on a agarose gel (1.5%) containing ethidium bromide (EtBr) (1µl). The samples were then electrophoresed (10V/cm; 1x TAE buffer). DNA was visualized with a UVP transilluminator.

Fragmentation was further confirmed using the FITC BrDU Flow kit. The FITC BrDU Flow kit (BD Sciences) was used as per manufacturers protocol. Following incubation with the extracts; the

cells were trypsinized then labelled with BrDU and stained with staining buffer (50 µl). Thereafter the cells were fixed and permeabilized using BD cytofix/cytoperm buffer (60 µl). The cells were then incubated and following incubation cells were treated with DNase to expose BrDU epitopes, after which immunofluorescent staining was carried out with fluorochrome conjugated anti-BrDU.

3.5.4 Cell cycle changes : Caspase-3 activation

The PE caspase 3 apoptosis kit (BD Pharmagen) was used as per manufacturers protocol. The cells were seeded in plates (24 well) and left to adhere overnight. They were subsequently treated with the methanolic extracts of *C. asiatica* (125 µg/ml, 250 µg/ml and 500 µg/ml) and *E. rhinocerotis* (125 µg/ml, 250 µg/ml and 500 µg/ml). As well as with the methanolic extracts of *C. asiatica*-band 2 (250 µg/ml) and *E. rhinocerotis*-band 1 (125 µg/ml). At day three, the cells were trypsinized, washed twice with PBS (1 X), resuspended in BD Cytofix/Cytoperm solution (50 µl) and then incubated on ice (20 min). The cells were then pelleted and the Cytofix/Cytoperm solution was discarded. Thereafter the cells were washed twice with BD perm/wash buffer (100 µl) and resuspended in this buffer together with the antibody (10 µl) and incubated (30 min; RT). The cells were finally washed with perm wash buffer (100 µl) and resuspended in DB perm/wash buffer (50 µl) ready to be analyzed by flow cytometry.

CHAPTER 4: RESULTS

4.1 Extraction of crude extracts from *C. asiatica* and *E. rhinocerotis*

The yield of extract from dried plant material is shown in Table 3. The highest amount obtained was from the aqueous extract followed by the hexane extract for both *C. asiatica* and *E. rhinocerotis*. The methanolic and ethanolic extract were in the range of 5-6 %.

Table 3: Yields of material from crude extract of *C. asiatica* and *E. rhinocerotis*

Plant species	Date of collection	Area	Dry weight (g)	Plant part Extracted	Yield (%)			
					Aqueous	Methanolic	Ethanolic	Hexane
<i>C. asiatica</i>	May 2009	Pinetown (KwaZulu Natal)	10	Leaves	8.3	5.7	5.5	5.8
<i>E. rhinocerotis</i>	September 2009	Western Cape	10	Leaves and young stems	7.2	5.1	5.2	5.5

4.2 Anti-oxidant activity of extracts of *C. asiatica* and *E. rhinocerotis*

The aqueous, ethanolic, methanolic and hexane extracts of both *C. asiatica* and *E. rhinocerotis* were tested for their anti-oxidant activity. This was carried out to determine which extract of the two plants had the highest free radical scavenging capacity. Their values indicated large variation in anti-oxidant activity. The flavanoid, Rutin, was used as the positive control.

C. asiatica showed the same activity as Rutin for the ethanolic extract and the least activity was found with the aqueous extract. The same trend was found for *E. rhinocerotis*, it showed a 100 % activity with the ethanolic extract and the lowest activity with the aqueous extract. The results for all the extracts are illustrated in Figure 20.

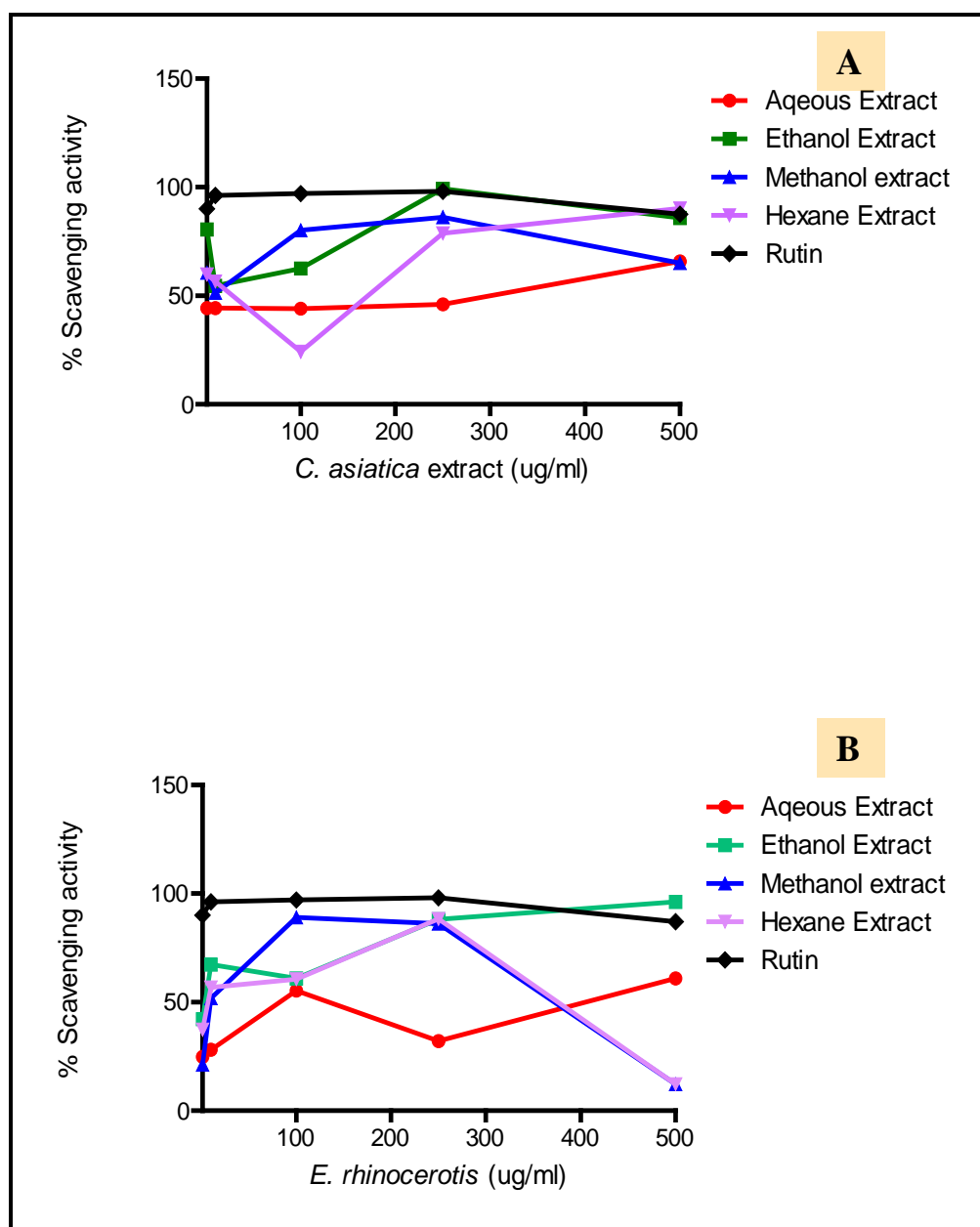


Figure 20: Antioxidant activity of the extracts of (A) *C. asiatica* and (B) *E. rhinocerotis*.

4.3 Viability of cells exposed to extracts

The main aim of the cell viability assay was to determine which of the extracts of *C. asiatica* and *E. rhinocerotis* produced the most toxic effect (i.e. rendered the cells least viable) on the CaCO-2 cell line and was not toxic to PBMC. The effect was observed on day 1, 3 and 5 at a range of concentrations (500 $\mu\text{g/ml}$ - 3,17 $\mu\text{g/ml}$) for the aqueous, ethanolic, methanolic and hexane extracts. These results were also compared to the effect due to taxol.

4.3.1 *C. asiatica*

At day one the aqueous, methanolic, ethanolic and hexane extracts of *C. asiatica* and taxol showed an increase in cell viability of the CaCO-2 cells up to a concentration of 100 µg/ml and thereafter a decrease in cell viability (Fig. 21A). At 250 µg/ml 80 % of the cells were non-viable as observed with the methanolic extract (Fig. 21A).

The PBMC on day one, showed a significant decrease in cell viability from 61 µg/ml onwards. The highest cell viability is noted with the ethanolic extract of *C. asiatica* at a concentration of 61 µg/ml (150 %). There was an average cell viability of the ethanolic extract from 100-500 µg/ml. This concentration produced an average of 100 % cell viability (Fig. 21B).

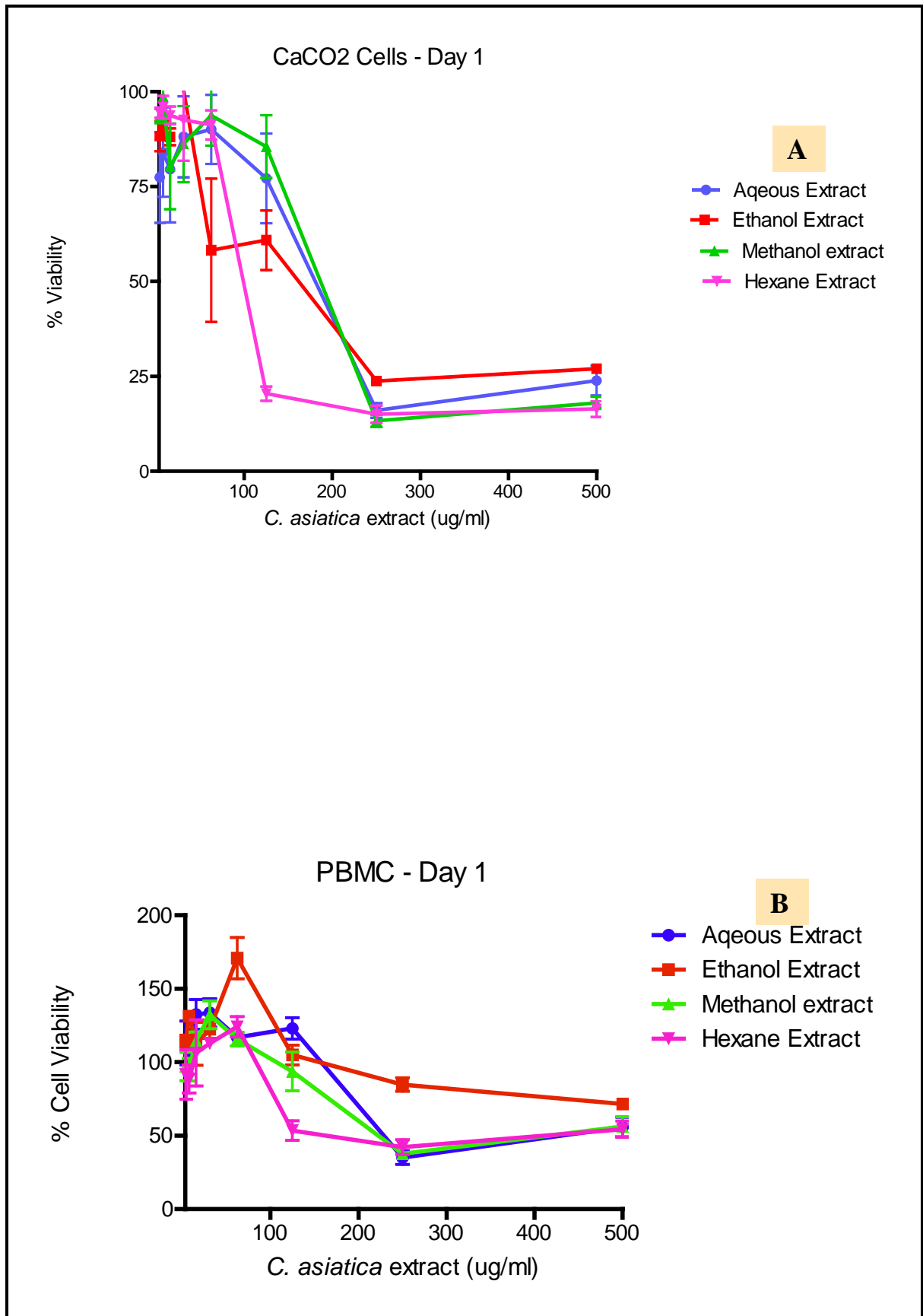
CaCO-2 cells on day three showed a noticeable drop in percentage cell viability from 125 µg/ml. This was noticed in all the treatments of *C. asiatica*. The most effective cytotoxic extract was noticed with the hexane, with a trend similar to taxol (Fig 23). The hexane extract produced an average 25% cell viability between 100-500 µg/ml (Fig. 21C).

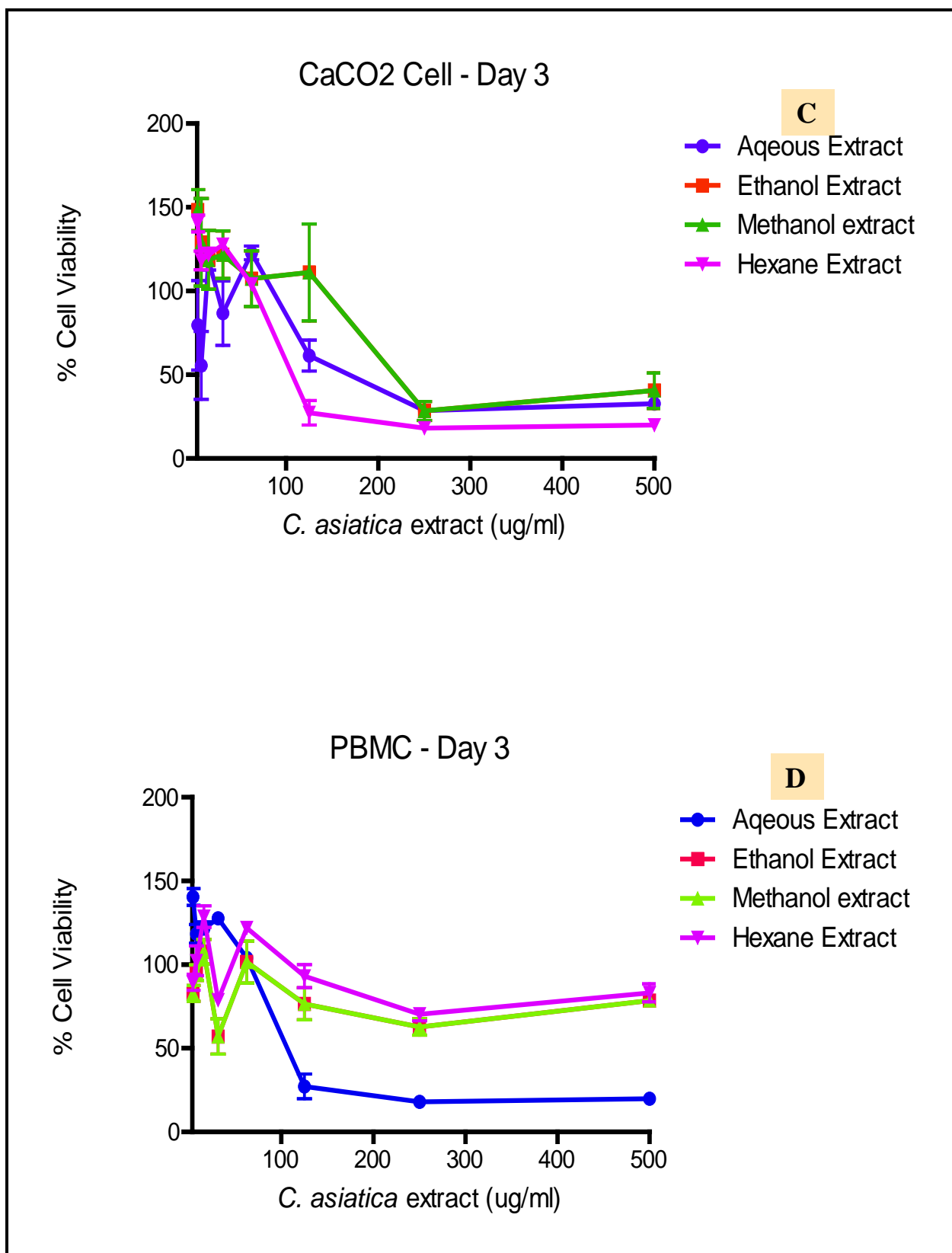
PBMC at day showed a significant increase in cell viability in the methanolic and hexane extract from 3.17-61 µg/ml, thereafter a decrease in percentage cell viability was noticed (Fig. 21D). The highest cell viability was noted with the hexane extract that produced an average 80 % cell viability at a concentration of 250 µg/ml -500 µg/ml.

On day five there was a decrease in percentage cell viability in CACO-2 cells. This trend is seen from concentrations between 150 µg/ml to 500 µg/ml. The most significant decrease in cell viability was in the hexane extract of *C. asiatica* from a concentration between 250-500 µg/ml. At

500 µg/ml, the cell viability is 30 %. This is a 5 % decrease in cell viability in contrast with taxol (Fig 23), which at 500 µg/ml produces a 35 % cell viability (Fig. 21E).

The PBMC treated with the different extracts of *C. asiatica* on day five, the highest cell viability was in the methanolic extract of *C. asiatica* at a concentration of 3.17 µg/ml (≥ 150 %). There was an average cell viability of the hexane extract from 250-500 µg/ml. This concentration produced an average of 80 % cell viability (Fig. 21F).





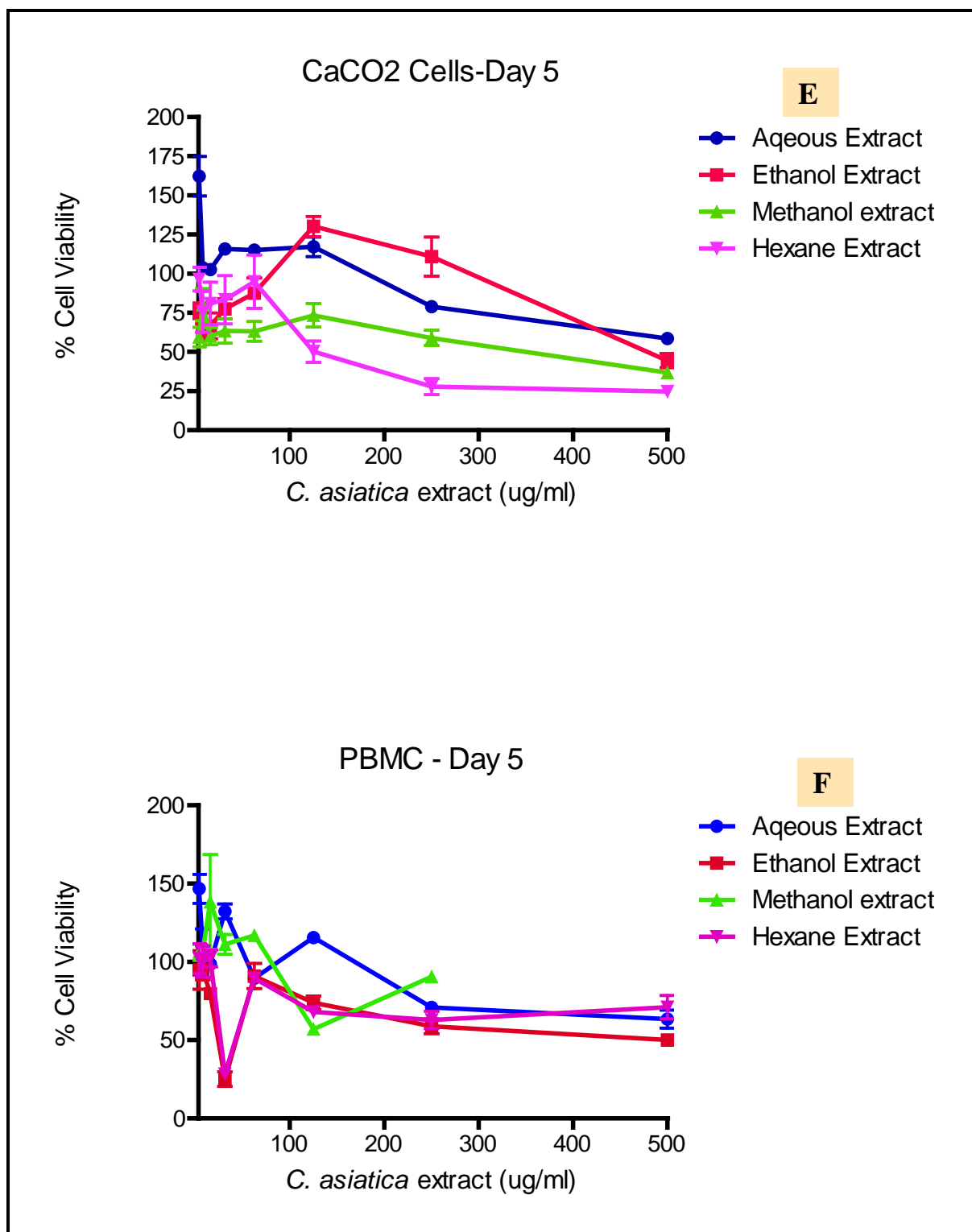


Figure 21: Viability of CaCO-2 cells and PBMC treated with the different extracts of *C. asiatica* at day 1 (Fig. 21A and 21B), day 3 (Fig. 21C and 21D) and Day 5 (Fig. 21E and 21F).

4.3.2 *E. rhinocerotis*

On day one, the aqueous, methanolic, ethanolic and hexane extracts of *E. rhinocerotus* showed a decrease in the cell viability at all the concentrations tested ie., 3.17 µg/ml to 500 µg/ml. The highest decrease in viability of CaCO-2 cells was seen with hexane extract as 80% cells died (Fig. 22A).

PBMC treated with the extracts of *E. rhinocerotis* at day one showed an increase in cell viability with methanol, ethanol and hexane extracts at concentrations between 3.17 µg/ml to 61 µg/ml. The greatest cell viability produced from PBMC at day one was the ethanolic extract at 90 µg/ml, which produced a 170 % cell viability (Fig. 22B). The aqueous extract caused an increase in viability up to 125 µg/ml but declined thereafter.

At day three the CaCO-2 cell showed a decrease in percentage cell viability from a concentration of 61 µg/ml. But the most ardent effect was noticed with the cell treated with the hexane extract of *E. rhinocerotis* (Fig. 22C). This extract produced an average of 25 % cell viability at concentrations from 100 µg/ml to 500 µg/ml. A similar pattern was observed with the methanolic extract, producing an average of 20 % cell viability (250-500 µg/ml). This is similar to taxol (Fig 23), which produced an average of 10 % cell viability from 250-500 µg/ml.

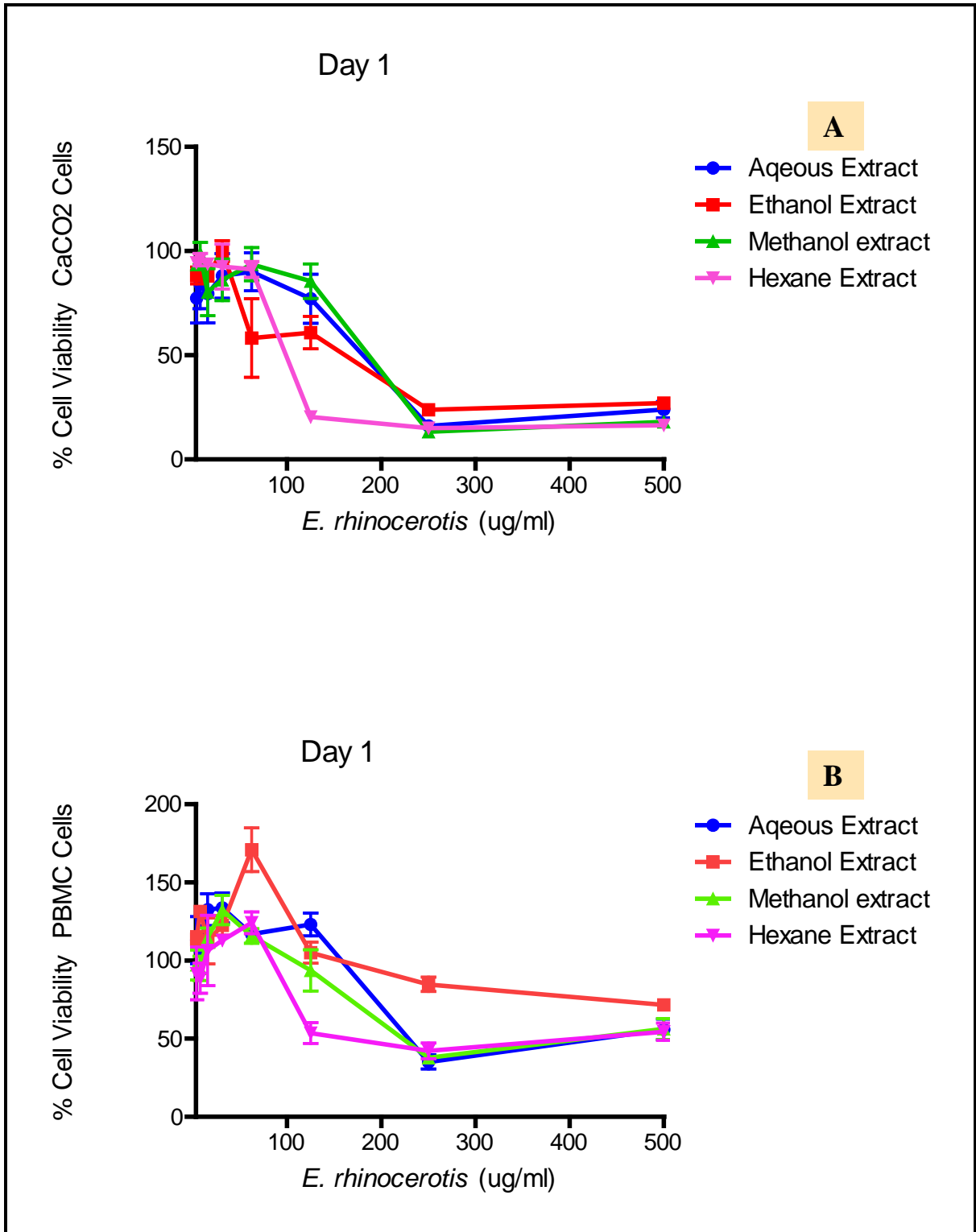
The PBMC on day three showed that the methanolic extract at 61 µg/ml produced a 125% cell viability. The ethanolic extract from a concentration of 125 µg/ml-500 µg/ml (Fig. 22D), produced an average of 100 % viability.

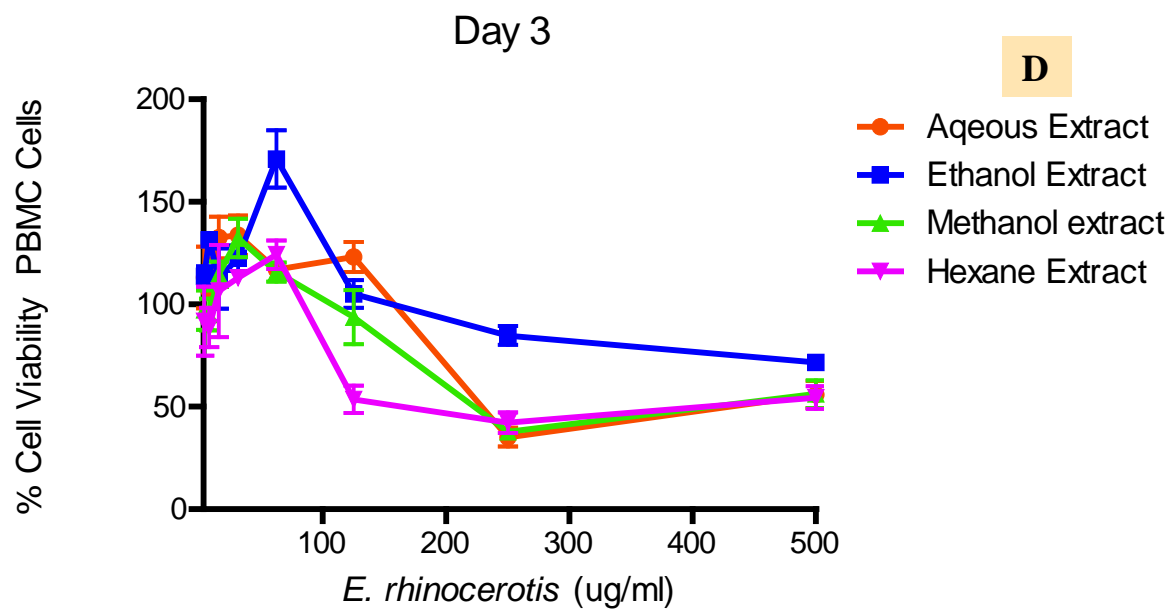
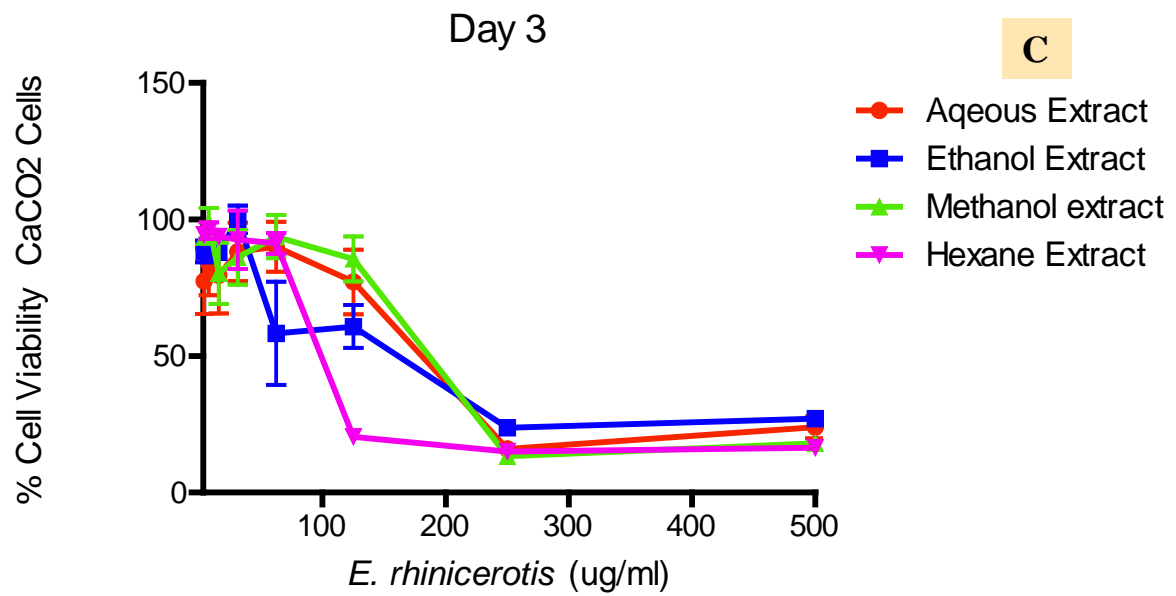
CaCO-2 cells on day five produced an increase in cell viability from 3.17 - 125 µg/ml. Thereafter the percentage cell viability began to decrease. The greatest cytotoxicity was observed with the

hexane extract of *E. rhinocerotis*. The hexane extract produced an average of 20 % cell death from 250-500 µg/ml (Fig. 22E). The methanolic extract from 250-500 µg/ml produced an average 60 % cell viability.

On day five the PBMC showed a significant increase in cell viability which was only noted in the aqueous and methanolic extracts of *E. rhinocerotis* at concentrations from 3.17-60 µg/ml (Fig. 22F). The greatest percentage cell viability was produced at with methanolic extract of *E. rhinocerotis* at a concentration of 3,17 µg/ml. This produced 175 % cell viability. Also at concentrations from 250-500 µg/ml, the methanolic extract produces an average of 80 % cell viability.

A comparison of cell viability of CaCO-2 and PBMC indicate that all the extracts are toxic to CaCO-2 cells and cause proliferation / no change in number of PBMC. To facilitate further experimentation CaCO-2 cells were exposed for 3 days to the methanolic extract (125 µg/ml, 250 µg/ml and 500 µg/ml) of *C. asiatica* and *E. rhinocerotis*.





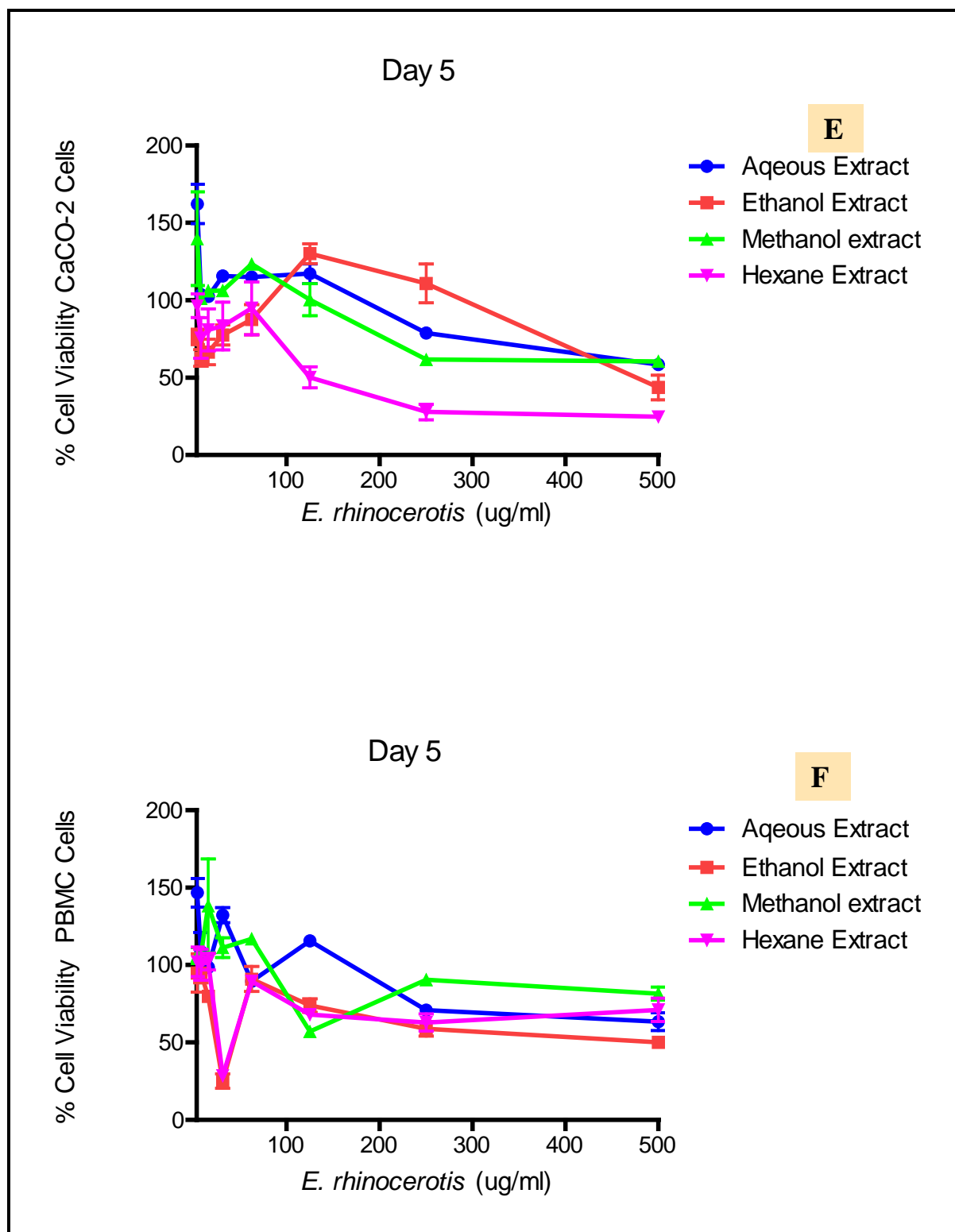


Figure 22: Effect of *E. rhinocerotis* extract, concentration and incubation period on cell viability.

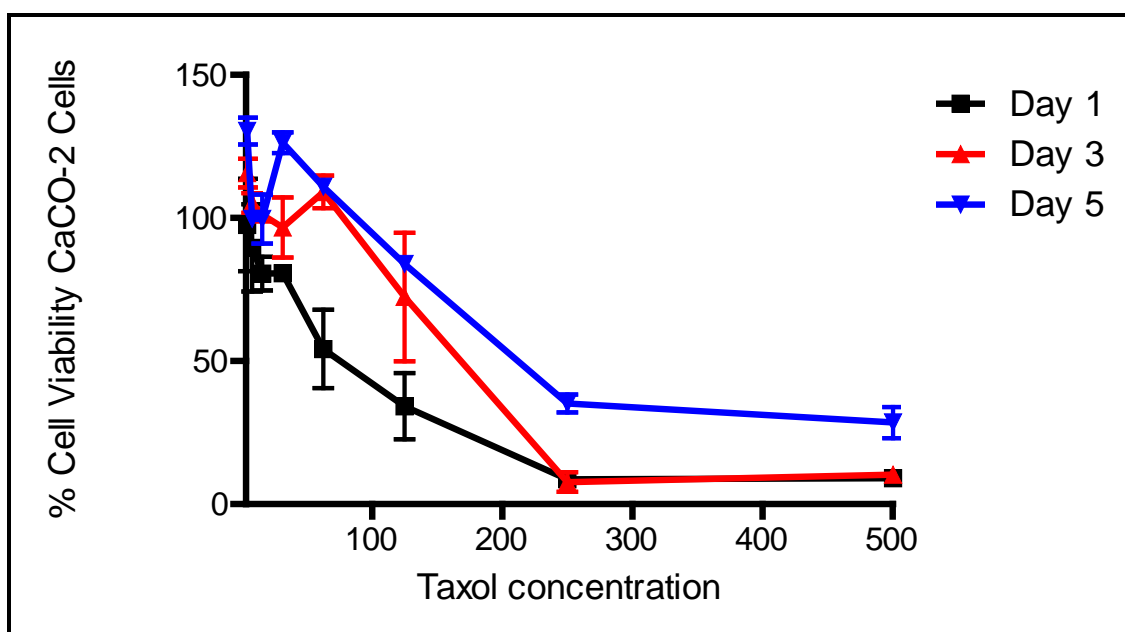
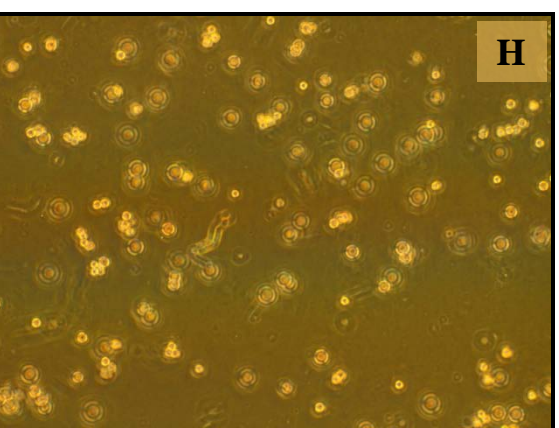
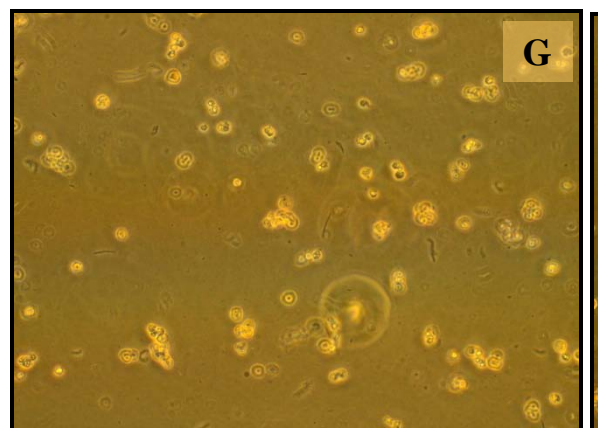
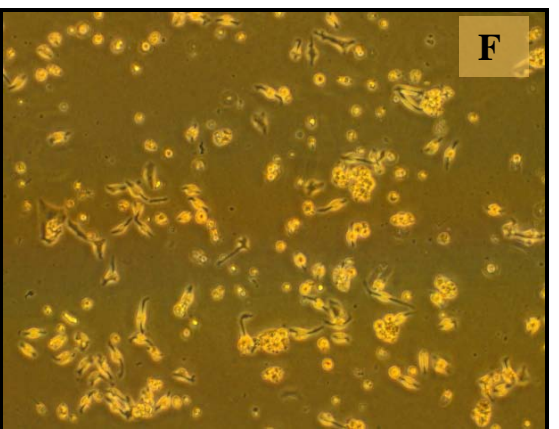
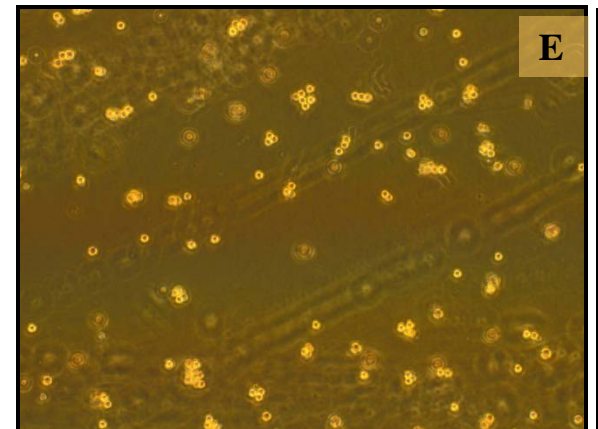
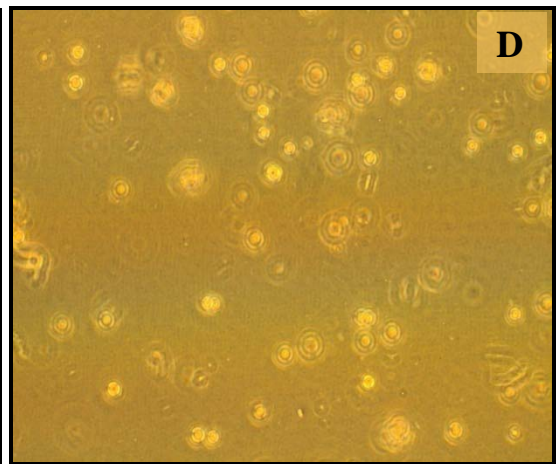
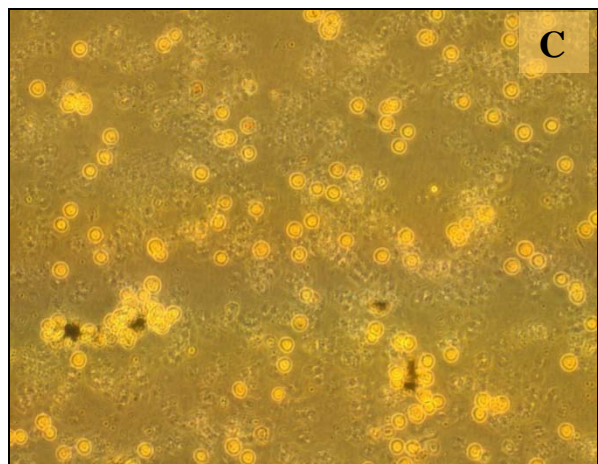
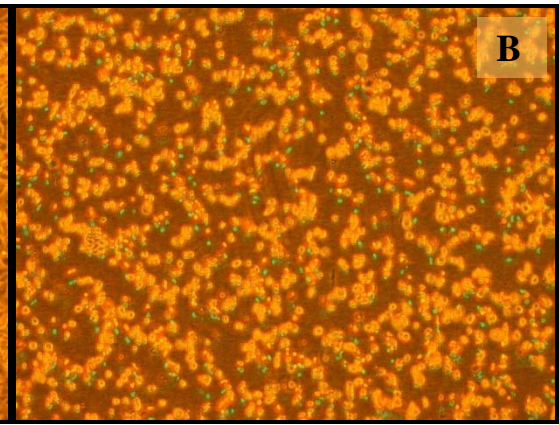
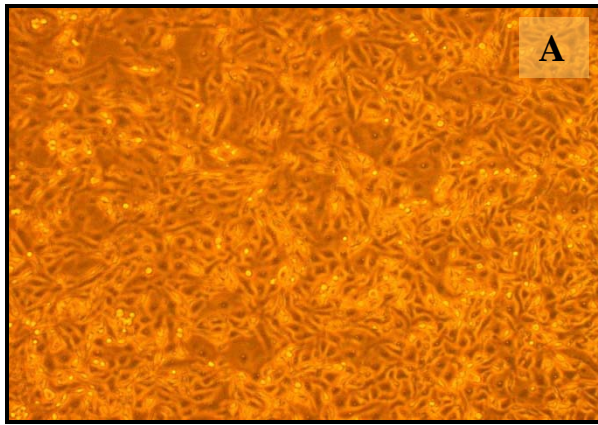


Figure 23: Effect of Taxol concentration and incubation period on CaCo-2 cell viability.

Light microscopic observations of *C. asiatica* showed an increase number of dead cells with an increase in concentration from 125 μg/ml - 500 μg/ml (Fig. 24D-F). The dead cells appeared rounded and detached with no clear morphology, whereas the control cells (unexposed CaCO-2) were attached and appeared translucent and healthy (Fig. 24A). Cells exposed to taxol, appeared detached and rounded, with a significant number of viable looking cells (Fig. 24C).

E. rhinocerotis also showed an increase in cell death as the concentrations increased from 125 μg/ml – 500 μg/ml (Fig. 25D-F). The monolayer of the untreated cells appeared spindle shaped (Fig. 23A) whereas cells exposed to taxol, appeared rounded and unhealthy (Fig. 25C).



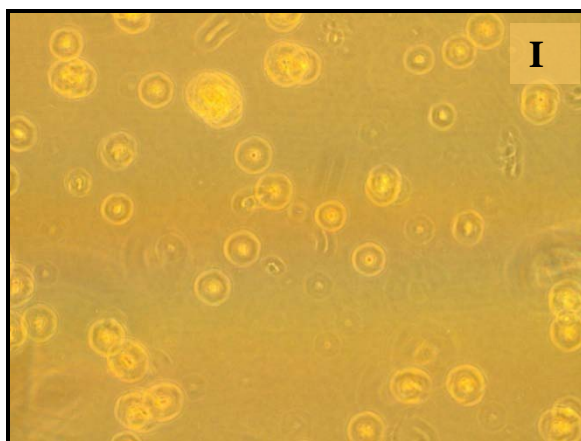


Figure 24: Microscopic observation of the morphology of CaCO-2 cells treated with 125 µg/ml - 500 µg/ml of *C. asiatica* and *E. rhinocerotis* methanolic extract. (A) the monolayer showed spindle shaped cells at 100 X , (B) de-attached cells appeared rounded at 100 X, (C) taxol 250µg/ml at 100 X magnification, (D) CaCO-2 treated with 500µg/ml *C. asiatica* at 100 X magnification, (E) CaCO-2 treated with 250 µg/ml *C. asiatica* at 100 X magnification , (F) CaCO-2 treated with 125µg/ml *C. asiatica* at 100 X magnification (G), CaCO-2 treated with 500 µg/ml *E. rhinocerotis* at 100 X magnification, (H) CaCO-2 treated with 250 µg/ml *E. rhinocerotis* at 100 X magnification and (I) CaCO-2 treated with 125µg/ml *E. rhinocerotis* at 100 X magnification.

4.4 Fractionation of the methonolic extracts of *C. asiatica* and *E. rhinocerotis*

The methanol extracts of *C. asiatica* and *E. rhinocerotis* were further fractionated by TLC and the bands were tested for cytotoxicity to CaCO-2 cells.

Methanol extract of 250 g plant material was prepared. After trying various solvents (results not shown) ethyl acetate: methanol: water (100:25:15) was found to give the most number of bands, with six major bands (Fig. 25). These bands were collected from the silica gel plates and after removing the silica, the fractions were filter sterilized and tested for toxicity. Band 2 (Fig. 25) showed the highest toxicity (87 %) of the CaCO-2 (Fig. 26A) and the least toxicity to the PBMC's (Fig 26B) at 250 µg/ml.

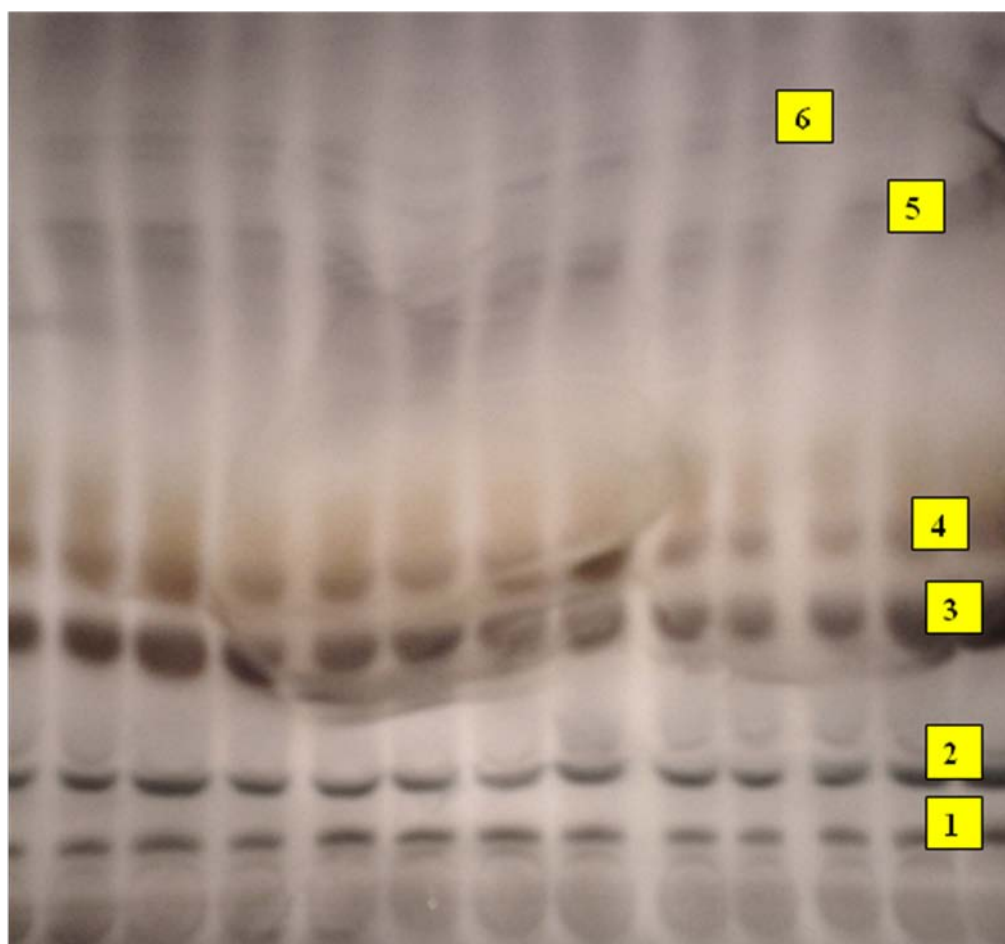


Figure 25: Thin layer chromatogram of the methanolic extract of *C. asiatica* using ethyl acetate: methanol: water (100:25:15).

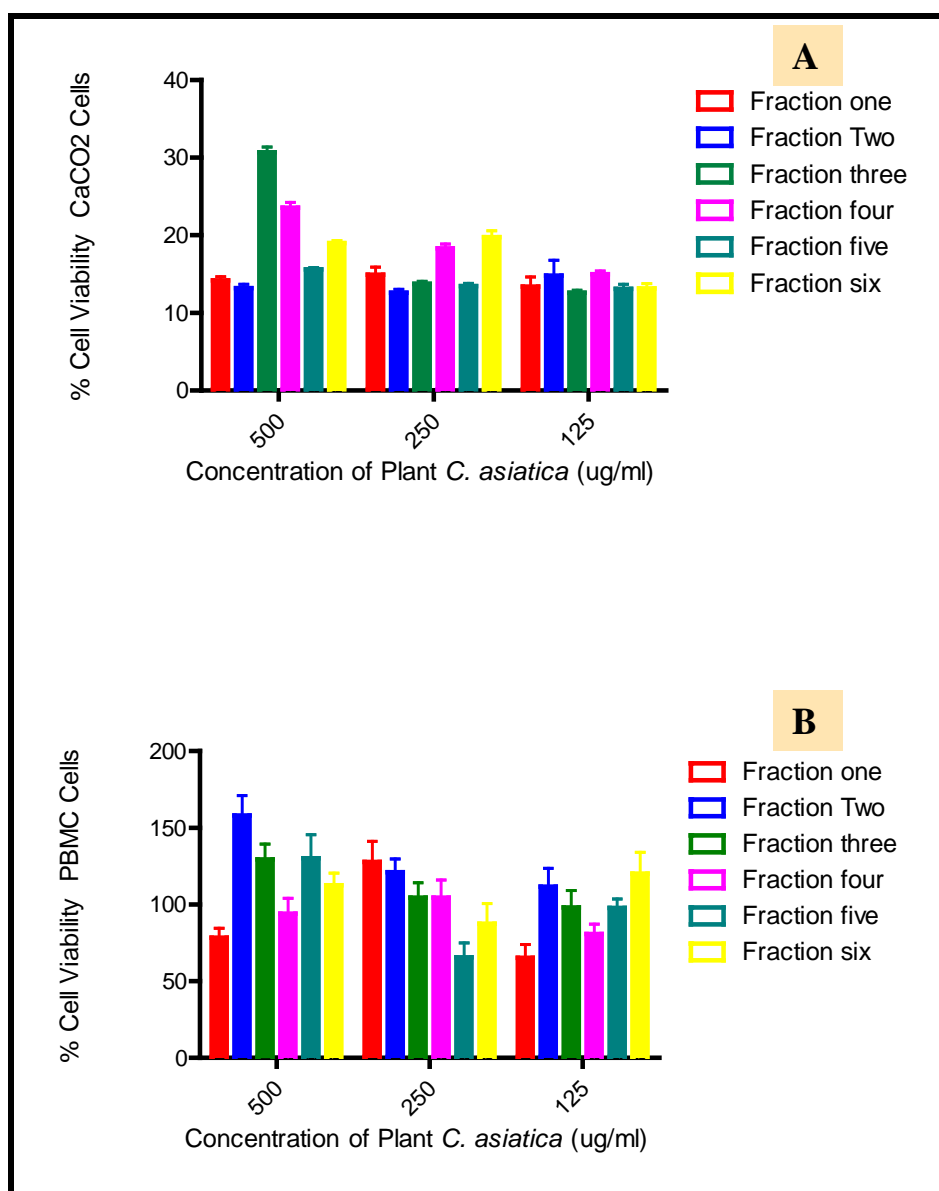


Figure 26: Percentage cell viability of (A) CaCO₂ cells and (B) PBMC treated with the fractions of *C. asiatica*.

The most efficient solvent system used in this study for the separation of extracts of *E. rhinocerotis* was hexane: ethyl acetate (6 : 4) and this gave four major bands. At 125 µg/ml Band one (fraction 1) resorted to 97,5 % toxicity (Fig 28A) to CaCO₂ cells and did not cause much change to PBMC (Fig. 28B).

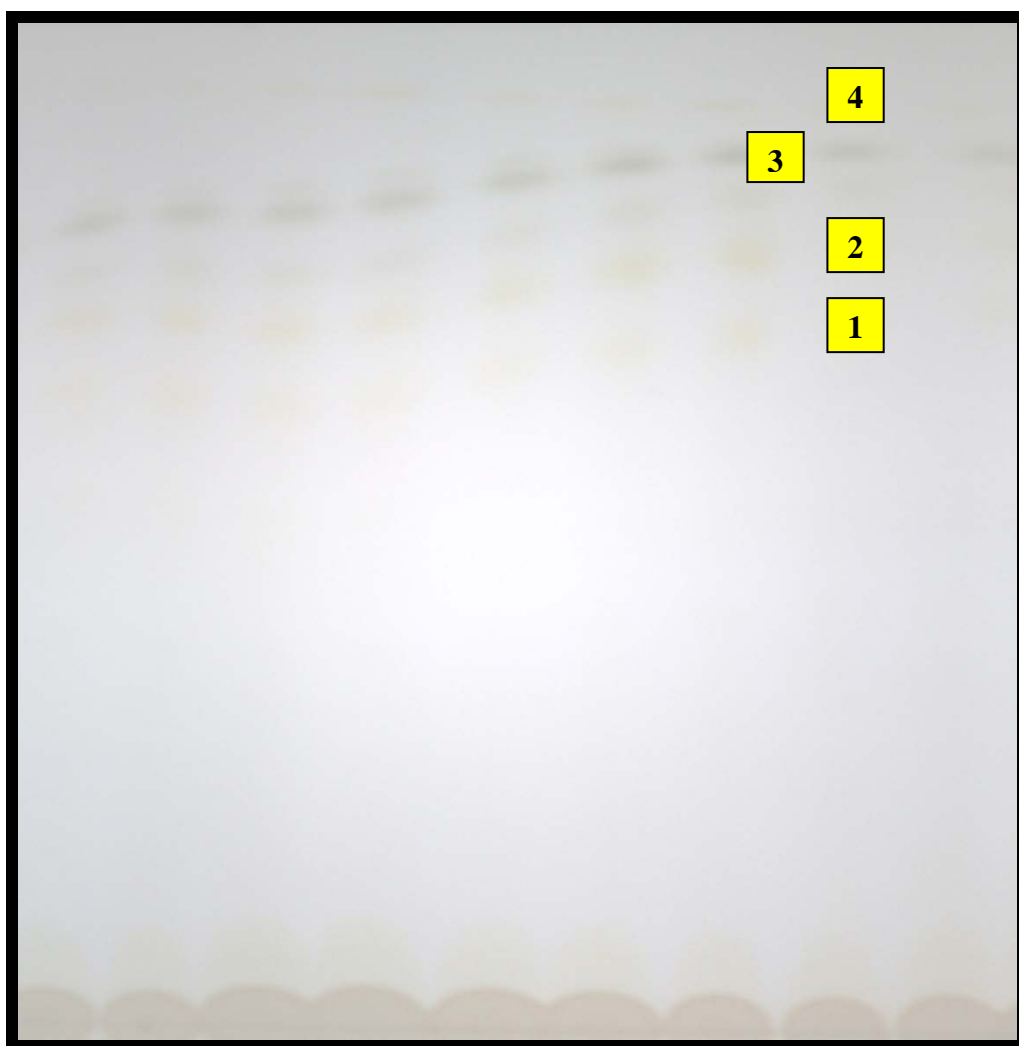


Figure 27: Thin layer chromatogram of the methanolic extract of *E. rhinocerotis* using hexane : ethyl acetate (6 : 4).

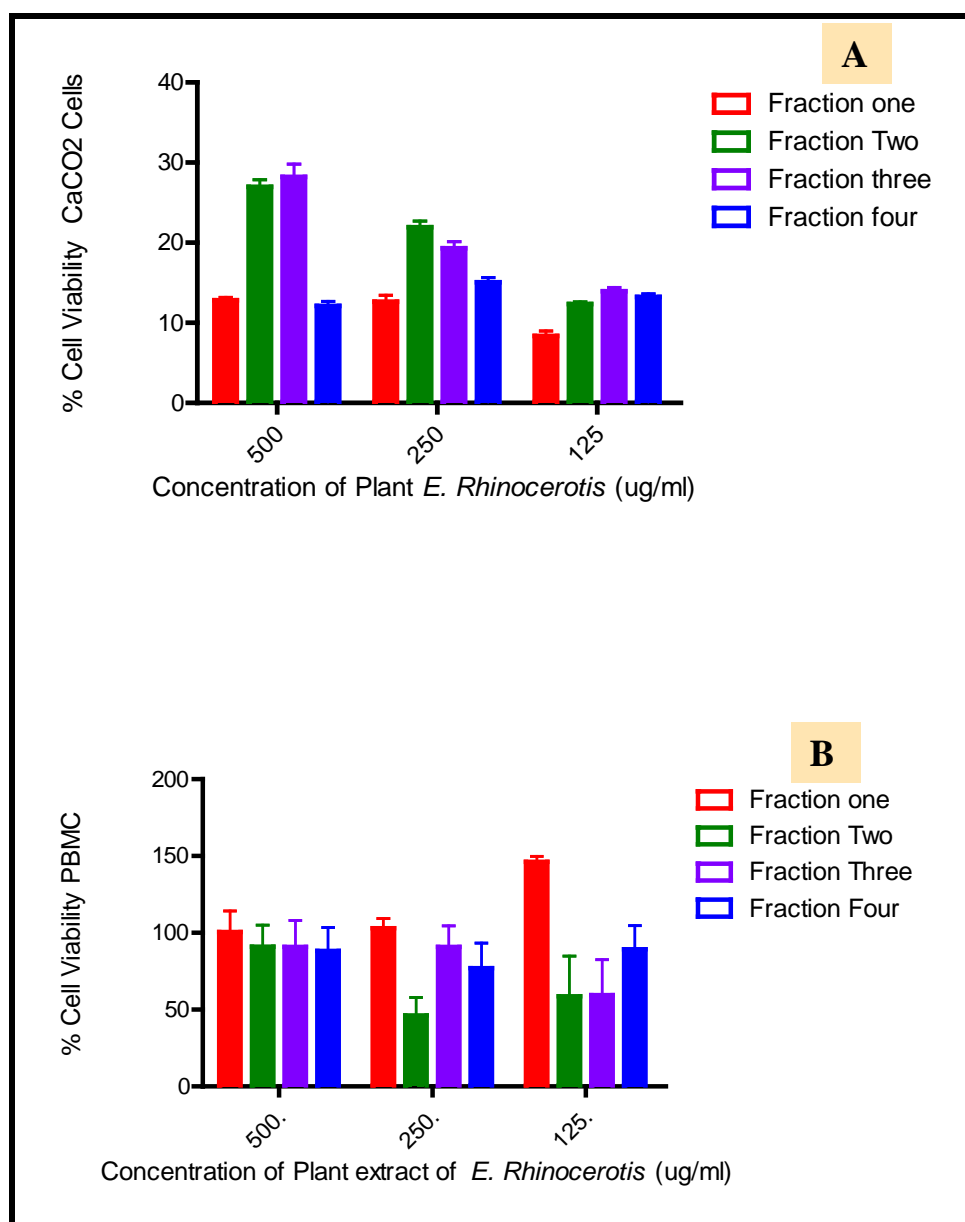


Figure 28: Viability of (A) CaCO-2 cells and (B) PBMC treated with the fractions of *E. rhinocerotis*.

Subsequent studies were conducted with bulking and cleaning fraction 2 from *C. asiatica* and Fraction 1 of *E. rhinocerotis*. Light microscopic observations of *C. asiatica* and *E. rhinocerotis* active fraction showed many dead cells. The cells appeared rounded and detached with no clear morphology (Fig. 29B and Fig. 29C), whereas the control (ie cells not exposed to any extract) were attached and appeared translucent and healthy. (Fig. 29A) Cells exposed to taxol, appeared detached and rounded, with a significant number of viable looking cells (Fig. 29B).

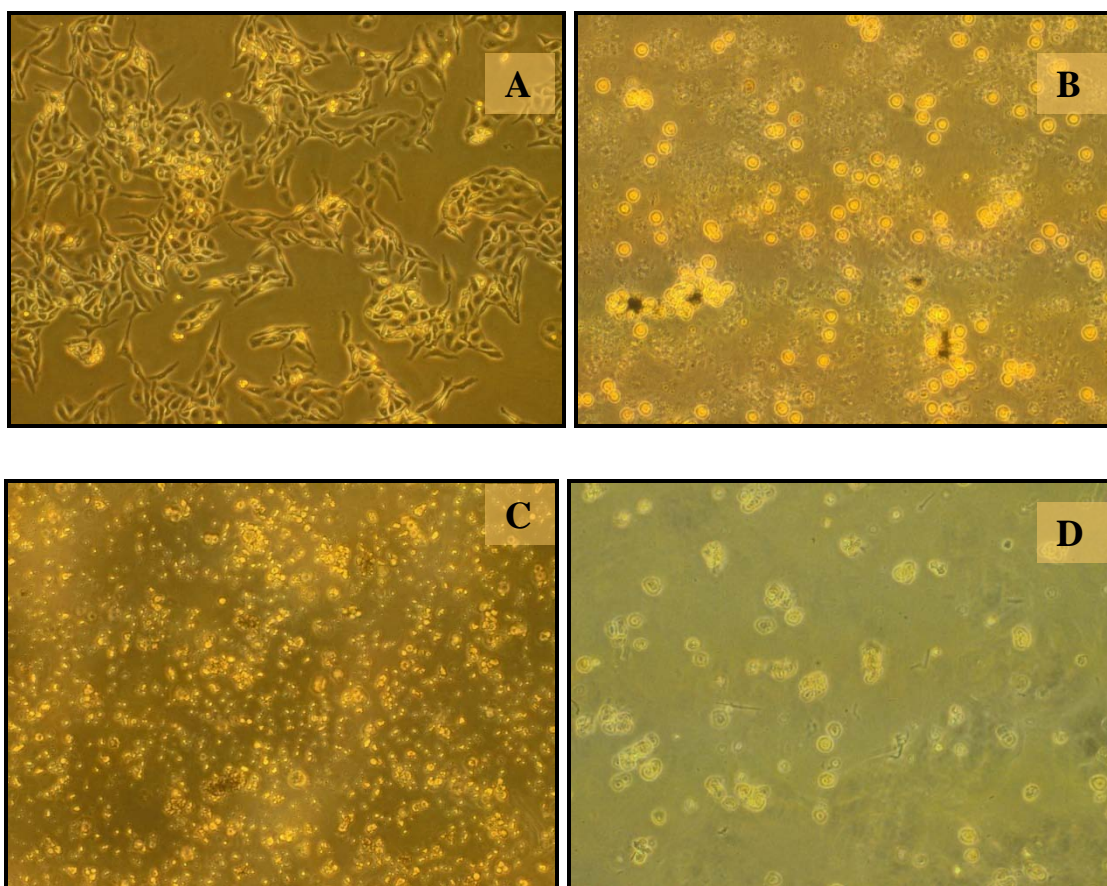


Figure 29: Microscopic observation of the morphology of CaCO-2 cells treated *E. rhinocerotis* and *C. asiatica* active fraction. (A) the monolayer showed spindle shaped cells at 100 X, (B) taxol 250 $\mu\text{g/ml}$ at 100 X magnification, (C) CaCO-2 treated with 250 $\mu\text{g/ml}$ *C.asiatica* active fraction 2 at 100 X magnification and (D) CaCO-2 treated with 125 $\mu\text{g/ml}$ *E.rhinocerotis* active fraction 1 at 100 X magnification.

4.5 Apoptotic activity of *C. asiatica* and *E. rhinocerotis* extracts and active fractions

4.5.1 Morphological changes

One of the key features of cancer cells is apoptosis. Acridine orange is a nucleic acid selective fluorescent cationic dye useful for cell cycle determination. It is cell-permeable, and interacts with DNA by intercalation or electrostatic attractions respectively. When bound to DNA, it is very similar spectrally to fluorescein, with an excitation maximum at 502 nm and an emission maximum at 525 nm (green). Ethidium bromide intercalates and stains DNA, when exposed to ultraviolet

light it will fluoresce with an orange colour. It will not stain healthy cells, it will only stain cells that have a permeable membranes (apoptotic cells).

The morphological features of apoptosis of CaCO-2 cells following treatment with the methanolic extracts of *C. asiatica* and *E. rhinocerotis* at concentration range from 125 µg/ml - 500 µg/ml using acridine orange-ethidium bromide staining showed membrane blebbing, dense chromatin and nuclei fragmenting into apoptotic bodies, as compared to the untreated control which did not exhibit these features (Fig. 30 and 31).

4.5.1.1 *C.asiatica*

Cells that were treated with the methanolic extracts *C. asiatica* (125 µg/ml) demonstrated a large number of non-viable apoptotic cells and non-viable normal cells as seen in Figure 30. This pattern is similar to the pattern observed with taxol. A much lower percentage of non-viable apoptotic cells and non-viable normal cells were observed with all the other concentrations of *C. asiatica*. However, in all the treatments of *C. asiatica* (125 µg/ml, 250 µg/ml and 500 µg/ml) and *E. rhinocerotis* (125 µg/ml, 250 µg/ml and 500 µg/ml), a large number of viable normal cells and viable apoptotic cells were observed.

Fraction 2 from the methanolic extract of *C. asiatica* showed many more non-viable apoptotic cells (features of late apoptosis) and few viable apoptotic cells (Fig. 30F) as compared to taxol which showed more dead cells (non-viable normal cells).

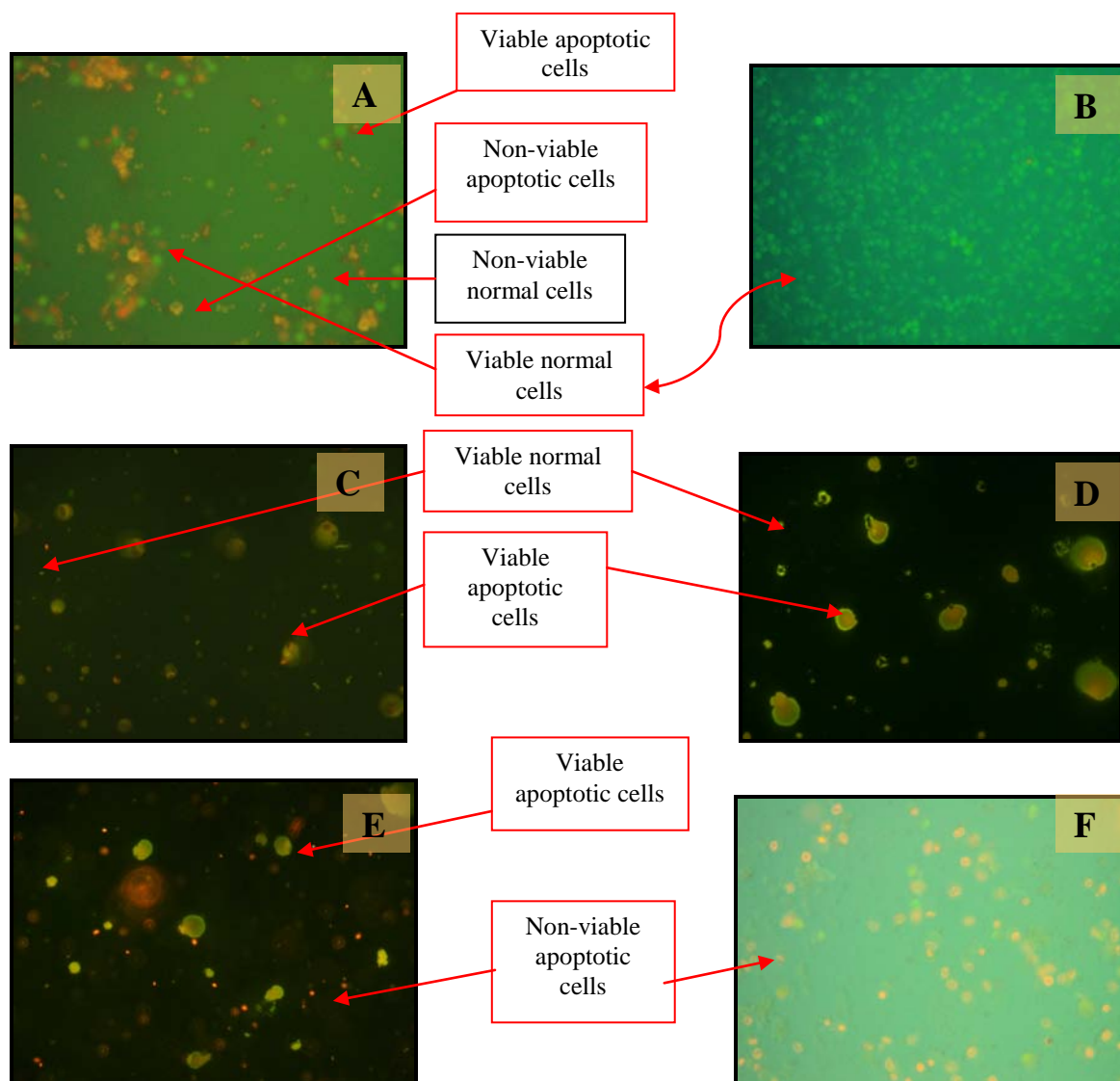


Figure 30: Fluorescence microscopic observation of the morphology of CaCO-2 cells treated with *C. asiatica* and taxol stained with acridine-orange ethidium bromide. (A) taxol 250 µg/ml at 100 X magnification (B) the monolayer, healthy cells at 100 X (C) CaCO-2 treated with 500 µg/ml with 200 X magnification, (D) CaCO-2 treated with 250 µg/ml with 200 X magnification, (E) CaCO-2 treated with 125 µg/ml with 200 X magnification and (F) CaCO-2 treated with the active fraction with 200 X magnification.

4.5.1.2 *E. rhinocerotis*

Cells that were treated with the methanolic extracts of *E. rhinocerotis*, at a concentration of 125 µg/ml similar to *C. asiatica*, demonstrated a large quantity of non-viable apoptotic cells and non-viable normal cells. This pattern is similar to the pattern observed with the positive control taxol. A much lower percentage of non-viable apoptotic cells and non-viable normal cells were observed with all the other concentrations of *E. rhinocerotis* (Fig. 31).

At Fraction 1 (125 $\mu\text{g/ml}$) of *E. rhinocerotis* displayed the most number of Non-viable apoptotic cells and non-viable normal cells. This is illustrated in Figure 32F. In comparison, taxol showed a higher percentage of dead cells (Fig. 31A).

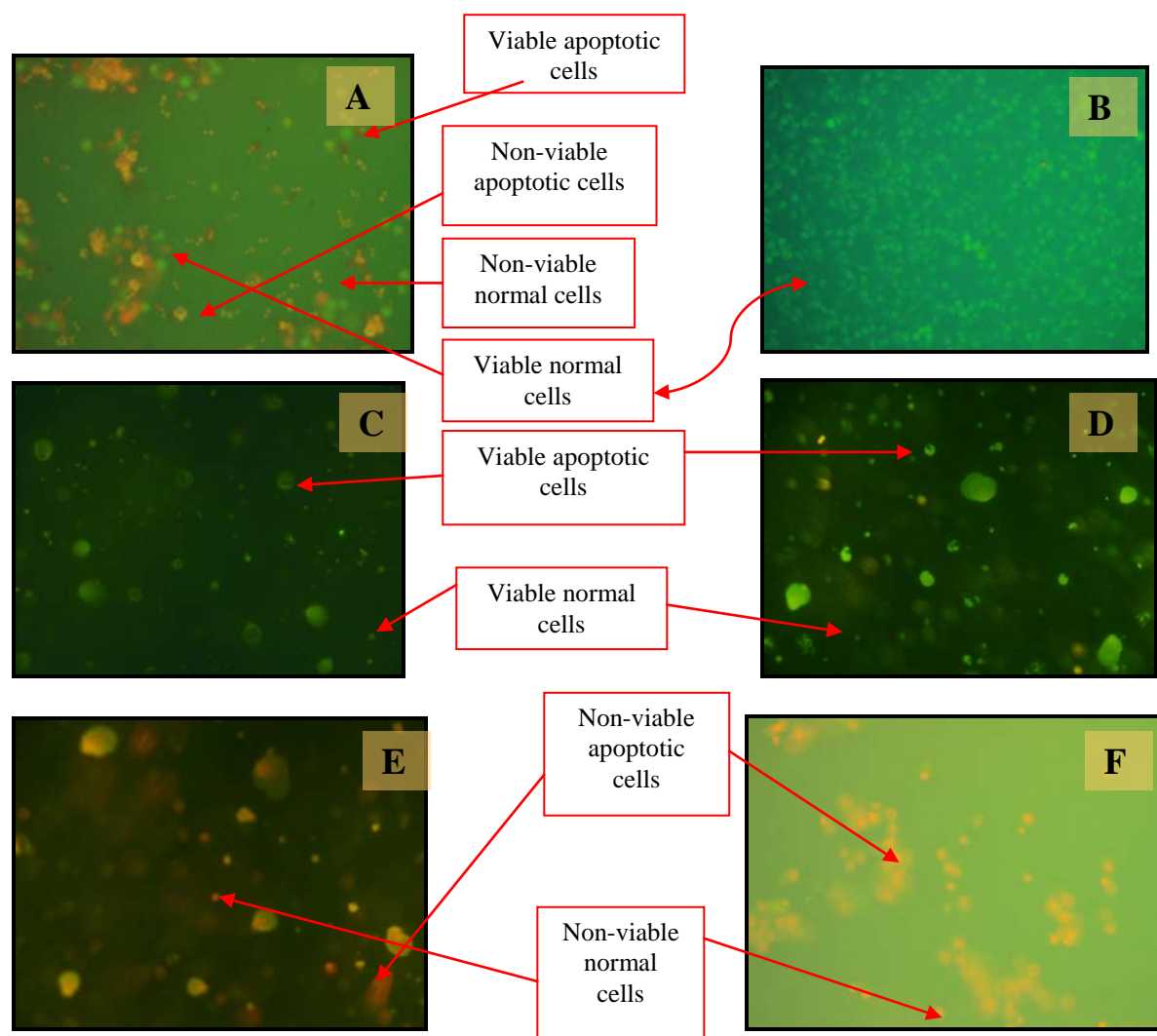


Figure 31: Fluorescence microscopic observation of the morphology of CaCO-2 cells treated with *E. rhinocerotis* stained with acridine-orange ethidium bromide. (A) taxol 250 $\mu\text{g/ml}$ at 100 X magnification (B) the monolayer healthy cells at 100 X, (C) CaCO-2 treated with 500 $\mu\text{g/ml}$ with 200 X magnification, (D) CaCO-2 treated with 250 $\mu\text{g/ml}$ with 200 X magnification, (E) CaCO-2 treated with 125 $\mu\text{g/ml}$ with 200 X magnification and (F) CaCO-2 treated with the active fraction with 200 X magnification.

4.5.2 Membrane changes

The characteristic externalization of the phosphatidylserine (PS) is a prominent marker of apoptosis. Annexin V-PE apoptosis detection assay allows for the detection and quantification of the externalized phosphatidylserine (PS) by FACS analysis. The Annexin V-PE Detection assay is based on the principle that annexin V, the 35-36 kDa Ca^{2+} dependent phospholipid binding protein, has a strong affinity for PS. Annexin V can also be conjugated to fluorochromes such as Phycoerythrin (PE) without affecting its affinity for PS, thus making annexin V-PE a sensitive probe for apoptosis detection. Externalized PS will thus be fluorescently labeled by annexin VPE (Thatte *et al.*, 2000). Results can be acquired and analyzed in the form of a histogram or scatter dot plot. For the results obtained in histogram form scatter dot blot the upper right quadrant represents the dead cells, upper left represents the late apoptotic cells, lower left represents the viable cells and lower right represents apoptotic cells.

CaCO-2 cells were plated in 24 well tissue culture plates as described in section 3.5.2. The cells were treated with the methanolic extracts and active fractions from *C. asiatica*, *E. rhinocerotis* and taxol, stained with annexin V-PE and analyzed on a FACScan instrument using CELLQuest PRO software (BD Biosciences). The cell fluorescence was measured by flow cytometry using the FL2 channel (565 to 605 nm) and a minimum of 10 000 events was acquired per sample.

4.5.2.1 *C.asiatica*

Only 3.72 % of the cells stained positive for the externalisation of phosphatidylserine in untreated control presented in Figure 33A. In cells treated with the methanolic extracts of *C. asiatica*, the most effective concentration was 125 $\mu\text{g/ml}$ (Fig. 32E) as it was observed that only 22.84 % of the

treated cells were viable as compared to 31.26 % at 500 μ g/ml and 38.75 % at 250 μ g/ml for the active fraction at 125 μ g /ml (Fig. 32F) there were 31.04 % viable cells (Table 4).

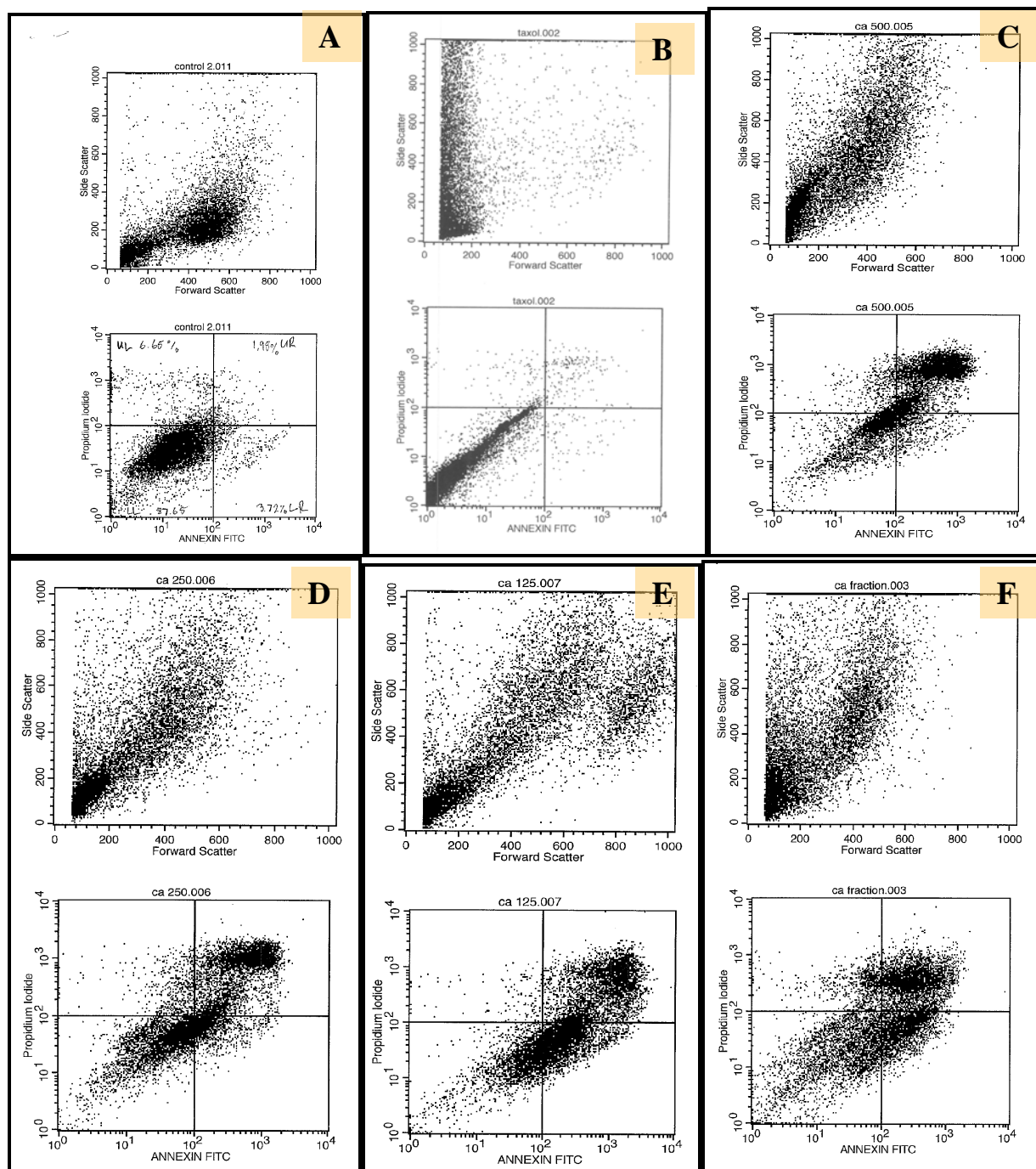


Figure 32: Flow cytometry analysis demonstrating the externalization of phosphatidylserine in CaCO-2 cells treated for 72h with *C. asiatica* methanolic extract and active fraction. Annexin V- PE Apoptosis Detection assay results are presented in a scatter plot form, denoting the percentage of cells that are necrotic (upper left), viable (lower left), apoptotic (lower right) and late apoptotic (upper right). (A) represents the untreated control, (B) represents the positive control (Taxol), (C) represents the CaCO-2 treated with 500 μ g/ml, (D) CaCO-2 treated with 250 μ g/ml, (E) CaCO-2 treated with 125 μ g/ml (F) and CaCO-2 treated with the active fraction.

Table 4: CaCO-2 cells treated with *C. asiatica* and stained with Annexin V-FITC/PI

Treatment	Viable (%)	Apoptotic (%)	Late Apoptotic (%)	Necrotic (%)
Negative Control	87.65	3.72	1.98	6.65
Positive control: taxol	7.38	0.86	1.90	1.69
500 µg/ml	31.26	6.29	55.96	6.49
250 µg/ml	38.75	14.21	41.97	5.07
125 µg/ml	22.84	36.85	39.06	1.25
Fraction 2 (250 µg/ml)	31.04	24.61	35.77	8.58

4.5.2.2 *E. rhinocerotis*

In cells treated with the methanolic extracts of *E. rhinocerotis*, the most effective concentration was 500 µg/ml (Fig. 33C). This concentration had a percentage cell viability of only 15.05 % as compared to 24.29 % (250 µg/ml) and 45.30 % (125 µg/ml). A very important result that was noted was the low percentage of necrotic cells observed for all of the treatments. This percentage was similar to the percentage seen with the non-treated cells.

However these results were slightly different in CaCO-2 treated with *E. rhinocerotis*'s active fraction (Fig. 33 F), where a high percentage of cells were viable (59.45 %). *E. rhinocerotis*'s active fraction also displayed a very low percentage of apoptotic and late apoptotic bodies as compared to *C. asiatica*'s active fraction. 31.60 % of necrotic CaCO-2 cells were noted in treatment *E. rhinocerotis*'s active fraction (Table 5).

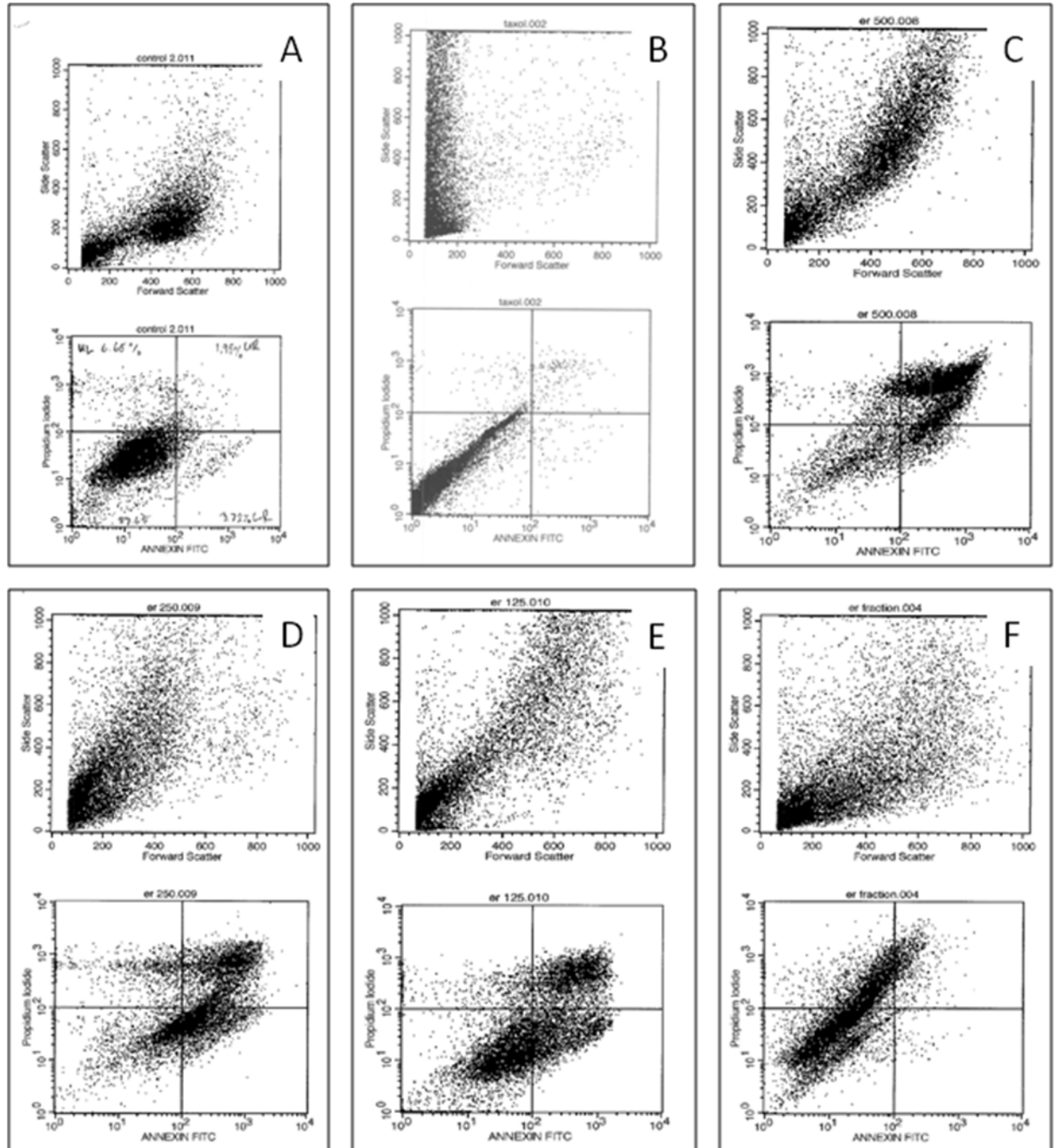


Figure 33: Flow cytometry analysis demonstrating the externalization of phosphatidylserine in CaCO-2 cells treated for 72h with *E. rhinocerotis* methanolic extract and active fraction. Annexin V- PE Apoptosis Detection assay results are presented in a scatter plot form, denoting the percentage of cells that are necrotic (upper left), viable (lower left), apoptotic (lower right) and late apoptotic (upper right). (A) represents the untreated control, (B) represents the positive control (Taxol), (C) represents the CaCO-2 treated with 500 $\mu\text{g/ml}$, (D) CaCO-2 treated with 250 $\mu\text{g/ml}$, (E) CaCO-2 treated with 125 $\mu\text{g/ml}$ and (F) CaCO-2 treated with the active fraction

Table 5: CaCO-2 treated with *E. rhinocerotis* and stained with Annexin V-FITC/PI

Treatment	Viable (%)	Apoptotic (%)	Late Apoptotic (%)	Necrotic (%)
Negative Control	87.65	3.72	1.98	6.65
Positive control: taxol	7.38	0.86	1.90	1.69
500 µg/ml	15.05	10.13	67.85	6.97
250 µg/ml	24.29	31.97	35.94	7.80
125 µg/ml	45.30	24.32	25.17	5.21
Fraction 1 (125 µg/ml)	59.45	0.82	8.13	31.60

4.5.3 Nuclear changes

The purpose of DNA fragmentation during apoptosis is still not clear, some may believe that it might be to facilitate the breakdown of DNA upon uptake of apoptotic cells by phagocytes, and others believe that it might be to destroy the information content of the cell and thus act as an irreversible step in the process (Mqoqi *et al.*, 2000).

To confirm DNA fragmentation another method i.e. immunofluorescent staining (BrDU) will be used because the extraction method together with gel electrophoresis did not provide any conclusive results. In this study immunofluorescent staining of incorporated bromodeoxyuridine (BrDU) and flow cytometric analysis was used to detect the total percentage of DNA that was fragmented. BrDU is incorporated to the DNA that has positively degraded into smaller fragments.

4.5.3.1 *C. asiatica*

Agarose gel electrophoresis of the DNA from the cells treated with the methanolic extract of *C. asiatica* (Fig. 34) and with the active fraction (Fig. 37) showed features of laddering (i.e. smearing) in lane 3 (125 µg/ml). In the active fraction laddering was seen in lane 2.

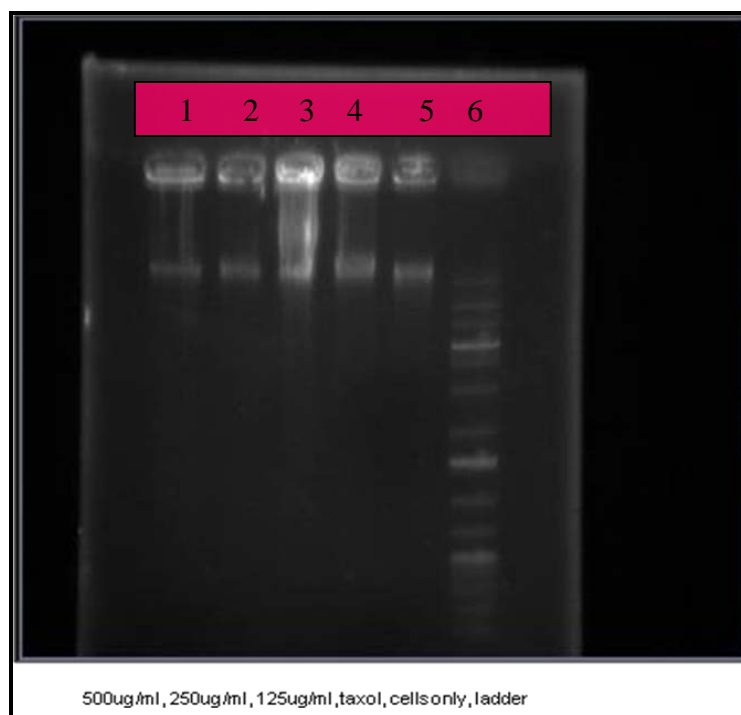


Figure 34: CaCO-2 treated with *C. asiatica* observed on a gel.

CaCO-2 cells treated with extracts of *C. asiatica*, the highest fragmentation was seen at a concentration of 125 µg/ml (Fig. 35E) demonstrating only a 6.4 % fragmentation. From the table below it is clear that the positive control taxol, did not incorporate the BrDU very effectively. Hence, it may be assumed that a very diminutive percentage of DNA fragmentation has occurred. From Table 6 (summary of BrDU) it is clear that after incubation with the active compounds from *C. asiatica*, a large number of DNA fragments were observed in comparison to the methanolic extract.

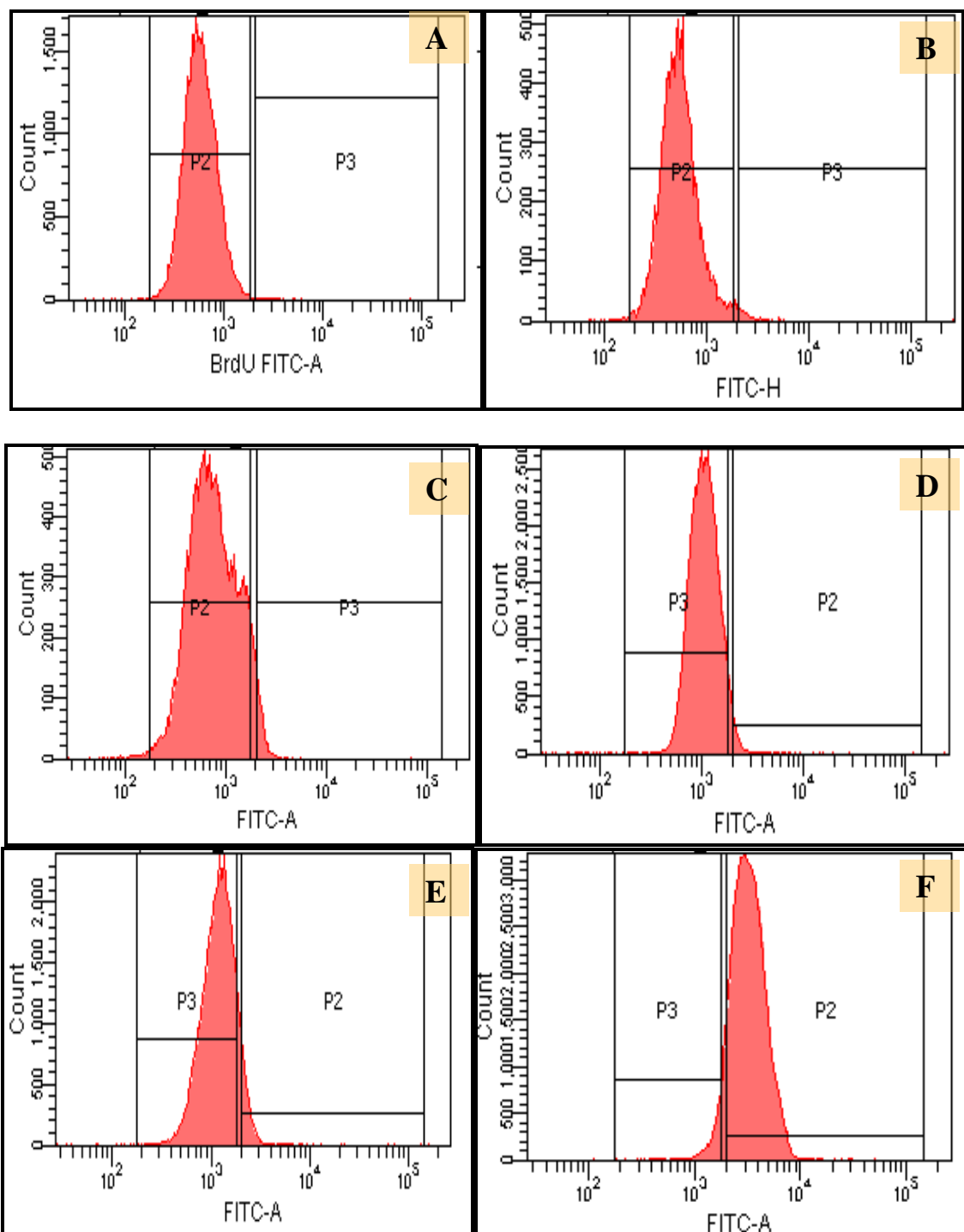


Figure 35: Flow cytometry analysis demonstrating the incorporation of BrdU upon DNA fragmentation in CaCO-2 cells treated for 72h with *C. asiatica* methanolic extract and active fraction. FITC-BrdU assay results are presented in a histogram, (A) represents the untreated control, (B) represents the Taxol, (C) represents the CaCO-2 treated with 500 µg/ml, (D) CaCO-2 treated with 250 µg/ml, (E) CaCO-2 treated with 125 µg/mL and (F) CaCO-2 treated with the active fraction

Table 6: Summary of CaCO-2 cells treated with *C.asiatica* with BrDU

Treatment	P 1 (% gated)	P 2 (% Negative for DNA fragmentation)	P 3 (% Positive for DNA fragmentation)
Negative Control	20.1	99.7	0.1
Positive control: taxol	24.2	97.7	1.1
500 µg/ml	19.0	93.2	2.7
250 µg/ml	60.7	94.9	1.7
125 µg/ml	58.5	86.4	6.4
Fraction 2 (125 µg/ml)	85.0	5.1	89.3

4.5.3.2 *E. rhinocerotis*

The CaCO-2 cells that were treated with *E. rhinocerotis* revealed a similar banding pattern to *C. asiatica*, with slight smears in lane 1, 2 and 4 (Fig. 36). Whereas, lane 3 was observed to have shown a compelling smear with light fragmenting. The CaCO-2 cells that were treated with *E. rhinocerotis* active fraction as observed in Lane 3 (Fig. 37) with a light smear and no fragmentation was seen. Taxol, also produced smearing and not fragmentation. This smearing pattern is evidence of necrosis and not apoptosis. This could have occurred because the DNA was extracted at a later stage of apoptosis when the DNA fragments were already engulfed by phagocytosis. However this needs to be further investigated.

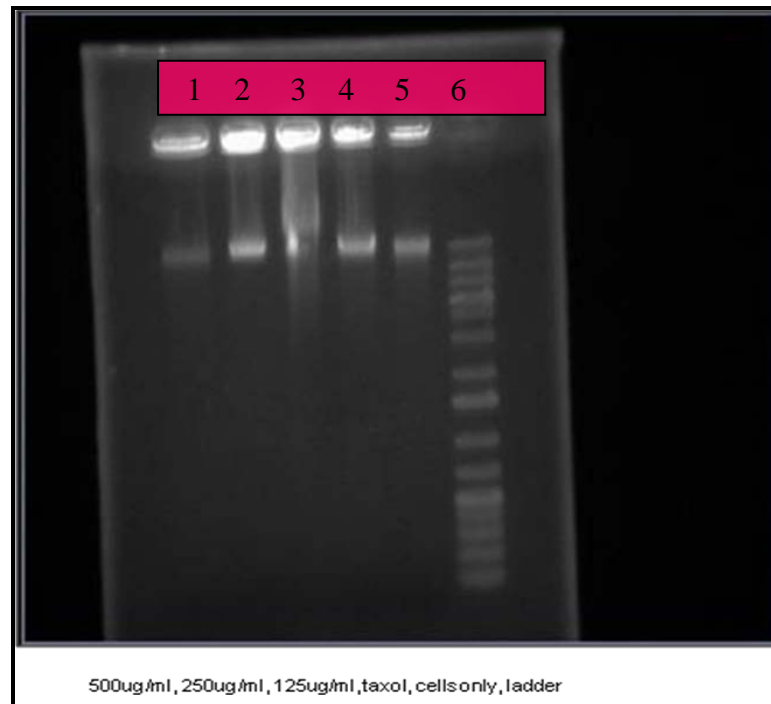


Figure 36: CaCO-2 treated with *E.rhinocerotis* observed on a gel.

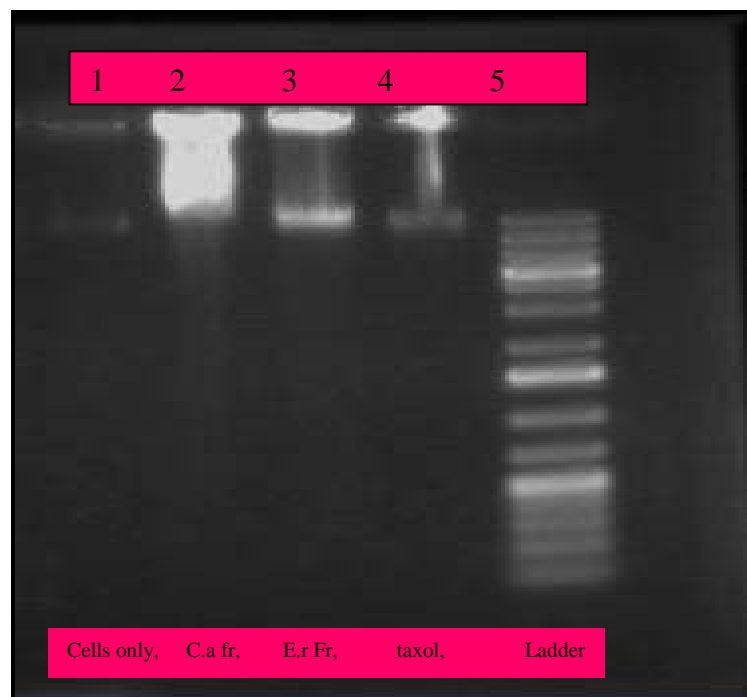


Figure 37: CaCO-2 treated with *C. asiatica* and *E. rhinocerotis* active fractions.

In CaCO-2 cells treated with extracts of *E. rhinocerotis*, a significant increase was seen with the incorporation of BrDU, with the highest fragmentation observed at a concentration of 500 $\mu\text{g/ml}$ producing 27 % positive DNA fragmentation (Fig. 38C). From Table 7 below one can deduce that the results produced by BrDU for positive DNA degradation is fairly lower than expected. The

active compound from *E. rhinocerotis* produced a considerable increase in the incorporation of BrdU as compared to the positive control taxol. These results indicate that these active compounds are able to positively degrade DNA into smaller fragments thus allowing apoptosis to occur in these cancer cell lines.

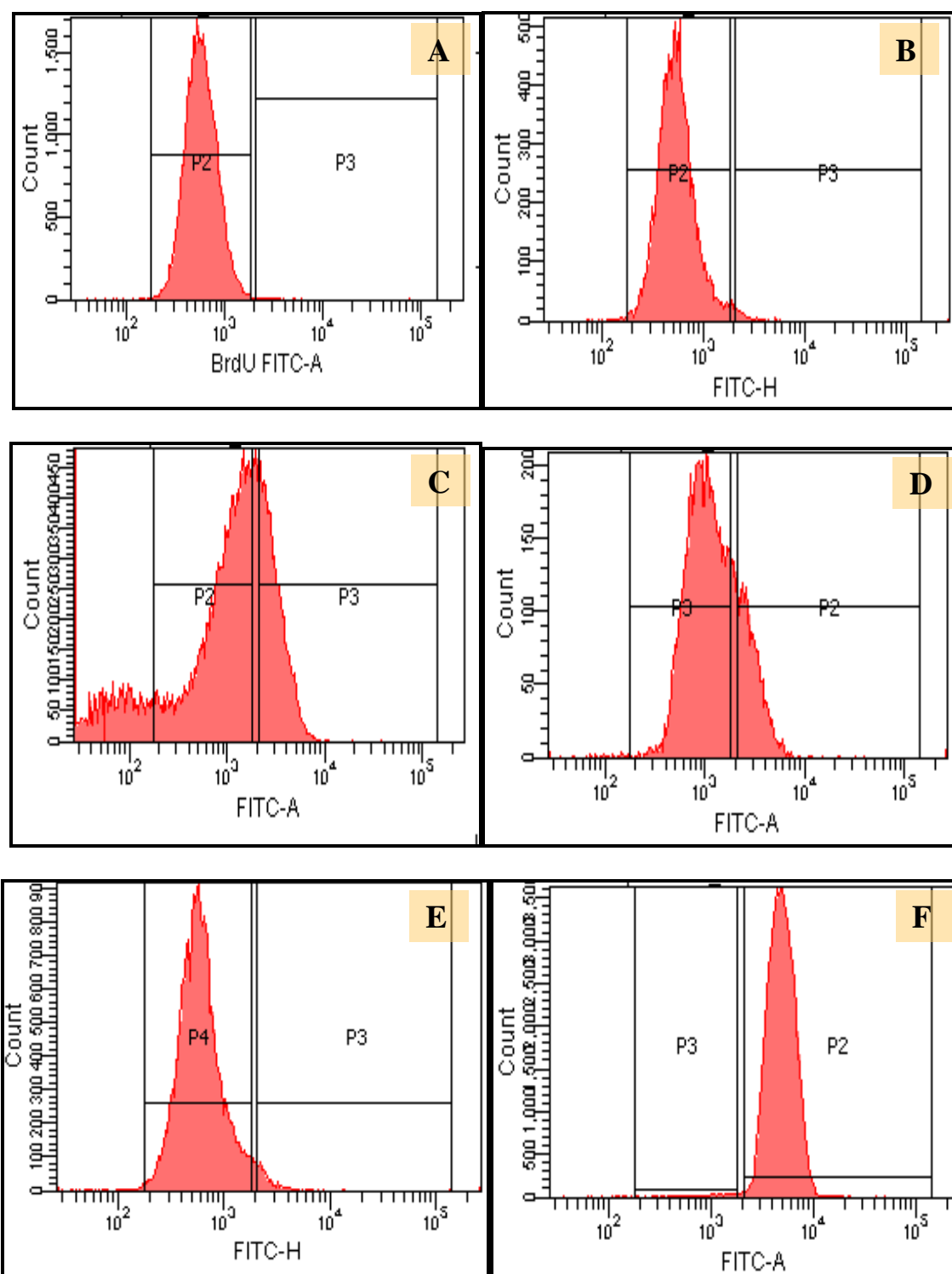


Figure 37: Flow cytometry analysis demonstrating the incorporation of BrdU upon DNA fragmentation in CaCO-2 cells treated for 72h with *E. rhinocerotis* methanolic extract and active fraction. FITC-BrdU assay results are presented in a histogram, (A) represents the untreated control,

(B) represents the taxol, (C) represents the CaCO-2 treated with 500 µg/ml, (D) CaCO-2 treated with 250 µg/ml (E), CaCO-2 treated with 125 µg/ml and (F) CaCO-2 treated with the active fraction

Table 7: Summary of CaCO-2 cells treated with *E. rhinocerotis* with BrDU

Treatment	P 1 (% gated)	P 2 (% Negative for DNA fragmentation)	P 3 (% Positive for DNA fragmentation)
Negative Control	20.1	99.7	0.1
Positive control: taxol	24.2	97.7	1.1
500 µg/ml	26.2	51.8	27.0
250 µg/ml	68.2	72.0	21.5
125 µg/ml	48.2	95.5	2.6
Fraction 1 (125 µg/ml)	80.0	1.0	98.8

4.5.4 Cell cycle changes of apoptosis: Caspase 3 activation

The caspase family of cystein proteases plays a key role in apoptosis and inflammation (reviewed in section 1.2.3.2.4). According to Watt *et al.* (1999) caspase 3 was found to degrade a variety of cytoplasmic and nuclear proteins and activate nucleases thereby inducing degradation of DNA. The active form is located in the nucleus and the inactive is localized in the mitochondria and cytoplasm. The active PE caspase 3 Detection assay allows for the detection and quantification of active caspase 3 by FACS analysis (BD Bioscience). This assay is based on the principle that active caspase 3 is recognized by monoclonal antibodies that were specifically made against the cleaved form of caspase 3. The monoclonal antibodies are conjugated with the fluorochrome PE (Bunpo *et al.*, 2004). Consequently, the apoptotic cells that contain active caspase-3 will fluoresce.

Cells were plated in 24-well tissue culture plates as described in section 3.4.5. Thereafter the cells were treated, permeabilized and stained with PE-conjugated polyclonal active caspase-3 antibody, the cells were analyzed on a FACScan instrument.

4.5.4.1 *C. asiatica*

In the above aspect of my study, the percentage of apoptotic induction and the release of active caspase 3 of CaCO-2 cells following treatment with the methanolic extracts of *C. asiatica* (Summarized in Table 8), only activated a slight amount of caspase 3. The highest amount of caspase activated here was only 21.7 % (with 47.8 % negative and altogether 73.3 % gated) observed at a concentration of 125 µg/ml (Fig 39E). This suggested that *C. asiatica* induce apoptosis more intensely through a different apoptotic pathway. As observed in the untreated cells (Fig. 39A), a large quantity (75.2 %) was negative for caspase 3 activity and only 0.3 % showed positive caspase 3 activity. Taxol showed only an 8.9 % caspase 3 positive activity and a 41.5 % negative caspase 3 activity (Fig. 39B).

From Figure 39F, it is indicated that after incubation with the active compound from *C. asiatica*, activated caspase 3 activity. This fraction showed much more positive effect than taxol. In cells treated with *C. asiatica* active fraction, here to a significant percentage of caspase 3 activity was noted (64.3 % positive, 20.8 % negative and 91.6 % gated). This result also showed improved caspase 3 activity from the other methanolic extracts of *C. asiatica*, which did not demonstrate very positive results. This shows that the methanolic extract of *C. asiatica* does not activate caspase 3 to a large extent; however the active fraction of *C. asiatica* definitely induces the activation of caspase 3 activities.

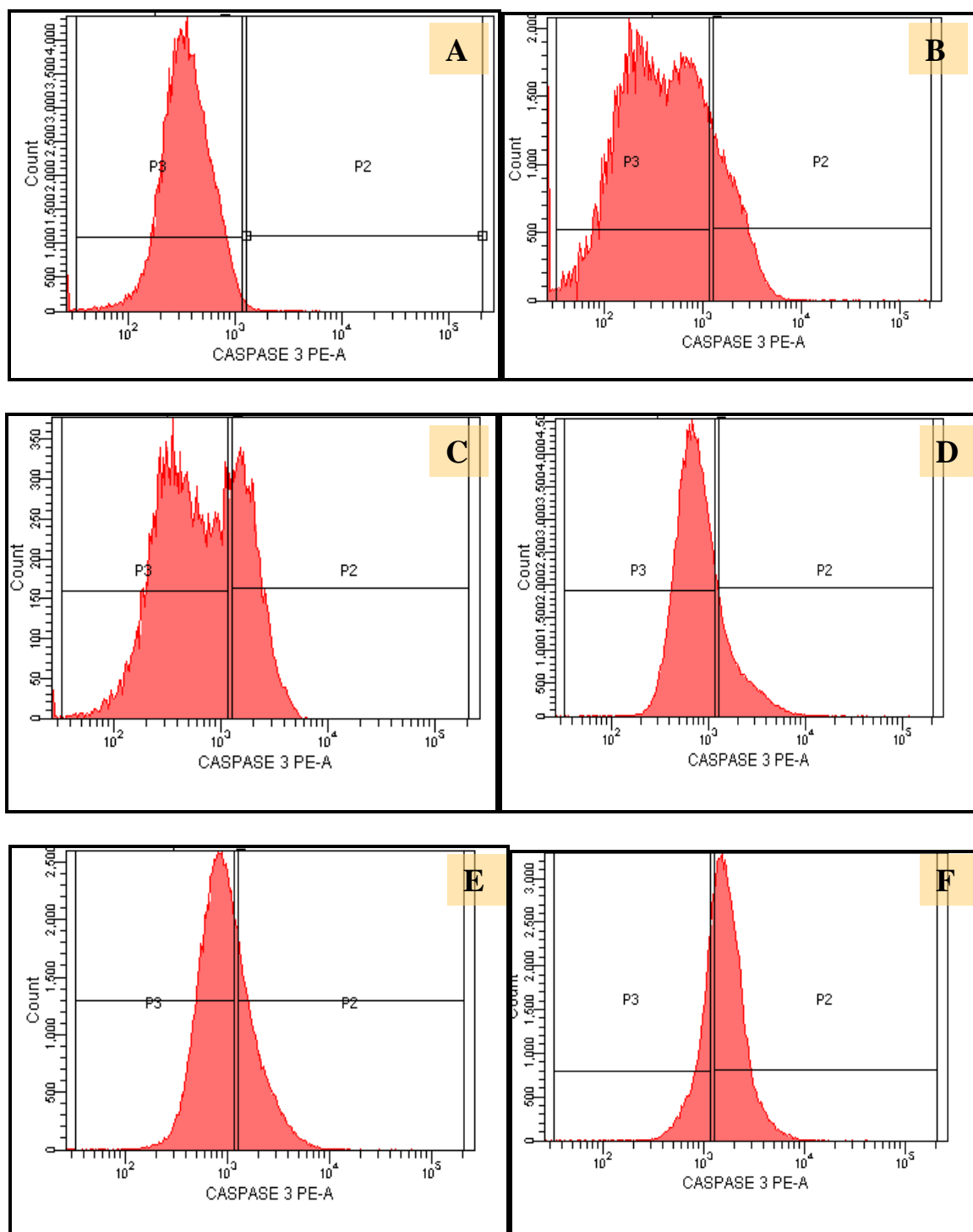


Figure 38: Flow cytometry analysis demonstrating the release of active caspase 3 in CaCO-2 cells treated for 72h with *C. asiatica* methanolic extract and active fraction. PE caspase 3 assay results are presented in a histogram, (A) represents the untreated control, (B) represents Taxol, (C) represents the CaCO-2 treated with 500 µg/ml, (D) CaCO-2 treated with 250 µg/m, (E) CaCO-2 treated with 125 µg/ml and (F) CaCO-2 treated with the active fraction

Table 8: CaCO-2 cells treated with *C. asiatica* that activated caspase 3

Treatment	P 1 (gated)	P 2 (Positive for caspase 3)	P 3 (Negative for caspase 3)
Negative Control	76.1	0.3	75.2
Positive control: taxol	52.2	8.9	41.5
500 µg/ml	30.2	8.9	20.3
250 µg/ml	89.2	7.2	68.7
125 µg/ml	73.3	21.7	47.8
Fraction 2 (125 µg/ml)	91.6	64.3	20.8

4.5.4.2 *E.rhinocerotis*

Cells incubated with methanolic extracts of *E. rhinocerotis* showed a much greater caspase 3 activity (Table 9). However the most significant caspase 3 activity was seen at a concentration of 500 µg/ml (Fig. 40C), at this concentration 77.8 % was positive for active caspase 3 (9.9 % was negative and altogether 91.1 % was gated).

E. rhinocerotis isolated active fraction (Fig. 40F) demonstrated the highest caspase 3 activity of 79.5 % (3.7 % negative and altogether 84.1 % gated), this result was even more promising than the methanolic extracts.

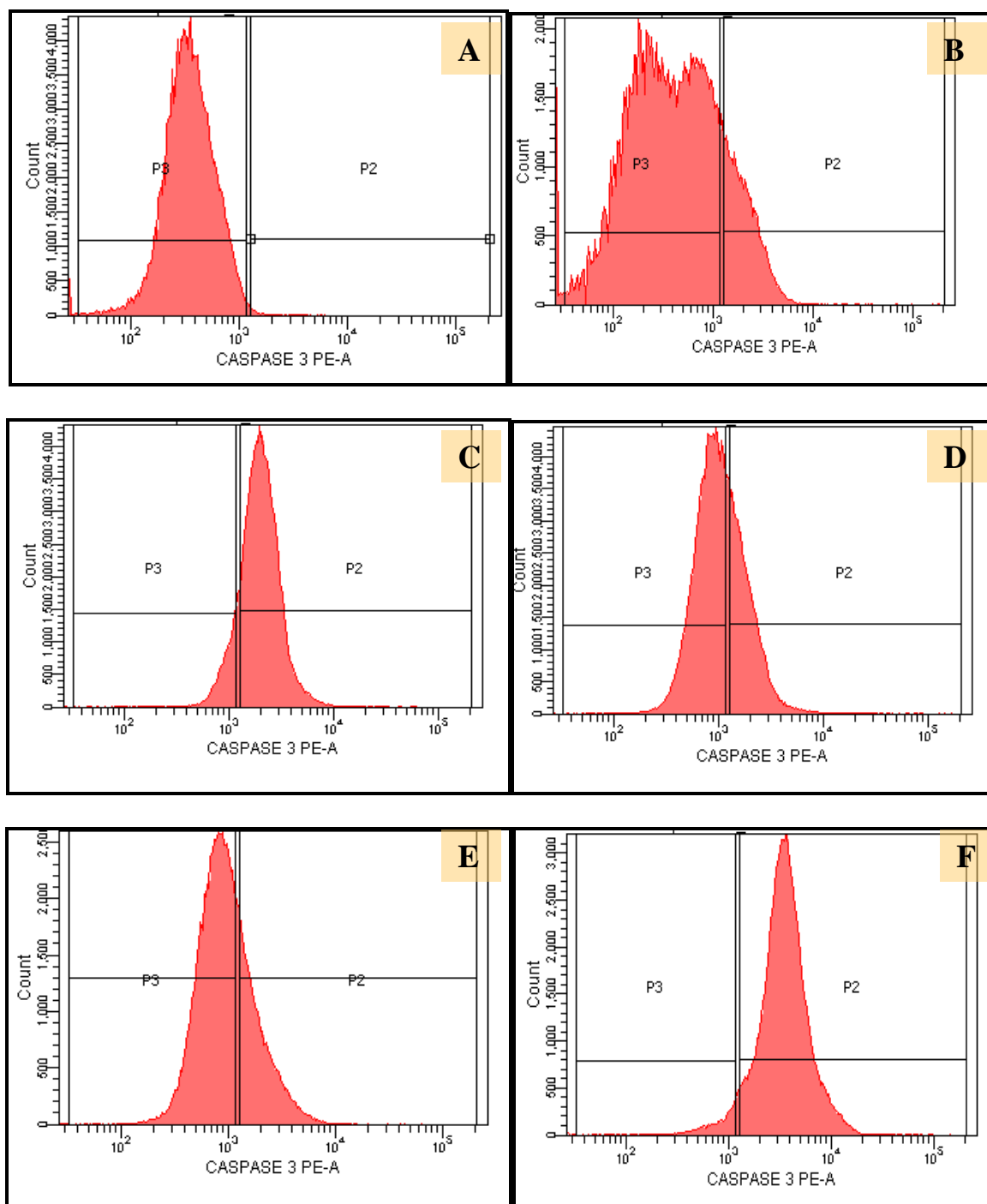


Figure 39: Flow cytometry analysis demonstrating the release of active caspase 3 in CaCO-2 cells treated for 72h with *E. rhinocerotis* methanolic extract and active fraction. PE caspase 3 assay results are presented in a histogram, (A) represents the untreated control, (B) represents taxol, (C) represents the CaCO-2 treated with 500 µg/ml, (D) CaCO-2 treated with 250 µg/ml, (E) CaCO-2 treated with 125 µg/ml and (F) CaCO-2 treated with the active fraction.

Table 9: CaCO-2 cells treated with *E. rhinocerotis* that activated caspase 3

Treatment	P 1 (gated)	P 2 (Positive for caspase 3)	P 3 (Negative for caspase 3)
Negative Control	76.1	0.3	75.2
Positive control: taxol	52.2	8.9	41.5
500µg/ml	91.1	77.8	9.9
250µg/ml	85.5	29.9	50.5
125µg/ml	66.9	30.9	32.6
Fraction 1 (125µg/ml)	84.1	79.5	3.7

CHAPTER 5: DISCUSSION

There are as yet no extremely effective drugs for the treatment of most cancers with exerting severe side effects. A successful anticancer drug should kill or debilitate cancer cells without causing extreme damage to normal cells (Monks *et al.*, 2002). This model situation is attainable by inducing apoptosis in cancer cells. Natural compounds isolated from medicinal plants, as rich sources of novel anticancer drugs, have been of increasing interest. Thus, the focus of this study was to screen extracts of two South African plants i.e. *C. asiatica* and *E. rhinocerotis* for the presence of possible fractions that induce apoptosis.

5.1 Antioxidant activity

In carcinogenesis, reactive oxygen species are responsible for initiating the multistage carcinogenesis process starting with DNA damage and accumulation of genetic events in one or few cell lines which leads to progressively dysplastic cellular appearance, deregulated cell growth, and finally carcinoma (Hanif *et al.*, 1997). The antioxidant activity assay was carried out to assess the ability of the medicinal plant extracts to scavenge free radicals *in vitro* by the DPPH method (Choi *et al.*, 2002) in this study.

The ethanolic and methanolic extract of *C. asiatica* showed a higher level of anti-oxidant activity than the aqueous, ethanolic and hexane extract. The ethanolic extract showed a 95 % radical scavenging activity which was higher than that observed for Rutin. These results correlate with those of Brand-Williams *et al.* (1995) who also showed a high scavenging activity. For *E. rhinocerotis* the ethanolic extract also showed a 100 % free radical scavenging activity, however this was seen at a higher concentration of 500 µg/ml. The antioxidant activity of this plant material has not been reported before.

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) participate in processes, contributing to cancer progression but also playing important roles in endogenous defenses, working to eliminate and controlling the spread of transformed cells. Due to recent increases in the number of cancer patients using antioxidant supplements, a greater understanding of both the damaging and protective actions of ROS in carcinogenesis is crucial to further advances in cancer treatment (Heinecke, 2003).

5.2 Cell viability

Cytotoxicity and cell viability assays have been used expansively to screen potential anti-cancer drugs. These assays measure cell death or inhibition of cell growth. The rationale is that potential anti-cancer drugs will either inhibit cancer cell growth or kill cancerous cells (Cardellina *et al.*, 1993). In this study the XTT assay was used to determine which of the extracts (aqueous, ethanolic, methanolic or hexane) of *C. asiatica* and *E. rhinocerotis* produced the most toxic effect on the CaCO-2 cell line and caused a proliferative effect on the immune cells (PBMC).

The XTT assay showed that the methanolic extract after 3 days of incubation from 125-500 µg/ml produced the most amount of toxicity in CaCO-2 cells and caused the PBMC unaffected or proliferate. This extract caused a 70 % cell death of CaCO-2 cells at 250 µg/ml while produced a 80 % cell viability in PBMC. Similarly, Babu *et al.* (1995) reported that the methanolic extract of *C. asiatica* inhibited proliferation of transformed tumor cells (Ehrlich ascites tumor cells :IC₅₀ 75 µg/ml).

The XTT assay also demonstrated that the methanolic extract of *E. rhinocerotis* was most effective against CaCO-2 cells and least toxic towards PBMC. A concentration from 250-500 µg/ml produced an average cell death of 80 %. Being Non-toxic to the PBMC at certain concentrations

indicate that cytotoxicity induced were specific to the cell line tested (CaCO-2) as cytotoxicity is not constantly induced in all the cell lines.

5.3 Activity of selected fractions of the methanolic extracts of *C. asiatica* and *E. rhinocerotis*

Fractionation involves the separation of a mixture of metabolites based on their molecular structures and intermolecular forces (Rabson *et al.*, 2005). To maintain a reasonable level of purity, only the fraction with the highest activity was further processed and assessed for apoptotic activity.

Using ethyl acetate : methanol : water (100 : 25 : 15) as a solvent system, six major bands were found from the methanolic extract of *C. asiatica* and were isolated using preparative TLC method. Band 2 from *C. asiatica* showed the highest toxicity of the CaCO-2 cells and the least toxicity to the PBMC's. At a concentration of 250 µg/ml *C. asiatica* killed 87,5 % of the CaCO-2 cells. Park *et al.* (2005) isolated asiatic acid from *Centella asiatica*, and reported that it decreased viability and induced apoptosis in human melanoma SK-MEL-2 cells in a time and dose manner.

For *E. rhinocerotis* (methanolic extract) four bands were obtained using hexane : ethyl acetate (6 : 4) as a solvent system and these were collected by preparative TLC. Band 1 of *E. rhinocerotis* extract demonstrated high toxicity of the CaCO-2 cells and the least toxicity to the PBMC's. At a concentration of 125 µg/ml of *E. rhinocerotis* extract killed 97,5 % of CaCO-2 cells. Essack, (2006) used ethyl acetate : hexane (1 : 3) as the mobile phase to separate the aqueous extract of *E. rhinocerotis* and identified the molecule 6-(4'-hydroxyphenyl)-2,3-di(R)tetrahydro-4H-pyran-4-one.

All the bands obtained were fractioned from the methanolic extracts of the two plants, therefore the crude extracts had lower activity than the separated bands (higher in concentration).

5.4 Morphological changes in cells during apoptosis

For more than 150 years, morphological features played the primary role in the description of cell death. But, during the past few decades cell death has been characterized on the molecular level, which obviously improved current insights into morphological transitions. Often cell death expose both apoptosis and necrosis. The design of cell death can be particularly perplexing since numerous drugs cause apoptosis as a result of drug-induced secondary effects on a cell. All this makes the classification of the characteristic features of apoptosis and necrosis enormously difficult.

The frequency of apoptotic induction of CaCO-2 cells following exposure of the methanolic extracts and active fractions of *C. asiatica* and *E. rhinocerotis* was determined by fluorescence microscopy of nuclear stains, acridine orange and ethidium bromide.

In cells treated with methanolic extracts of *C. asiatica* at different concentrations as well as the active fraction of *C. asiatica*, showed a large population of non-viable apoptotic cells. This indicates late apoptosis. Babykutty *et al.* (2008) reported the treatment of methanolic extract of *C. asiatica* below 41 µg/ml did not show a significant evidence of cell death even after 24 h. Treatment with higher concentrations of methanolic extract of *C. asiatica* for 48 h resulted in the formation of viable and non-viable apoptotic bodies.

Cells that were treated with the methanolic extracts and active fraction of *E. rhinocerotis*, demonstrated a large quantity of non-viable apoptotic cells and non-viable normal cells. This pattern is similar to the pattern observed with taxol. However in the cells treated with the active

fraction, a large number of dead cells were observed, which were higher than the positive control, taxol. This was not documented previously. Both the active fractions from *C. asiatica* and *E. rhinocerotis* showed a higher concentration of apoptosis than the methanolic crude extracts. This could be due to the fact that the active fraction will be present at a higher concentration on its own whereas, in the crude extract its very dilute.

5.5 Membrane changes in cells during apoptosis

Detection of apoptotic cell death in cells and tissues has become of paramount importance in many fields of modern biology, including cancer biology. Most methods exploit properties of dying cells that are more or less specific for the apoptotic process. The Annexin-V FITC detection kit was intended to perceive specific changes in the cell surface membrane which are signature events of early apoptosis.

The methanolic extracts from 125 µg/ml - 500 µg/ml and band 2 of *C. asiatica* subjected to Annexin-V revealed that at 125 µg/ml, 36.85 % of the cell population underwent early apoptosis. This was greater than the effect caused by the active fraction. It was previously reported by Babbykutty *et al.* (2008), that bright green annexin FITC staining was imparted to membrane of the apoptotic cells (47.8 %), indicating the early stages of apoptosis. The nuclei of cells with later stages of apoptosis exhibited red color of propidium iodide, signifying its condensed status. Control cells (1.5 %) were negative for annexin FITC staining, when incubated with the methanolic extract of *C.asiatica* and tested on MCF-7 cell line.

CaCO-2 cells treated with the extracts of *E. rhinocerotis* produced an average population of apoptotic cells whereas, cells treated with active fraction of *E. rhinocerotis* produced only a small percentage of apoptotic cells (0.8 % early and 8.13 % late apoptosis) and 31.60 % of necrotic cells.

Essack, (2006) reported that cells treated with the aqueous extract of *E. rhinocerotis* stained positive (71 %) for externalized phosphatidylserine respectively, compared to the negligible 2 % of the cells in the untreated control.

5.6 Nuclear changes in cells during apoptosis

DNA fragmentation plays a key role in the cell death system since it takes place very early in the apoptotic process. The degradation of DNA start with the generation of high molecular weight fragments of about 300 kb, which are fragmented more to about 50 kb and more fragmentation takes place in the end giving rise to 10 to 40 kb fragments which in the end give rise the oligosomal DNA fragments (Collins *et al.*, 1997). These such fragments are detectable on agarose gels as distinctive “DNA ladders” when extracted from apoptotic cells.

Agarose gel electrophoresis of the DNA from the cells treated with the methanolic extract and active fraction of *C. asiatica* reveals smearing with slight fragmentation. This pattern is confirmation of necrosis rather than apoptosis. Bunpo *et al.* (2004) reported that an isolate of *C.asiatica* i.e asiatic acid, produced an apoptotic pattern on DNA gel electrophoresis. The classic laddering pattern of inter-nucleosomal DNA fragmentation was observed in asiatic acid-treated cells (SW480), indicating that an irreversible apoptotic death had been induced. Bunpo *et al.* (2004) previously reported that the aqueous extract of *C. asiatica* significantly decreased the total number of cells, accompanied by a decrease in the 5-bromo-2'-deoxyuridine-labeling index and an increase in the induction of apoptotic cells in the colonic mucosa

The CaCO-2 cells that were treated with *E. rhinocerotis* revealed a similar banding pattern to *C. asiatica*, showing dark smearing pattern and light fragmentation. However, this light fragmentation is not conclusive that apoptosis has occurred. This smearing pattern is evidence of necrosis and not

apoptosis. This could have occurred due to the fact that the DNA was extracted at a later stage of apoptosis where the DNA fragments were already engulfed by phagocytosis. In CaCO-2 cells treated with extracts and active fraction of *E. rhinocerotis*, also showed similar pattern to *C. asiatica* with the active fraction producing a large percentage of DNA fragmentation. Essack, (2006) reported that 5 mg/ml aqueous extract *E. rhinocerotis* respectively induce DNA fragmentation in 75 % of the cells, compared to the 2 % of the cells in the untreated cells.

5.7 Cell cycle changes in cells during apoptosis: Caspase-3 activation

Apoptosis proceeds through an ordered series of steps. The caspase family of proteases may be divided into initiator caspases that are activated through regulated protein-protein interactions and effector caspases that are activated proteolytically by an upstream / initiator caspase. During apoptosis a family of enzymes, the caspases mediate important proteolytic occurrences. Caspases are synthesized as enzymatically inert zymogens that contain three domains namely, an *N*-terminal prodomain, a p20 domain and a p10 domain. Caspases are usually activated by proteolytic cleavage between these domains. Caspase 3 is a prevalent caspase that is ultimately responsible for the majority of apoptotic processes. It causes the cleavage or degradation of several important substrates. Therefore, we examined the effects of the extracts on caspase 3 activation.

CaCO-2 cells treated with the methanolic extracts and active fraction of *C. asiatica*, showed a moderate release of caspase when exposed to the extracts at different concentrations. This could have been attributed to the induction of apoptosis through a different pathway. Whereas cells exposed to the active fraction revealed a great percentage of caspase 3 release.

CaCO-2 cells treated with methanolic extracts and active fraction of *E. rhinocerotis* demonstrated a great percentage of caspase 3 release but the cells treated with the active fraction showed greatest

percentage (79 %) of caspase 3. Essack, (2006) previously reported the aqueous extract of *E. rhinocerotis*, showed 50 % of cells (MCF-7) stained positive for active caspase 3, respectively, compared to 2 % of the cells in the untreated cells.

CHAPTER 6: CONCLUSION

It is concluded that the methanolic extracts and active fractions from *C. asiatica* and *E. rhinocerotis* possess intoxicating anti-tumor effect on human colon cancer cells (CaCO-2). Both the extracts and the fractions targets the late apoptotic cycle. However, both the fractions from the methanolic extracts produced a much more potent effect than the methanolic extracts as a whole. Preliminary identification of these active fractions were also undertaken in this study (results not included), but due to time limitation the complete identification of these compounds were not achieved. Future studies can focus on the characterization of the active fractions.

The current study provides evidence of potential anti-tumor activity of *C. asiatica* and *E. rhinocerotis*. These may exercise its anti-carcinogenic effects associated with two elementary processes: suppression of cell proliferation and induction of apoptosis on human colon cancer cells *in vitro*. However, the effective biochemical component of the fractions and how it hinders the proliferation and induces apoptosis of CaCO-2 cells remain indefinite. Whilst this study is an *in vitro* investigation, *in vivo* experiments can provide further insight in the role of these plants as a potential anti-cancer agent. Further research is required to determine the effectiveness of these plant products in treating cancers.

REFERENCES

1. Adlercreutz, H., Fotsis, T., Heikkinen, R., Dwyer, J.T., Woods, M., Goldin, B.R., and Gorbach, S. L. (1982). Inhibition of the human aromatase by mammalian ligans and isoflavonoids phytoestrogens. *Lancet* 2, 1295–1299.
2. Babu, T. D., Kuttar, G., and Padikkla, J. (1995). Cytotoxic and antitumor properties of certain taxa umbelliferae. *Journal of Ethnopharmacology* 48, 53-57.
3. Babykutty, S., Padikkala, J., Sathiadevan, P.P., Vijayakurup, V., Azis, T.K.A., Srinivas, P. and Gopala, S. (2008). Apoptosis Induction of *Centella Asiatica* on Human Breast Cancer Cells. *African Journal of Traditional, Complementary and Alternative Medicine* 6, 9–16.
4. Berezney, R. (1991). The nuclear matrix: a heuristic model for investigating genomic organization and function in the cell nucleus. *J Cell Biochem* 47, 109-123.
5. Bhattacharjee, S.K. (2008). *Handbook of medicinal plants* 5th revised edition, Pointer publishes, Jaipur, India.
6. Boik, J. (2001). *Natural Compounds in cancer therapy. Promising non-toxic anti-tumor agents from plants and other natural sources.*
7. Brand-Williams, W., Cuvelier, M.E., and Berset, C. (1995). Use of a free radical method to evaluate antioxidant activity. *Lebensmittel Wissenschaft and Technologie* 28 (1), 25–30.

8. Bunpo, P., Kataoka, K., Arimochi, H., Nakayama, H., Kuwahara, T., Bando, Y., Izumi, K., Vinitketkumnien, U., and Ohnishi, Y. (2004). Inhibitory effects of *Centella asiatica* on azoxymethane-induced aberrant crypt focus formation and carcinogenesis in the intestines of F344 rats. *Food Chem Toxicol.* 42, 1987–1997.
9. Cardellina, J.H., Gustafson, K.R., Beutler, J.A. (1993). NCI intramural research on HIV inhibitory and antitumor plant natural products. In *Human Medicinal Agents from Plants*. Kinghorn AD, Balandrin MF (eds) Am Chem Soc Symp Ser 534. ACS: Washington, DC; 218±227.
10. Chaube, S., and Murphy, M.L. (1995). The Teratogenic Effects of Recent Drugs Active in Cancer Chemotherapy. *Advanced Teratology* 3, 181-237.
11. Choi, C.W., Kim, S.C., Hwang, S.S., Choi, B.K., Ahn, H.J., Lee, M.Y., Park, S.H. and Kim, S.K. (2002). Antioxidant activity and free radical scavenging capacity between Korean medicinal plants and flavonoids by assay guided comparison. *Plant Science* 163, 1161-1168.
12. Collins, J.A., Schandl, C.A., Young, K.K., Vesely, J., and Willingham, M.C. (1997). Major DNA fragmentation is a late event in apoptosis. *J Histochem Cytochem* 45, 923-934.
13. Cordell, G.A., Beecher, C.W., and Pezzut, J.M. (1991). Can ethnopharmacology contribute to development of new anticancer drug? *Journal of ethnopharmacology* 32, 117-133.
14. Cragg, G.M. (1998). *Paclitaxel (Taxol)*: a success story with valuable lessons for Natural Product Drug discovery and development. John Wiley & Sons, Inc., New York.

15. Danziel, J.M (1937). The useful plants of west tropical Africa. *Nature* 12, 34-47
16. Dekker, T.G., Fourie T.G., Elmare, M., Snykers, F.O., van der Scgyf, C.J. (1988). Studies of South African plants Part 7: Rhinocerotinoic acid labdane diterpene with anti-inflammatory properties from *Elytropappus rhinocerotis*. *South Afr. J. Chem.* 41, 33-35
17. Duke, J.A. and Ayensu, E.S. (1985). Medicinal Plants of China. *Nature* 312, 456-478.
18. Essack, M. (2006). Screening extracts of indigenous South African plants for the presence of anti-cancer compounds. A thesis submitted in for the degree of Masters in Science in the Department of Biotechnology, Faculty of Science, University of the Western Cape.
19. Freshney, R.I. (1987). Culture of Animal Cells: A Manual of Basic Technique, Second Edition. Alan R. Liss, New York.
20. Garcia, M., Jemal, A., Ward, E. M., Siegel, R.L. and Thun, M. J. (2007). Global Cancer Facts & Figures. Atlanta, GA: American Cancer Society.
21. Gupta, R. and Flora, S. J. (2006). Effect of *Centella Asiatica* on arsenic induced oxidative stress and mental distribution in rats. *Journal of Applied Toxicology* 26, 213-222.
22. Hagmar, B., Ryd, W., and Skomedal, H. (1991). Arabinogalactan blockade of experimental metastases to liver by murine hepatoma Invas. *Metast* 11, 348-355.
23. Hanahan, D., and Weinberg, R.A. (2000). The hallmarks of cancer. *Cell*. 100, 57-70.

24. Hanif, R., Qiao, L., Shiff, S.J., and Rigas, B. (1997). Curcumin, a natural plant phenolic food additive inhibits cell proliferation and induces cell cycle changes in colon adenocarcinoma cell lines by prostaglandin- independent pathway. *Journal of natural products* 56, 1123-1129.
25. Hausen, B. M. (1993). *Centella asiatica* (Indian pennywort), an effective therapeutic but a weak sensitizer. *Journal of natural products* 56, 1123-1129.
26. Horobin, R.W., and Kiernan, J.A. (2002). *Conn's Biological Stains. A Handbook of Dyes Stains and Fluorochromes for Use in Biology and Medicine*, 10th Edition, Oxford.
27. Hsu, S.H., Tsai, T.R., Lin, C.N., Yen, M.H., and Kuo, K.W. (1996). The rhamnose moiety of solamargine plays a crucial role in triggering cell death by apoptosis. *Biochem Biophys. Res. Commun* 229, 1–5.
28. Hurtwitz, H., Fehrenbacher, L., and Novotny, W. (2000). Genetic alterations during colorectal tumor development. *New England journal of medicine* 11, 319: 525.
29. Hutchings, A.H., Scott, G., and Cunningham, A. (1996). *Zulu Medicinal Plants*. *Bothalia* 30, 100–114.
30. Inamdar, P.K., Yeole, K.D., Cibogare, A.B. and Desouza, N.J. (1996). Determination of biologically active constituents in *Centella asiatica*. *Journal of chromatography* 742, 127-130.

31. Kamatoua, G.P.P., Makungab, N.P., and Viljoena, A.M. (2008). South Africa *Salvia* species: A review of biological activities and phytochemistry. *Journal of Ethnopharmacology* 13, 1-9.
32. Kamuhabwa, A., Nshimo, C., and Witte D. (2000). Cytotoxicity of some medicinal plant extracts used in Tanzanian traditional medicine. *Journal of Ethnopharmacology* 70, 143-149.
33. Kasper, D.L., Braunwald, E., and Fauci, A. (2004). *Harrison's Principles of Internal Medicine* 16th Edition. McGraw-Hill Professional.
34. Kaufmann, S.H., and Vaux, D.L. (2003). Alterations in the apoptotic machinery and their potential role in anticancer drug resistance. *Oncogene* 22, 7414-7430.
35. Kintzios, S.E., and Barderaki, M.G. (2004). *Plants that fight cancer*. Churchill Livingstone, London.
36. Kiuchi, F., Shibuya, M., and Sankawa, U. (1982). Inhibitors of prostaglandin biosynthesis from *Alpinia officinarum*. *Chem. Pharm. Bull* 30, 754–757.
37. Levyns, M.R. (1935). A revision of *Elytropappus* Cass. *Journal of South African Botany* 1: 89-103.

38. Low, A.H., Kee, N., Mnonopi, N., Davids, H., Naudel, R.J., and Frost, C.L. (2008). Antithrombotic/anticoagulant and anticancer activity of selected medicinal plants from South Africa. *African Journal of Biotechnology* 7, 217-223.
39. Mander, M. (1998). Marketing of indigenous medicinal plants in South Africa. Food and Agriculture Organization of the United Nations, Rome.
40. Mills, S., and Bone, K. (2000). *Principles and practice of phytotherapy modern herbal medicine*. Churchill Livingstone, London.
41. Monks, N.R., Bordignon, S.A.L., Ferraz, A., Machado, K.R., Faria, D.H., Lopes, R.M., Mondin, C.A., de Souza, I.C.C., Lima, M.F.S., da Rocha, A.B., and Schwartsmann, G. (2002). Anti-tumor screening of Brazilian plants. *Pharmaceutical Biology* 40 (8), 603–616.
42. Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 65, 55-63.
43. Mqoqi, N., Kellert, P., Sitas, F., and Jula, M. (2000). Incidence of histologically diagnosed cancer in South Africa 1998-1999. *J Res* 1, 240-259.
44. Nagata, S. (2000). Apoptotic DNA Fragmentation, *Experimental Cell Research* 256, 12–18.
45. Narula, J., Haider, N., Virmani, R., DiSalvo, T.G., Kolodgie, F.D., and Hajjar, R.J. (1996). Apoptosis in myocytes in end-stage heart failure. *N Engl J Med* 33, 1182-1189.

46. Park, B.C., Bosire, K.O., Lee, Y.S. and Kim, J.A. (2005). Asiatic acid induces apoptosis in SK-MEL-2 human melanoma cell. *Cancer letters* 218, 81-90.
47. Perera, F.P and Weinstein, I.B. (2002). Molecular epidemiology and carcinogen-DNA adduct detection: new approaches to studies of human cancer causation. *J Chronic Dis* 35, 581–600.
48. Pettit, G.R., Pierson, F.H. and Herald, C.L. (1994). *Anticancer Drugs From Animals, Plants and Microorganisms*, John Wiley & Sons, Inc., New York.
49. Pinto, M., Robine-Leon, S., and Appay, M.D. (1983). Enterocyte-like differentiation and polarization of the human colon carcinoma cell line Caco-2 in culture. *Biol Cell* 47, 323-30.
50. Rabson, A., Roitt, I.M., and Delves, P.J. (2005). *Really essential medical immunology* 2nd edition, John Wiley & Sons, Inc., New York.
51. Reddy, B.S., Wang, C.X., Samaha, H., Lubet, R., Steele, V.E., Kelloff, G.J., and Rao, C. V. (1997). Chemoprevention of colon carcinogenesis by dietary perillyl alcohol. *Cancer Res* 57, 420–425.
52. Saklani, A and Kutty, S.K. (2007). *Plant-derived compounds in clinical trials*. Department of Natural Products, National Institute of Pharmaceutical Education and Research (NIPER), Punjab, India.
53. Schiff, P.B., Fant, J., and Horwitz, S.B. (1979). Promotion of microtubule assembly in vitro by taxol. *Nature* 277, 665-667.

54. Schiff, P.B., and Horwitz, S.B. (1981). Taxol assembles tubulin in the absence of exogenous guanosine 5'-triphosphate or microtubule-associated proteins. *Biochemistry* 20, 3247-3252.
55. Shukla, A., Rasik, A.M. and Dhawan, D.N. (1999). Asiaticoside induced elevation of antioxidant levels in healing wounds. *Phytotherapy research* 13, 50-54.
56. Steenkamp, V., and Gouws, M.C. (2006). Cytotoxicity of six South African medicinal plant extracts used in the treatment of cancer. *South African Journal of Botany*, 72: 630-633.
57. Stennicke, H.R. and Salvesen, G.S. (2000). Caspases-Controlling Intracellular Signals by Protease Zymogen Activation. *Biochimica Biophysica Acta*, 1477, 299-306.
58. Su, Z., Lin, J., Prewett, M., Goldstein, N.I., and Fisher, P.B. (1995). Anticancer Res 15, 1841-1848.
59. Thatte, U., Bagadey, S., and Dahanukar, S. (2000). Modulation of programmed cell death by medicinal plants. *Cellular and molecular biology* 46, 199-214.
60. Vacca, A., Ribatti, D., Iurlaro, M., Merchionne, F., Nico, B., Ria, R., and Dammacco, F. (2002). Docetaxel versus paclitaxel for antiangiogenesis. *Journal of hematotherapy & stem cell research* 11, 103-118.
61. Valenzuela, M.T., Siles, E., Villaobos, L., Jones, L., Guerrero, R., and Ruize de Almodovar, B.R. J. (2000). Cancer. *Celluar and Molecular Biology* 78, 1594-1501.

62. Van Cruchten, S., and Van Den Broeck, W. (2002). Morphological and biochemical aspects of apoptosis, oncosis and necrosis. *Anat Histol Embryol* 31, 214-223.
63. Van Wyk, B.E., van Oudtshoorn, B., and Gericke, N. (2000). Medicinal plant of South Africa, Vol 1, Briza Publications, Pretoria
64. Vogelstein, B., and Kinzler, K.W. (2004). Cancer genes and the pathways they control. *Nat Med* 10, 789-799.
65. Watt, J.M. (1999). African plants potentially useful in mental health,. *Lloydia* 30, 1–22.
66. Willingham, M.C. (1999). Cytochemical Methods for the Detection of Apoptosis. *The Journal of Histochemistry & Cytochemistry* 47, 1101-1109.
67. World Health Organization (2008). Regional office for western Pacific, research guidelines for evaluating the safety and efficacy of herbal medicines, Manila.
68. Yoon, Y., Kim, Y.O., Lim, N.Y., Jeon, W.K., and Sung, H.J. (1999). Shikonin, an ingredient of *Lithospermum erythrorhizon* induced apoptosis in HL60 human premyelocytic leukemia cell line *Planta Med* 65, 532–535.
69. Yoshida, M., Fuchigami, M., Nago, T., Okabe, H., Matsunaga, K. and Fujioka, T. (2005). Antiproliferative constituents from umbelliferae plants. *Biology and Pharmacology bulletin* 28, 173-175.

