Selective induction of apoptosis by 7-methyljuglone, its derivatives and isolated compounds from *Foeniculum vulgare* Mill. on human cancer cells

by

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List of abbreviations

Acquired Immunodeficiency Syndrome	AIDS
Adenosine diphosphate	ADP
Adenosine triphosphate	ATP
Afrikaans	Afr.
Basal cell carcinoma	BCC
Base pairs	bp
Before Christ	B.C.
Benign prostatic hypertrophy	BPH
Calcium	Ca^{2+}
Carbon dioxide	CO_2
Chloroform	$CHCl_3$
Chronic myelogenous leukaemia	CML
Colon carcinoma	COLO-205
Dalton's lymphoma ascetic	DLA
Death effector domains	DEDS
Deoxyribonucleic acid	DNA
Dysplastic oral keratinocyte	DOK
Ehrlich ascites carcinoma	EAC
English	Eng.
Fas ligand	FasL
Fifty percent inhibitory concentration	IC_{50}
Gas chromatography	GC
Gas chromatography-mass spectrometry	GC-MS
Glutathione (oxidized from)	GSSG
Glutathione (reduced form)	GSH

Hematologic remission	CHR
High-performance liquid chromatography	HPLC
Homoharringtonine	HHT
Human colon carcinoma	Caco-2
Human gastric adenocarcinoma	MK-1
Human immunodeficiency virus	HIV
Human papillomavirus	HPV
Human prostate cancer	LNCaP
Human uterine carcinoma	HeLa
H.G.W.J. Schwelckerdt Herbarium	PRU
Interleukin converting enzyme	ICE
Intraperitoneal	i.p.
Kilo-base pair	kb
Kilograms	kg
Lymphocytic leukaemia	P-388
Mammary gland epithelial, pleural effusion adenocarcinoma	MCF-7
Methanol	MeOH
Murine melanoma	B16F10
Micro mol	µM
Multi Drug Resistance	MDR
Minimal inhibitory concentration	MIC
Murine lymphocytic leukaemia	P-388
Murine melanoma	B16F10
Naphthoquinones	NQ
National Cancer Institute	NCI
Metastases	M
Nicotinamide adenine dinucleotide phosphate	NADPH

Mitochondrial permeability transition pore	MPTP
Node involvement	N
Non-small cell lung cancer	NSCLC
Oesophagus carcinoma	SNO
Peripheral blood mononuclear cells	PBMCs
Prostate epithelial carcinoma	DU-145
Prostate Specific Antigen	PSA
Squamous cell carcinoma	SCC
Sodium 3'-[1-(phenyl amino-carbonyl)-3,4-tetrazolium]-bis-[4-methoxy-6-nitro) benzene sulfonic acid hydrate	XTT
Squamous oesophageal carci- noma	WHCO3
Surveillance Epidemiology and End Results	SEER
Tuberculosis	TB
Tumour	T
Tumour necrosis factor	TNF
Tumour node metastases	TNM
TUNEL	TdT-mediated dUTP nick end labeling
United States	US
United States of America	USA
United States Department of Agriculture	USDA
Vero cells	African green monkey kidney cells
World Health Organization	WHO
Zearalenone	ZEA

Abstract

A naphthoquinone, 7-methyljuglone and some of its 5-hydroxy, 5-acetoxy-, 5-alkoxy- and 1,2,4,5-tetra-O-acetate derivatives were tested for their activity in four human cancer cell lines: breast adenocarcinoma, cervical epithelial carcinoma, oesophageal carcinoma and prostate epithelial carcinoma. Compound 2,5-dihydroxy-7-methyl-1,4-naphthoquinone was found to be the most effective one (exhibited a fifty percent inhibitory concentration (IC_{50}) in the range of 5.3 to 14.7 µM), while the parent compound 7-methyljuglone was less active than several of these derivatives. The IC_{50} values of 5-hydroxy-6-methyl-1,4-naphthoquinone were found to be between 19.1 and 15.4 µM on the four cell lines. However this compound showed toxicity on peripheral blood mononuclear cells. Six derivatives were selected for mechanistic studies. Considering the findings from cell cycle analysis, caspase 3/7 activation and annexinV-FITC dual labelling, 5-hydroxy-6-methyl-1,4-naphthoquinone was found to have antitumour effect by inducing apoptosis. Two derivatives namely, '8-fluoro-5-hydroxy-7-methyl-1,4-naphthoquinone' and '2,5-dihydroxy-7-methyl-1,4-naphthoquinone' were found to be not toxic on peripheral blood mononuclear cells suggesting their action is specific for tumour cells. Compound 2,5-dihydroxy-7-methyl-1,4-naphthoquinone was found to induce apoptosis through caspase 3/7 activation. In view of the enhanced potencies associated with these derivatives, these analogues may hold considerable therapeutic potential for the treatment of leukaemia cancers.

The ethanol extracts of seven plant species (ethnobotanically selected) were also tested for their cytotoxicity, assayed by the XTT assay, against four human cancer cell lines at concentrations ranging from 0.78 to 100 µg/ml. Of all the ethanol extracts, *Foeniculum vulgare* was found to have the best activity on HeLa cells, which exhibited an IC_{50} value of 19.97± 0.048 µg/ml. Therefore, it was selected for isolation of the bioactive principles. The extract of *Foeniculum vulgare* was fractionated using column chromatography with hexane and ethyl acetate at different ratios as eluent. Two known compounds, '4-methoxycinnamyl alcohol' and 'syringin' were isolated. The IC_{50} values of '4-methoxycinnamyl alcohol' and 'syringin' were found to be 7.82 ± 0.28 µg/ml and 10.26 ± 0.18 µg/ml respectively on HeLa cells. Both compounds were tested for their cytotoxicity against U937 cells and also on

peripheral blood mononuclear cells. At the concentrations of 10 and 100 µg/ml '4-methoxycinnamyl alcohol' showed similar cell proliferation as that of the positive control 'cisplatin'. 'Syringin' however, had much lower cytotoxicity on the U937 cells than '4-methoxycinnamyl alcohol'. IC_{50} was found to be 91.14 ± 0.63 µg/ml. Both 'syringin' and '4-methoxycinnamyl alcohol' were not cytotoxic at concentrations of 1 and 10 µg/ml on the PBMCs as compared to cisplatin. '4-Methoxycinnamyl alcohol' was selected based on its activity on the cancer cells, for further investigation with regard to its mechanism of action. On gel electrophoresis it did not show a typical ladder pattern, instead a characteristic smear resulted which indicated necrosis.

Two best derivatives of 7-methyljuglone ('8-fluoro-5-hydroxy-7-methyl-1,4-naphthoquinone' and '2,5-dihydroxy-7-methyl-1,4-naphthoquinone') and the ethanol extract of *F. vulgare* warrant further investigation to be considered for their potential as anticancer agents.

CHAPTER 1

Introduction

Chapter 1

Introduction

1.1   Background

Since the earliest times naturally occurring substances from plants, animals and minerals provided a source of medicine for man. For a long time man has exploited particularly the plant kingdom, which has proved to be very useful for treating most of our ailments. During the course of history, experimentation has succeeded in distinguishing those plants which have beneficial effects from those that are toxic or merely non-effective (Lall, 2000). Throughout the centuries, humans have found through trial and error ways to relieve their pain and sickness. Every cultural group has responded by developing a medicinal system and making use of natural products to cure various aliments because of the fear of disease and death (Ellis, 1986). These traditions may seem strange and magical, others appear rational and sensible, but all of them are attempts to overcome illness and suffering and enhance quality of life (Finimh, 2001).

Plants have adapted to the diverse habitats of the world through their physical and biomedical modifications. For thousands of years plants have been used traditionally as a source of treatment for various ailments throughout the world among all human races (Ellis, 1986). Plants have always provided an important source of medicines, and were first used in folk medicine. In ancient times in various cultures worldwide people have always been using holistic means of healing. In contrast to the frequent assumptions, the medicines used by traditional healers are surprisingly effective.

Historically the development of many important classes of drugs relied on natural products that have served as templates (McChesney, 1993). Six major fields of study contribute to the studies on natural products. These include: ethnoecology, traditional agriculture, cognitive ethnobotany, material culture, traditional phytochemistry and palaeoethnobotany. Ethnobotany is defined by Cotton (1996) as all the studies, which describe local people's interaction with the natural environment, as well as all the studies, which concern the mutual relationships between plants and traditional people. This definition is a very broad description of a large range of subjects such as ethnomedicine, ethnotaxonomy, ethnoecology etc.

Ethnopharmacology on the other hand is known as the scientific evaluation of traditional medicine, which usually excludes spiritual and mythical aspects of plant use. The aim is here to determine if the plants that are used have any biologically active compounds. Scientists are not affiliated with the belief of the people. The ability to correlate the ethnobotanical reports with corresponding scientific studies could lead to the improved selection of plants for study in the healthcare system (Lall, 2000). Ethnopharmacology provides an alternative approach for the discovery of medicinal compounds.

The results obtained by researchers, ethnobotanists and scientists often justified the use of plants in folk medicine and is a serious basis for the improvement of the efficacy, safety and quality of the plant remedies used worldwide. Conventional western medicine accepts folk medicine only when their efficacy is confirmed (Philp, 2004).

1.2   The role of natural products in western medicine

Modern medicine has benefited significantly from anecdotal results of their empirical methodology (Lewis *et al.* 1995). Presently large numbers of illnesses are treated by choosing necessary candidates for a pharmacopoeia that is inadequate (Lewis *et al.* 1995). It is estimated that about 25% of all modern medicines are directly or indirectly derived from higher plants, and 11% of the drugs considered as basic and essential by the World Health Organization (WHO), are of plant origin. More than half of the world's 25 top selling pharmaceuticals, for 1991, owe their origin to a variety of natural source materials (Table

1.1). Two of these top selling pharmaceuticals cyclosporine and mevinolin) are natural products and 12 others are natural product derived (O'Neill, 1993).

Table 1.1 The world's 25 best selling pharmaceuticals in 1991 (Phillips & Drew, 1992).

Position 1991	Product	Therapeutic Class	Sales $m
1	Ranitidine	H2 antagonist	3,032
2	[a]Enalapril	ACE inhibitor	1,745
3	[a]Captopril	ACE inhibitor	1,580
4	[a]Diclofenac	NSAID	1,185
5	Atenolol	β-antagonist	1,180
6	Nifedipine	Ca^{2+}	1,120
7	Cimetidine	H_2 antagonist	1,097
8	[a]Mevinolin	HMGCoA-R inhibitor	1,090
9	[a]Naproxen	NSAID	954
10	[a]Cefaclor	β-lactam antibiotic	935
11	Diltiazem	Ca^{2+} antagonist	912
12	Fluoxetine	5HT reuptake inhibitor	910
13	Ciprofloxacin	Quinolone	904
14	Amlodipine	Ca^{2+}	896
15	[a]Amoxycillin/ clavulanic acid	β-lactam antibiotic	892
16	Acyclovir	Anti-herpetic	887
17	[a]Ceftriaxone	β-lactam antibiotic	870
18	Omeprazole	H^+ pump inhibitor	775
19	Terfenadine	Anti-histamine	768
20	[a]Salbutamol	β_2-agonist	757
21	[a]Cyclosporin	Immunosuppressive	695
22	[a]Piroxicam	NSAID	680
23	Famotidine	H_2 antagonist	595
24	Alprazolam	Benzodiazepine	595
25	[a]Oestrogens	HRT	569

[a]Natural product derived

The world market in 1997, for over the counter phytomedicinal products was United States (US) $ 10 billion, with an annual growth of 6.5% (Rates, 2001). The North American market for products of plant origin reached US$ 2 billion in 1997 (Rates, 2001). Fifty percent of the phytomedical products in Germany are sold on medical prescriptions. Well-established herbal medicine industries are found in China and India (Rates, 2001). About 60% of the medicines currently available on the market that are derived from natural products are mainly from higher plants as well as most of those in the trial stages (Calixto, 2000). Higher plants therefore, contribute about one quarter of the prescriptions dispensed. Ethnomedicines represent an important class of natural therapeutics that are used and accepted worldwide. Numerous examples from medicine impressively demonstrate the innovative potential of natural compounds and their impact on drug discovery and development. Traditional healers today still use plants in their crude form of herbal remedies although Western technologies have transformed these plant products into more palatable forms. The search for new plant derived drugs and the recognition and validation of traditional medicine could lead to new strategies to control various diseases. Current literature suggests that traditional healers, operating within many rural communities where local products are more accepted, should become the subject of intense research to establish new ways of strengthening collaboration between the traditional healers and modern health care providers (Ndubani & Hojer, 1999). Natural products play a significant role as source of drug leads in the discovery and understanding of cellular pathways that are a vital component of innovation in the drug discovery process (Guallo *et al.*, 2006).

Development of naturally derived drugs (pharmaceuticals) to clinically active agents took 30 years until in the 1990s. New drugs originating from natural sources in the areas of cancer and infectious diseases are 60% and 75%, respectively (Guallo *et al.*, 2006), between 1981 and 2002, and 23 new drugs (derived from natural products) between 2001 and 2005, were introduced for the treatment of disorders such as bacterial and fungal infections, cancer, diabetes, dyslipidemia, atopic dermatitis, Alzheimer's disease and genetic diseases such as tyrosineamia and Gaucher disease (Table 1.2) (Lam, 2007). Furthermore, a total of 136 natural–product-derived drugs have undergone various stages of clinical development that might be used to treat human diseases in all major therapeutic areas in the future (Lam, 2007). Natural products also had a major impact on cancer chemotherapy. Newman *et al.*, reported that more than 60% of the approved drugs for

cancer treatment are natural products or derived from natural products (Newman *et al.*, 2003). Currently more than 30% of compounds of microbial origin are undergoing various stages of clinical development as anticancer agents. Marine organisms led to the discovery of two novel anticancer agents which are in Phase I clinical studies (Lam, 2007).

Table 1.2 Drugs derived from natural products launched in Europe, Japan and the United States 2001-2005 (Lam, 2007).

Year	Generic name (trade name)	Natural product	Indications
2001	Caspofungin (Cancidas®)	Pneumocandin B	Antifungal
2001	Pimecrolimus (Elidel®)	Ascomycin	Atopic dermatitis
2001	Telithromycin (Ketek®)	Erythromycin	Antibacterial
2002	Amrubicin hydrochloride (Calsed®)	Doxorubicin	Anticancer
2002	Biapenem (Omegacin®)	Thienamycin	Antibacterial
2002	Ertapenem (Invanz™)	Thienamycin	Antibacterial
2002	Fulvestrant (Faslodex®)	Estradiol	Anticancer
2002	Galantamine (Reminyl®)	Galantamine	Alzheimer's disease
2002	Micafungin (Funguard®)	FR901379	Antifungal
2002	Nitisinone (Orfadin®)	Leptospermone	Antityrosinaemia
2003	Daptomycin (Cubicin™)	Daptomycin	Antibacterial
2003	Miglustat (Zavesca®)	1-deoxynojirimycin	Type 1 Gaucher disease
2003	Mycophenolate sodium (Myfortic®)	Mycophenolic acid	Immunosuppression
2003	Pitavastatin (Livalo®)	Mevastatin	Dyslipidemia
2003	Rosuvastatin (Crestor®)	Mevastatin	Dyslipidemia
2004	Everolimus (Certican™)	Sirolimus	Immunosuppression
2004	Talaporfin sodium (Laserphyrin®)	Chlorophyll and L-aspartic acid	Anticancer
2005	Doripenem (Finibax®)	Carbapenem	Antibacterial
2005	Extenatide (Byetta®)	Incretin	Anti-diabetic
2005	Paclitaxel nanoparticles (Abraxane®)	Taxol	Anticancer
2005	Pramlintide acetate (Symlin®)	Amylin	Anti-diabetic
2005	Tigecycline (Tigacil®)	Tetracycline	Antibacterial
2005	Zicontide (Prialt™)	MVIIA	Pain management

There are several advantages to screen natural products for drug discovery that outweigh their limitations and some are more important, 'quantifiable' advantages: (i) Natural products offer unmatched chemical diversity together with their structural complexity as well as biological potency; (ii) A complementary region of chemical space is occupied by natural products as compared to that of synthetic compounds; (iii) In combinatorial chemistry natural products are used as templates which enables the creation of libraries of analogs, which might have enhanced drug-like properties (e.g. pharmacokinetics, solubility); they also might increase our understanding of the genetics and biosynthesis of natural products so that the guidelines of natural product biosynthesis can be optimized; (v) Compounds from natural products could possibly lead to the discovery and better understanding of the disease process and pathways involved by using these targets, (vi) Synthetic drugs are typical due to their structural modifications where natural products can go strait from 'hit' to a drug (Lam, 2007).

Cancer represents one of the most severe health problems worldwide and two fields that are of utmost importance in drug discovery and clinical therapy is the development of new anticancer drugs and more effective treatment strategies (Altmann & Gertsch, 2007). Today most research is focused on cancer-specific mechanism and its corresponding molecular targets as well as for improved cytotoxic agents in the functional inhibition of cellular microtubules for better treatment strategies. It is crucial for further advances in cancer therapy.

1.3   Global use of plants as medicine

Throughout the past decade the practice of traditional healers served as an increasing global interest for their use of medicinal plants to treat illness (Akerele, 1994). Presently a medicinal plant (used to treat disease) is defined as a plant that has pharmacological activity where an edible plant is used as food in daily life (Park & Pezzuto, 2002). According to the WHO, the lack of access to modern medicine and poverty forces 65-80% of the world's population living in developing countries to depend solely on plants for primary health care. The use of plant products ranged from three to 80% based on prior research and this research varied in different geographical areas (WHO, 1993).

1.4   Medicinal plant use in Africa

Throughout the history and even today mankind was provided with plants to serve as herbal remedies for many illnesses or diseases, they continue to be an important part in developing countries as therapeutic remedies in primary health care (Tshikalange *et al.*, 2005). It is estimated by the WHO that between 60-90% of Africa's population relies on medicinal plants totally or partially to meet their health care needs. Herbal medicines or traditional remedies play a major role in the culture, traditions and religious life of African people (Fennell *et al.*, 2004; Steenkamp, 2003). This is true also for South Africa where up to 60% of the population consult an estimated 200 000 traditional healers, especially in rural areas where traditional healers are more accessible than Western doctors (Taylor *et al.,* 2001). Traditional healers are most commonly known by the Zulu people as 'inyangas' or 'herbalists' and 'isangomas' or 'diviners', but the distinction between the two has become blurred, with both using herbal medication (Van Wyk *et al.*, 1997). Traditional healers or

practitioners are also known in Xhosa as 'ixwele' or 'amaquira', in Sotho as 'ngaka' and in Venda as 'nanga', 'mungome' or 'maine' (Mabogo, 1990; Van Wyk *et al.*, 1997; Steenkamp, 2003). It is often argued that traditional healers operate closer to people and that they are indispensable health care providers in many rural communities of Africa where modern medicine is not readily available (Ndubani & Hojer, 1999). The traditional healer or practitioner relies on symptomatic diagnosis of disease and pays special attention to the use of herbs in treating various diseases (Mabogo, 1990). The plant-part used depends on the nature and state of the disease and varies from one species to another; it varies also from practitioner to practitioner (Mabogo, 1990; Steenkamp, 2003). In urban areas remedies are purchased by the local people at muti markets or shops. In KwaZulu Natal it is estimated that approximately 80% of the population use plant medicine only. Annually more than 20 000 tons of plant material is harvested processed and sold as traditional medicine. The disadvantage of this is that overexploitation of wild populations and useful medicinal plants become inevitable. In South African healthcare 4000 plant taxa are ethnomedicinally used in traditional medicine (Fennell *et al.*, 2004). The possibility of serious toxicity can arise in few of these plants. Other dangers also exist with the misadministration or dosage, misidentification, especially of toxic plants, mutagenic effects and the potential of genotoxicity that follow prolonged use of some of the popular herbal and traditional remedies (Fennell *et al.*, 2004).

The Southern African flora consists of just about 25 000 species of higher plants whereof 3 000 species are used medicinally, of which approximately 350 species are commonly used and traded (Taylor *et al.,* 2001). Detailed analysis of the pharmacological properties of medicine used traditionally brought to light the presence of innumerable acids, alkaloids, flavonoids, terpenoids, oils, gums, resins, fats etc. in medicinal plants. Some of these ingredients with a specific pharmacological action have been identified by pharmacologists. The traditional use of medicinal plants and the pharmacological activity of extracts previously investigated show a viable approach to pharmaceutical research in the areas of various diseases such as arthritis, tuberculosis (TB), cancer, diabetes, bacterial and viral infections (Lall, 2000). Many South African medicinal plants have been known to possess potentially valuable therapeutic agents. It is known that traditional healers use indigenous medicinal plants to treat many illnesses including cancer. Despite this, in South Africa, plants used for the treatment of cancer are rarely reported on (Steenkamp & Gouws,

2006). For many South Africans, traditional medicines have become a way of life and are part of the cultural and religious life of these people, especially in the rural areas but also in urban areas (Steenkamp, 2003). It has been estimated that between 12 to 15 million South Africans, use traditional remedies to treat diseases and heal wounds from as many as 700 different plants. When selecting plants used to treat cancer it has been recommended that ethnopharmacological usages such as immune and skin disorder, inflammatory, infectious, parasitic and viral diseases be taken into account, since these reflect disease states bearing relevance to cancer or a cancer symptom (Steenkamp & Gouws, 2006).

It is essential to have new anti-cancer agents that can be produced from local source substances such as natural vegetation. Traditional/herbal/plant medicines have been used in the rural areas for centuries by local healers. These medicines give a good lead for discovering new drugs with antibacterial, antifungal and antitumour properties. Plant products have been shown to be valuable sources of novel anti-cancer drugs therefore, large-scale projects should be implemented to test compounds from potentially useful medicinal plants (Mans *et al.*, 2000). There is also a considerable scientific and commercial interest in the continuing discovery of new anticancer agents from all natural product sources including from plants (plant secondary metabolites) (Mann, 2002). Anticancer therapeutics has gained enormous attention by scientists from the enormous pool of synthetic, biological and natural products that spawn a prolific output, all over the world (Mukherjee *et al*, 2001). With the introduction of molecular biological models into phytopharmacology and new target directed pharmacological screening methods, research is essential to get more detailed information on the underlying mechanisms of action of multivalent herbal plant preparations, extracts and compounds as well as their synergistic effects which will help to integrate more plant extract preparations into the concept of modern medicine (Wagner, 1999).

1.5   Plants as a source of anti-cancer agents

Historically plants have been valuable sources used in the treatment of cancer (Hartwell, 1982) and many other diseases. Hartwell published a long list of more than 3000 plants that are being used in the treatment of cancer. In many instances the cancer is undefined

and is reported on symptoms that apply to the skin or other visible conditions that sometimes correspond to cancerous conditions (Cragg & Newman, 2005). This is a problem because cancer is poorly defined in traditional medicine and folklore. Despite these observations, an essential role have been played by plants as a source of effective anti-cancer agents, and natural sources derived from plants, marine organisms and micro-organisms account for over 60% of currently used anti-cancer agents (Cragg & Newman, 2005). Nevertheless, a well reputable armamentarium of valuable chemotherapeutic agents have come from approximately five decades of systemic drug discovery and development, together with several significant achievements in the treatment and management of human cancer (Mans *et al.*, 2000). In reality, chemotherapy effectiveness has to endure different confounding factors that include systemic toxicity due to lack of specificity, rapid drug metabolism, and both intrinsic and acquired drug resistance, furthermore the most unpredictable factor affecting chemotherapy is multidrug resistance since tumour cells are very adaptable (Johnstone *et al.*, 2002).

In the 1950s the search for anti-cancer agents from plants sources started to intensify. It was also in this time when the discovery and development of vinblastine and vincristine (vinca alkaloids), and the isolation of the cytotoxic podophyllotoxins took place (Cragg & Newman, 2005). This led to the intensive plant collection from temperate regions in the 1960s plus the discovery of novel chemotypes such as the taxanes and camptothecins, which showed a range of cytotoxic activities (Cassady & Douros, 1980; Cragg & Newman, 2005).

Revival of plants and other organisms took place when new screening technologies were developed in the 1986, now the focus was on the tropical and sub-tropical regions of the world (Cragg & Newman, 2005). Even though several anticancer agents are now in the preclinical development no new clinical agents from plants have reached the stage of general use (Cragg & Newman, 2005).

There are many plant-derived anticancer agents which are in clinical use these days. These include the vinca alkaloids vinblastine and vincristine (Figure 1.1 a) that were isolated in minute quantities from *Catharanthus roseus* G. Don. (Apocynaceae) (Figure 1.1 b). Vincristine possesses a formyl group where vinblastine has a methyl group and despite

these small differences their toxicological properties and spectra of antitumour activities differ (Hill, 2001).

During the search for potential oral hypoglycaemic agents it was found that extracts of *C. roseus* reduced white blood cell counts and caused bone marrow depression in rats, and afterwards in mice studies these extracts were active against lymphocytic leukaemia (Cragg & Newman, 2005). Combinational chemotherapy regimes use vincristine as a key component for the treatment of acute lymphocytic leukaemia, a number of children's solid tumours, acute childhood leukaemia, Hodgkin and non-Hodgkin lymphomas as well as multiple myeloma, breast and small-cell lung cancer in adults (Hill, 2001). Vinblastine, alternatively, is an essential component of curative chemotherapy regimens used for testes germ cell cancers and advanced Hodgkin disease and it is also frequently used to treat carcinoma of the bladder, breast and Kaposi's sarcoma through combinational therapy with other anti-cancer drugs (Hill, 2001). Vinblastine and vincristine remain in wide spread clinical usage even today. Analogs produced via synthetic modification could possibly have activity against other tumour types, less toxicity and side effects (Lee, 1999). Several semi-synthetic analogs were made and the most recent are vinorelbine and vindesine (the C-3 amidoanalog of 4-deacetyl vinblastine). Vindesine when compared with other natural vinca alkaloids has less neurotoxicity, but causes complete remission in acute lymphocytic childhood leukaemia and adult nonlymphocytic leukaemia (Lee, 1999). These semi-synthetic analogs are used in combination with other cancer chemotherapeutic drugs and used against a variety of cancers, breast, lung and highly developed testicular cancers as well as Kaposi's sarcoma (Cragg & Newman, 2005).

(a)   (b)
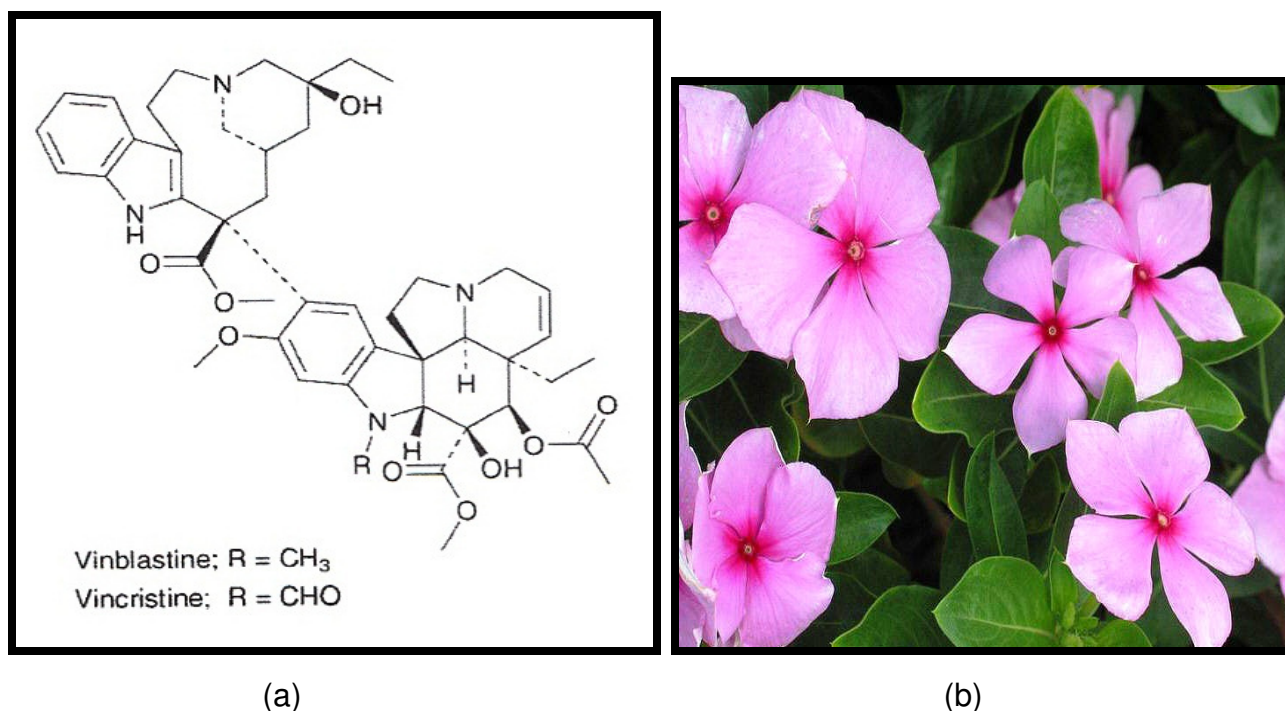
Figure 1.1 (a) Natural alkaloids 'Vinblastine' and 'Vincristine' isolated from (b) *Catharanthus roseus* (Cragg & Newman, 2005) (http://www.biologie.uni-regensburg.de/Botanik/Schoenfelder/kanaren/images/Catharanthus_roseus.jpg).

Another two semi-synthetic derivatives are etoposide (Figure1.2 a) teniposide (Figure1.2 b) of the parent compound epipodophyllotoxin (Figure 1.2 c) (natural product), which is an isomer of podophyllotoxin (Figure 1.2 d). Etoposide and teniposide are clinically used for the treatment of testicular, lymphomas, leukemias and bronchial cancers, but their use is limited due to problems such as drug resistance, poor bioavailability and myelosuppression therefore they need further structural modification. The medicinally used *Podophyllum* species (Podophyllaceae) from the Indian subcontinent, *Podophyllum peltatum* Linnaeus (commonly known as the American mandrake or Mayapple), and *Podophyllum emodii* Wallich, have been used extensively for the treatment of skin cancers and warts throughout history (Cragg & Newman, 2005). In 1880 the major active constituent, 'podophyllotoxin' was first isolated and only in the 1950s its correct structure was reported. Podophyllotoxin functions as a mitotic inhibitor by binding reversibly to tubilin and it inhibits microtubule assembly (Lee, 1999). Many ligans directly related to podophyllotoxin (podophyllotoxin-like) were reported; several of them were dropped from the clinical trials, due to their unacceptable toxicity and lack of efficacy (Cragg & Newman, 2005). Etoposide and its thiophene analog teniposide are structurally related to podophyllotoxin, but at C-4 they

have opposite stereochemistry (β in etoposide and teniposide, α in podophyllotoxin), different substituents (glycosyl in etoposide and teniposide, OH in podophyllotoxin) and at C-4' (OH in etoposide and teniposide, OMe in podophyllotoxin) (Lee, 1999). The antitumour action of etoposide and its analogs is to inhibit DNA topo II (an essential enzyme) and, then increase DNA cleavage. Etoposides other actions include covalent protein binding by a bio-oxidized E-ring orthoquinone, and metal- and photo induced cleavage of DNA caused by hydroxyl radicals formed from metal-etoposide complexes (Lee, 1999).

(a)   (b)

(c)   (d)
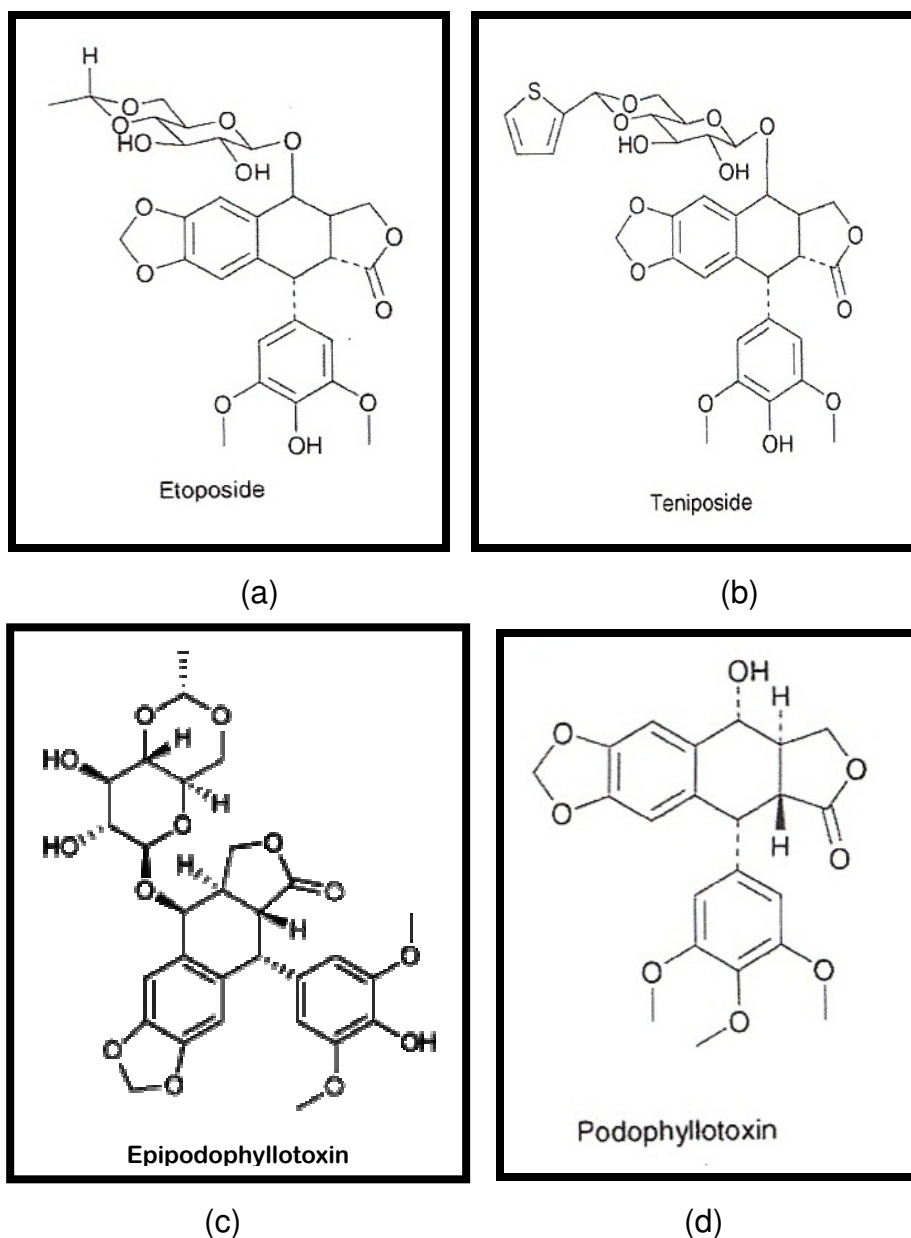
Figure 1.2 Semi-synthetic derivatives of epipodophyllotoxin, an isomer of podophyllotoxin (a) etoposide and (b) teniposide which are clinically active (c) epipodophyllotoxin (d)

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podophyllotoxin
(http://www.nature.com/nchembio/journal/v3/n8/images/nchembio.2007.10-COMP19.gif)
(Cragg & Newman, 2005).

The taxanes were more recently added to the armamentarium of plant-derived chemotherapeutic agents (Kingston, 2005) Paclitaxel (taxol®) (Figure 1.3 a) was isolated originally by Wani and his co-workers in 1971, from the bark of *Taxus brevifolia* Nutt. (Taxaceae) (Pacific Yew) (Figure 1.3 b), and today paclitaxel, together with several key precursors (the baccatins), are found in the leaves of various *Taxus* species (Cragg & Newman, 2005). Paclitaxel is used alone or in combination with other cancer drugs primarily for the treatment of ovarian, breast, and non-small cell lung cancer (NSCLC). Furthermore it showed efficacy against Kaposi sarcoma, potential treatment of multiple sclerosis, psoriasis and rheumatoid arthritis. It has a unique mode of action; it acts as a mitotic inhibitor by promoting the assembly of microtubules (Lee, 1999) and interacts with the polymerized form of αβ-tubulin (Altmann & Gertsch 2007). Taxol induces apoptosis in proliferating cells through cell cycle arrest at G2/M. From early on, supply was a major obstacle in the development of paclitaxel since it is present only in minute quantities. It led to the synthesis of 'Taxotere', a form of 10-deacetyl-baccatin III which is more readily available. This compound was isolated from the European yew tree (*Taxus baccata* L.). A major renewable natural source were made available to this important class of drugs through the semi-synthetic conversion of baccatins to paclitaxel, and biologically active paclitaxel related analogs, such as docetaxel (Taxotere®) (Cragg & Newman, 2005). Docetaxel is used in the treatment of breast cancer and NSCLC, and has microtubule-stabilizing properties (Altmann & Gertsch 2007). It has also shown efficacy in combination with other cancer drugs such as anthracyclins, prednisone and cisplatin, paclitaxel. Other *Taxus* species such as *Taxus canadensis* Marshall, *T. baccata* L. and other parts of *T. brevifolia* were used by the Native American tribes for non-cancerous conditions. Only one report for the use of cancer was found for *T. baccata* in the traditional Asiatic Indian (Ayurvedic) medicine system. Currently another 23 taxanes are in the preclinical stage, 9 are undergoing PhaseI/II clinincal development for new and improved anti-cancer agents, and several other second generation taxanes were selected for clinical development to improve their solubility and activity against drug resistant tumours (Cragg & Newman, 2005; Altmann & Gertsch 2007). Taxanes are popular antitumour agents but clinical

resistance poses a threat to their successful treatment. Effective conventional chemotherapy treatment of cancer is difficult due to multidrug resistance (Lucci *et al.*, 1999).

(a)   (b)
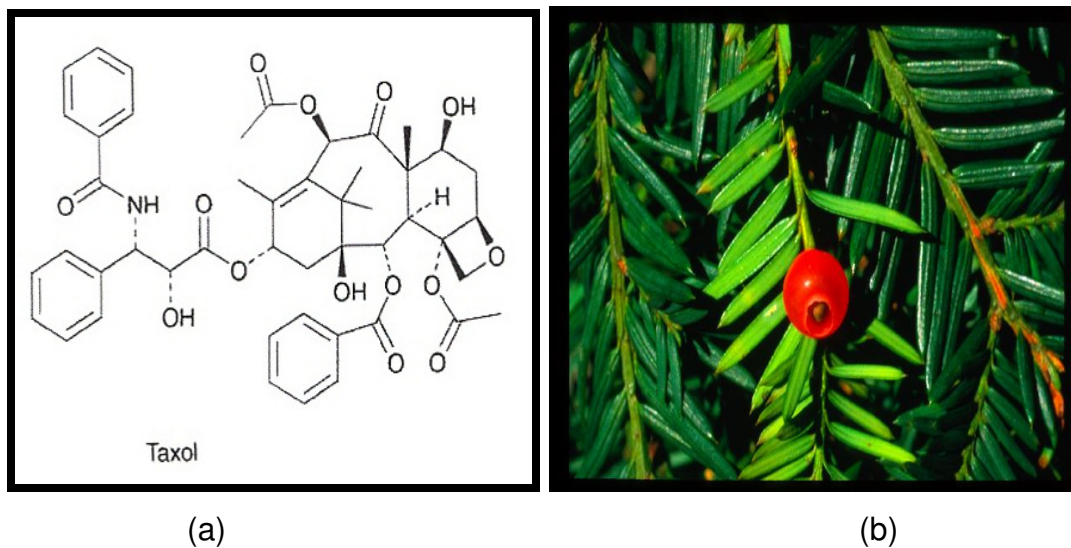
Figure 1.3 (a) Taxol isolated from (b) *Taxus brevifolia* (Cragg & Newman, 2005) (http://arnica.csustan.edu/boty3050/medicinal/taxus_brevifolia.jpg).

From *Camptotheca acuminate* Decne (Nyssaceae) 'camptothecin' (Figure 1.4), a natural alkaloid, was isolated. Camptothecin is another important addition to the anti-cancer drug armamentarium of clinically active agents. The National Cancer Institute (NCI) (US), advanced camptothecin (as sodium salt) to clinical trials in the 1970s. It caused severe bladder toxicity and was consequently dropped. Topotecan (used for treatment of ovarian and small cell lung cancers) and irinotecan (used for treatment of colorectal cancers) (CPT-11; Camptosar) were developed throughout extensive research and structural modification as more effective derivatives of camptothecin (Cragg & Newman, 2005; Tazi *et al.*, 2005; Lee, 1999). These compounds together with camptothecin were potent antitumour and DNA topo I inhibitory agents (Lee, 1999). Both derivatives are amine-hydrochloride salts. Topotecan was made to be 100-fold more water soluble than the parent compound camptothecin, which is poorly water soluble. Irinotecan was metabolized to be more potent, about 200 to 1000 times more than camptothecin, and *in vivo* is a phenolic topo I inhibitor. Several second- and third-generation camptothecins e.g. exatecan and diflomotecan are currently undergoing clinical trials (Tazi *et al.*, 2005). Five less toxic, water soluble synthetic 7-(acylhydrozono)-formyl camptothecins were found to be more

potent than camptothecin in causing protein-linked DNA breaks and DNA topo I inhibition (Lee, 1999).
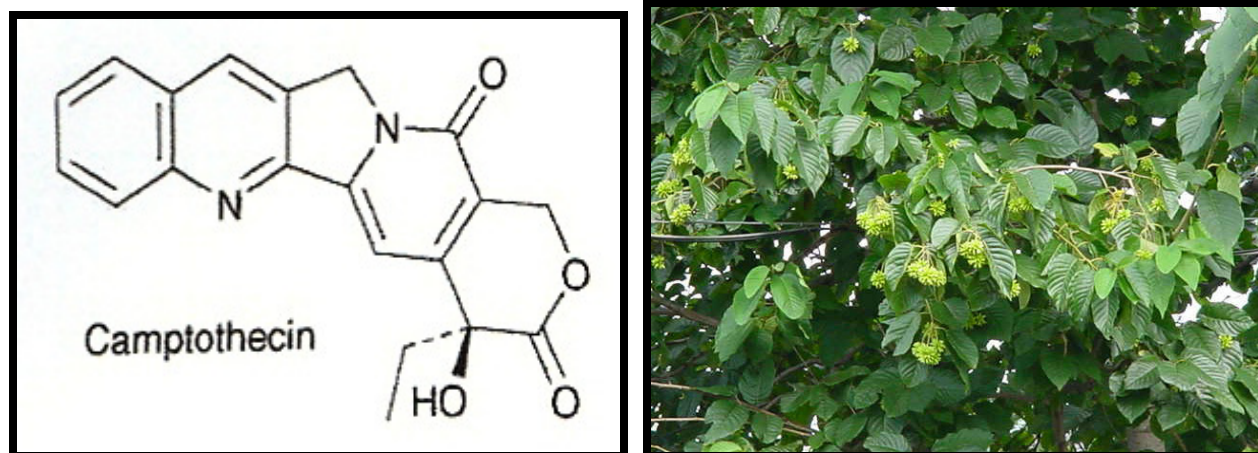
Figure 1.4 A natural alkaloid 'camptothecin' from *Camptotheca acuminate* (http://hortiplex.gardenweb.com/plants/jour/p/56/gw1007256/1452951021731136.jpeg) (Cragg *et al.*, 2005).

'Homoharringtonine' (HHT) (Figure 1.5 a) was isolated from *Cephalotaxus harringtonia* var. *drupacea* (Sieb and Zucc.) (Cephalotaxaceae) (Figure 1.5 b) (Itokawa *et al.*, 2005). In China, acute myelogenous leukaemia and chronic myelogenous leukaemia is treated effectively with the use of a harringtonine and HHT racemic mixture. In patients with chronic myelogenous leukaemia (CML) in the late chronic phase complete hematologic remission (CHR) has been reported by homoharringtonine and it is effective against various leukaemias (Cragg & Newman, 2005). Elliptinium, a derivative of ellipticine, was isolated from a Fijian medicinal plant *Bleekeria vitensis* A.C. Sm. (known for anticancer properties) and species of several genera of the Apocynaceae family, is marketed for treatment of breast cancer in France (Cragg & Newman, 2005). These plant derived agents are also clinically in use.
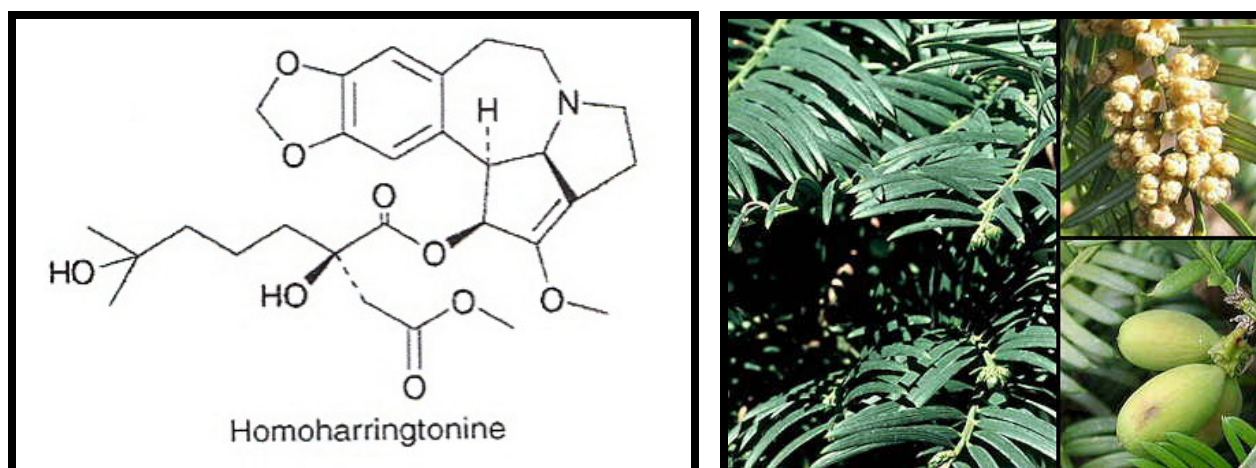
Figure 1.5 (a) 'Homoharringtonine' isolated from the Chinese tree (b) *Cephalotaxus harringtonia* var. *drupacea* (Cragg & Newman, 2005) (http://www.arbolesornamentales.com/Cephalotaxusharri.jpg).

By systematically screening plant extracts, clinically active agents with antitumour properties can be isolated so that their metabolism can be explained so that more active molecules might be synthesized (Hill, 2001).

1.6 Plant derived anticancer agents in clinical development

From *Dysoxylum binectariferum* Hook. f., Meliaceae family, rohitukine was isolated and this flavonoid structure formed the basis for a novel synthetic flavonoid structure, flavopiridol (Figure 1.6 a). During a structure activity study over 100 synthetic analogs were synthesized. These analogs were tested against a series of breast and lung carcinoma cell lines, in the course of these studies it was found that they have tyrosine kinase activity and potent growth inhibitory activity (Cragg & Newman, 2005). Flavopiridol showed the most potent activity. I*n vivo* (in mice) broad spectrum activity was found against human tumour xenografts. The NCI then selected it for preclinical and clinical studies in collaboration with the Hoechst Company; currently it is in 18 Phase I and Phase II clinical trials. Flavopiridol is effectively used alone or in combination with other anticancer agents to treat a broad range of tumours, leukemias, lymphomas and solid tumours (Cragg & Newman, 2005).

In the 1970s the NCI and United States Department of Agriculture (USDA) were working together with the South Africa Botanical Research Institute on a random collection

program. Combretastatins, a family of stilbenes, were isolated from the South African *Combretum caffrum* (Eckl. & Zeyh.) Kuntze, which was collected as part of that random collection program. The genera *Combretum* and *Terminalia* both belong to the family Combretaceae that are used for malaria, hepatitis and a variety of other diseases in Indian and African traditional medicine. Reportedly, several of the *Terminalia* species have been used for cancer treatment. The combretastatins act as anti-angiogenic agents, they cause tumour necrosis through vascular shutdown in tumours (Cragg & Newman, 2005). One of the water-soluble analogs of the combretastatins, A4 phosphate (CA4) (Figure 1.6 b), has shown promising activity in early clinical trials, and now several mimics are being developed of which three is in clinical trials and another 11 in the preclinical development. By combining medicinal and combinatorial chemistry a multitude of analogs were synthesized from this chemical class that served as a model which had a relatively simple natural product structure. All of them containing the crucial trimethoxy aryl moiety linked to substituted aromatic moieties through a variety of two or three atom bridges together with heterocyclic rings and sulfonamides (Li & Sham, 2002).

Flavopiridol
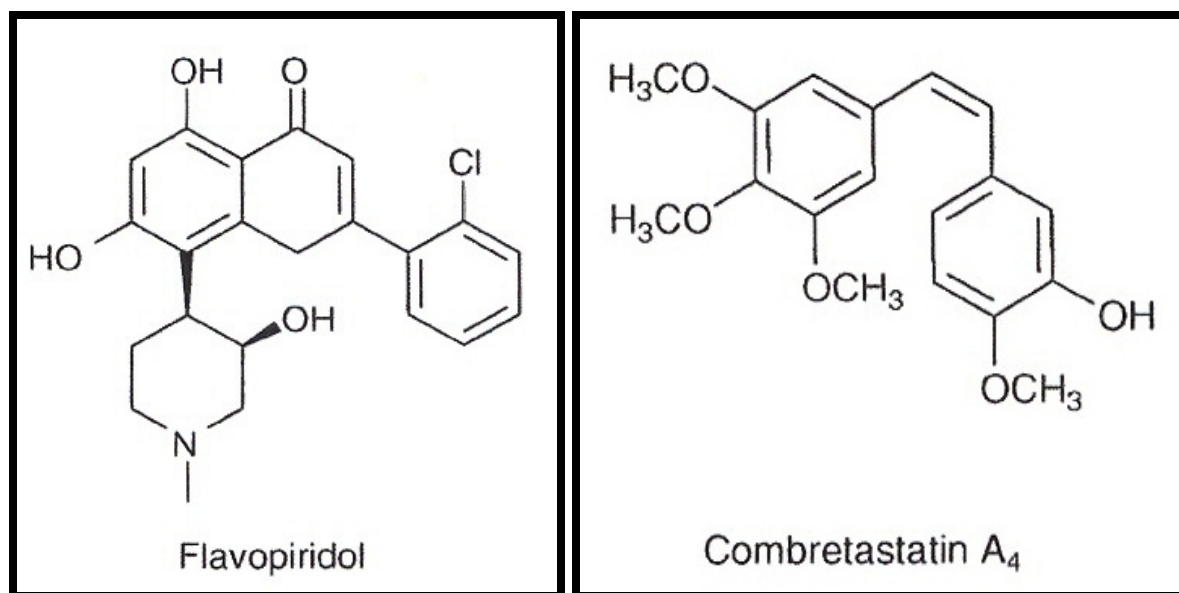
Combretastatin A4

(a)   (b)
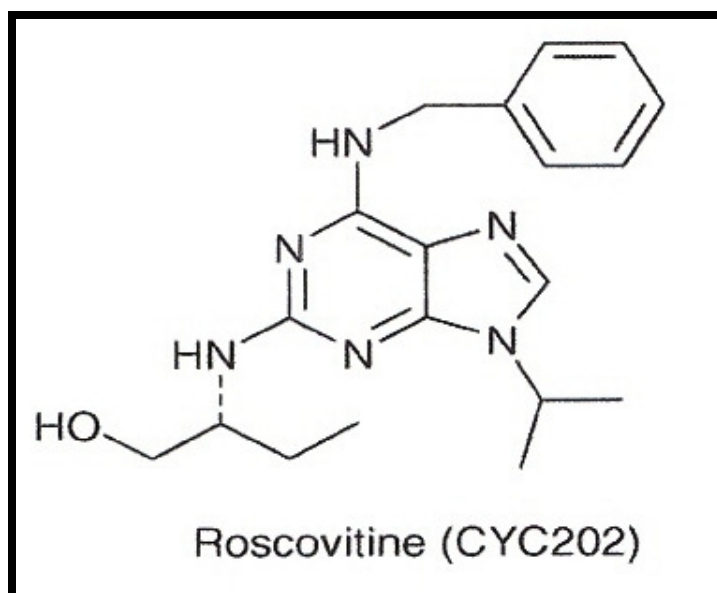
(c)

Figure 1.6 (a) A novel synthetic flavonoid 'flavopiridol' (b) One of the water-soluble analogs of the combretastatins, 'combretastatin' phosphate (CA4). (c) 'Roscovitine' derived from olomucine (Cragg & Newman, 2005).

Olomucine was first isolated from the cotyledons of *Raphanus sativus* L. (Brassicaceae) (radish) (Meijer & Raymond, 2003). Olomucine inhibit cycline-dependent kinases (Ddk), proteins which play a major role in cell cycle progression (Cragg & Newman, 2005). Roscovitine (derived from olomucine) (Figure 1.6 c) is a more potent inhibitor that resulted from chemical modification. In Europe, roscovitine is currently in Phase II clinical trials and further development was also taking place within this series of olomucine derived compounds which led to the development of 'purvalanols' (Cragg & Newman, 2005; Chang *et al.* 1999). Purvalanols is currently undergoing preclinical development because they are even more potent than the natural product olomucine and its synthetic derivative roscovitine.

Today a number of plant derived anticancer agents are available in the market. Their mode of action and target are well known (Table 1.3).

Table 1.3 Summary of anticancer agents derived from natural products (Fang, 2006).

Compounds	Drug name	Source	Cancer use	Mode of action	Arrested cell cycle
Doxorubicin	Adriamycin® Rubex®	_Streptomyces peucetius_ var. _caesius_ (Microbe)	Lymphoma, breast, ovary, lung and sarcomas	Topoisomerase II inhibition and DNA binding	G_2/M phase
Etoposide/Teniposide	Etopophos® VePesid®	_Podophyllum peltatum_ (Plant)	Testicular and small cell lung cancer	Topoisomerase II inhibition	S and G_2/M phase
Mitomycin C	Mutamycin®	_Streptomyces lavendulae_ (Microbe)	Gastric, colorectal, anal and lung cancer	DNA alkylation and cross linking	Non-specific
Paclitaxel/Docetaxel	Taxol®	_Taxus brevifolia_ (Plant)	Ovary, breast, lung, bladder, and head and neck cancer	Promotion of microtubule stabilisation	G_2/M phase
Topotecan/Irinotecan	Hycamtin®	_Camptotheca acuminate_ (Plant)	Ovarian, lung and paediatric cancer	Topoisomerase I inhibition	S and G_2/M phase
Vinblastine	Velban® Velbe®	_Catharanthus roseus_ (Plant)	Bladder, kidney, lung, leukaemia, prostate and germ-cell ovarian cancer	Microtubule assembly inhibition	M phase
Vincristine	Oncovin® Vincrex®	_Catharanthus roseus_ (Plant)	Leukemia, lymphoma, neuroblastoma and rhabdomyosarcoma	Inhibition of tubilin polymerization	M phase

1.7   Targeting natural products

Drug discovery and clinical therapy are extremely important for the development of more effective new anticancer drugs as targeted therapeutics with tailored treatment strategies and regimens.   Currently research is focused, due to progress in cancer biology, on cancer-specific mechanisms and molecular targets corresponding to that (Altmann & Gertsch, 2007). Natural products for cancer chemotherapy are often very potent but have limitations in terms of solubility in aqueous solvents and they show narrow therapeutic indices, this caused the termination of a large number of pure natural products such as bruceantin and maytansin (isolated form *Maytenus serrata*) (Cragg & Newman, 2005). Alternatively these agents should be investigated and developed as potential "warheads", by attaching these agents to monoclonal antibodies that are targeted specifically to epitopes on the tumours of interest (Sausville *et al.*, 1999). With the emergence of novel technologies revived interest in "old" agents could make it possible for them to be developed in to effective drugs. Dedicated research over a 20-30 years period for clinical agents such as topotecan, paclitaxel (taxol$^{®}$), irinotecan and the camptothecin derivatives eventually led to the confirmation of their efficiency.

Revived interest was also found for 'bruceantin' isolated from *Brucea antidysenterica* J.F. Mill. (Simaroubaceae), since it showed substantial activity against panels of leukaemia, lymphoma and myeloma cell lines, as well as in animal models bearing early and advanced stages of the same cancers, (Cragg & Newman, 2005). Previously, bruceantin showed activity in animal models. However, during its clinical trials no objective response was found. Further development was therefore ended. Currently there is strong evidence supporting further development of bruceantin for the treatment of haematological malignancies. Its activity was linked with the down-regulation of (c-MYC), a key oncoprotein (Cragg & Newman, 2005).

Development of new derivatives from natural products with improved antiproliferative profiles and chemo preventive activities is essential, but it can only happen if their molecular mechanism of action, their effects on cellular signalling process is entirely understood as well as their structure-activity relationships (Kuo *et al.*, 2005). Another vital clinical problem that needs to be tackled is drug resistance (Johnstone *et al.*, 2002).

1.8   Rationale for studying anticancer botanicals

Revival is taking place in medicinal botanicals (including herbal remedies) as part of complementary medicine for disease prevention and therapy as conventional medications have high costs, side effects and therapeutic limitations (Park & Pezzuto, 2002). Enthusiasm and exceptional growing public interest for botanicals are not only found in the United States where about 40% of the Americans are using alternative medicine, but also in other parts of the world.   Reduced risk of cancer was suggested by high consumption of fruits and vegetables in epidemiologic studies.   Therefore, the great interests and enthusiasm in naturally occurring phytochemicals for cancer chemoprevention (Park & Pezzuto, 2002).

Whole botanicals (extracts) are seen as effective and safe to the general public, but investigative and conceptual scientific evidence is difficult to obtain.   Extracts, herbal preparations or botanical medicine contain many compounds and pose significantly more conceptual challenges during research than that of a single compound, because they contains unknown components with unknown properties, the different components may act together as a barrier to the toxic effects of a single compound (buffer) and number of different compounds in combination may have synergistic activities (Vickers, 2002). They are also not subjected to the same regulatory standards than the other conventional medicine. Furthermore, possible drug interactions, recommended dosage and schedule create a lot of concern.

There are several reasons why whole botanical extracts, containing many unknown compounds, should be used or may benefit in anticancer treatment because there is the possibility is that whole botanical extracts can decrease the adverse effects as well as synergistic activity. There could also be the possibility of antagonistic activity due to multiple component interaction and competitive binding to common sites. Some have speculated that synergy results from the existence of "redundancy and back-up mechanisms found in the key regulatory and metabolic pathways of the cell" (Darzynkiewicz *et al.*, 2000). A number of different compounds in combination may have synergistic activity by targeting both primary and back-up mechanisms simultaneously (Darzynkiewicz *et al.*, 2000). The use of whole

plant botanicals or extracts could also reduce toxicity because of buffering taking place between the different constituents (Vickers & Zollman, 1999).

2.3 Problem statement

1. Are 7-methyljuglone and its derivatives, anticancer agents?
   If yes, then what is the mechanism involved?
2. Can anticancer properties of plants used traditionally for cancer treatment scientifically validated? Can bioactive principles of potent extracts be identified?

2.11 The aim of the study

The aims of the present study are as follows:

1. Investigation of anticancer activities of 7-methyljuglone and its derivatives on (breast adenocarcinoma (MCF-7), cervical epithelial carcinoma (HeLa), oesophageal carcinoma (SNO) and prostate epithelial carcinoma (DU145).
2. Scientifically validate the plants used traditionally in South Africa for cancer.
3. Identify the bioactive principle(s) from the most potent extract.
4. Evaluate the mechanism of action of potent compounds.

Chapter 1   This chapter provides a concise review of natural products being used to treat many ailments including cancer, plants and isolated compounds as a source of anti-cancer agents.

Chapter 2   A brief introduction to cancer with the current measures used to treat cancer as well as the different types of cell death: apoptosis, necrosis, oncosis, autophagy.

Chapter 3   Anticancer activity of the selected plant extracts on the following cell lines breast adenocarcinoma (MCF-7), cervical epithelial carcinoma (HeLa), oesophageal carcinoma (SNO) and prostate epithelial carcinoma (DU145).

Chapter 4   Cytotoxicity of 7-methyljuglone and its derivatives on the aforesaid cancer cells as in chapter 3 as well as U937 and peripheral blood mononuclear cells (PBMCs).

Chapter 5   Isolation and purification of the bioactive compounds of *Foeniculum vulgare* using column chromatography, thin layer chromatography, high performance liquid chromatography and spectral analysis such as nuclear magnetic resonance. In addition this chapter documents the cytotoxic activity of the purified compounds on cancer cell lines.

Chapter 6   Mechanism of action of potent anticancer compounds is dealt with in this chapter.

Chapter 7   In the discussion and conclusion all the results are brought together to give a more coherent picture of all the results.

Chapter 8   Acknowledgements

Chapter 9   Appendices

1.9   References

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CHAPTER **2**

Cell death and cancer

Chapter 2

Cell death and cancer

2.1   Cell death

Lately, researchers from a range of various fields of biology and medicine (such as cell and molecular biology, oncology, immunology, embryology, endocrinology, haematology, and neurology) took interest in of cell death (irreversible loss of vital cellular structure and function) as focus topic (Darzynkiewicz *et al.*, 1997).   Cell death, or "point-of-no-return" (Trump *et al.*, 1997), was also termed cell necrobiology since it is associated with a variety of life processes.   Humans and animals depend permanently on cell death as part of their development and it continues into adulthood (Raff, 1998).   For example: every minute of a mature humans live millions of cells die and cell death was balanced exactly by cell division so that we can remain the same size (Raff, 1998).   Other functions of cell death include the elimination of lymphocytes that have reached their age, it also plays an important role where it helps control cell numbers by removing excess cells: neutrophils (a type of white blood cell), and during fetal development it carves out cavities or divide digits to sculpt the different parts of the body.   A complex cascade of biological processes, also normally part of a cells life (pathways, enzyme systems, functioning of organelles, plasma membrane structure and function, modulation of transcriptional and translational activities etc) was activated in preparation for and during cell death (Darzynkiewicz *et al.*, 1997).   It is also vital to understand that every cell is programmed to die subsequent to a suitable stimulus (Trump *et al.*, 1997).

There are several different forms of cell death.   Studies of dying cells have demonstrated the need for precise definitions of various forms of cell death that include apoptosis, oncosis, necrosis and autophagy.   Cell death is generally divided into two different

mechanisms which are mutually exclusive and stand in sharp contrast, apoptosis (programmed cell death) and necrosis (accidental cell death) which are the two fundamental types of cell death (Table 2.1) (Darzynkiewicz *et al.*, 1997). Apoptosis and necrosis differ in their mechanism of induction.

Table 2.1 A comparison of apoptosis with necrosis, modified from Fang (2006).

Apoptosis	**Necrosis**
Trigger for cell death	
Intrinsic (pathway mitochondria-dependant)	Extrinsic (ligand receptor interaction)
Generated by signals within the cell	Triggered by death activators binding to receptors at the cell surface
Involves the participation of the mitochondria that release caspase-activating proteins.	Represented by tumour necrosis factor (TNF), family of receptors
Proteins of the Bc12 family proteins govern mitochondria-dependant apoptosis	TNF receptors utilize protein interaction modules known as death domains and death effector domains (DEDS) to assemble receptor-signalling complexes
	Receptor-signalling complexes recruit and activate cell death proteases, namely pro-caspase8 and 10.
Cell morphology	
Cell shrinks dramatically due to blebbing and produces apoptotic bodies	Cell swells and lose integrity of cell membrane due to influx of H_2O
Membranes and organelles remain intact	Outer membrane and organelle membranes rupture leading to non-specific release of damaging enzymes like DNAase and proteases
Nucleus condenses	Nucleus swells

Apoptosis	Necrosis
Cell morphology (continue)	
Pyknosis and karyorrhexis (dense condensation of chromatin)	Karyolysis preceded by irregular chromatin clumping
Single cells affected	Groups of cells affected
DNA degradation	
Apoptosis exhibits internucleosomal DNA degradation	Necrosis exhibits random DNA degradation
Caused by activation of specific nucleases	Caused by intracellular enzymes released upon organelle membrane rupture or caused by random enzymes
Inflammatory response	
No inflammatory response because membranes remain intact	Extensive inflammatory response due to release of intracellular material from the necrotic cell
Synthesis of anti-inflammatory agents occurs that results from actual upregulation of certain apoptotic gene products	Inflammation can further damage normal tissue
Activation of proteases	
Activation of caspases (cysteine proteases) that specifically cleave substrates with aspartic acid residue	Lyposomal rupture leads to release of many non-specific destruction of cell proteins
Genetic regulation	
Under strict genetic control	Induction by harsh cellular environment

Different forms of cell death are: 'apoptosis', 'necrosis', 'oncosis' and 'autopagy'. The pre-lethal response (reaction to injury prior to cell death often reversible) to cell death can be divided into two major categories known as apoptosis and oncosis, the pattern linking between these two types obviously depend on both the cell type and the injury (Trump *et*

al., 1997). The post-mortem autolytic and degradative cellular changes are termed necrosis; in fact there are anyhow two forms of necrosis: apoptotic necrosis and oncotic necrosis (Trump *et al.*, 1997). The necrotic step can either follow apoptosis or oncosis, these cells are termed late necrotic cells (Darzynkiewicz *et al.*, 1997). This makes cell death even more complex. According to Darzynkiewicz *et al.* (1997), studies of the proceedings that take place throughout both apoptosis and oncosis could be referring to the term cell necrobiology, whereas the post-mortem events of necrosis refer to cell necrology. The detailed description of all four forms of cell death is as follows:

2.1.1 Apoptosis

Kerr *et al.* (1972) was first to describe the distinct morphological features of programmed cell death, and coined the term apoptosis (Huppertz *et al.*, 1999). Apoptosis was derived from a Greek word which is put in plain words as "falling off" as used to described the leaves falling from trees. Generally it is accepted that cells have the ability to undergo an internally controlled cell suicide process known as apoptosis, which happens in response to an environmental agent, or given stimulus (Schwartzman & Cidlowski, 1993). Directed destruction of a cell by apoptosis can only happen if sufficient time was given to arrange a sequence of intracellular events (Huppertz *et al.*, 1999). Dying by apoptosis is therefore, an energy-dependant (Halestrap, 2005), tidy regulated and controlled death (quickly and neatly) that expends energy in the ATP form (Edinger & Thompson, 2004). Programmed cell death is used synonymously with apoptosis (multifocal single-cell death) as a type of "natural" death. The functional term, programmed cell death, is used to explain a cell death that is typical of life for a multicellular organism, alternatively, apoptosis (descriptive term) is used to explain a type of cell death that display a unique set of several morphological features that does not come for free (Martin *et al.,* 1994). However, the characteristic features of apoptosis together with the organization of multiple gene-directed energy-dependant biological processes that is crucial for apoptosis to take place (Cotter *et al.*, 1990) need to be observed during programmed cell death to classify it together with apoptosis. Therefore, to avoid misunderstanding, it is suggested that apoptosis and programmed cell death have to be used with care (Martin *et al.,* 1994).

The apoptotic mechanism of cell death or programmed cell death play a fundamental role in the normal development of tissues and organisms (Studzinski, 1995) through monitoring tissue homeostasis by balancing cell proliferation (opposite role to mitosis) (pathological cell accumulation occur in cancer) (Westphal & Kalthoff, 2003; Kerr *et al.*, 1993; Van Engeland *et al.*, 1998) and take place in a verity of settings following a range of microbiological and chemical injuries in various different organ systems (Trump *et al.*, 1997). It also affects numerous pathological and physiological processes (Schwartzman & Cidlowski, 1993), such as death of tumour cells and viral hepatitis (acidophilic bodies), alternatively to, embryonic tissue modelling and adult tissue turnover and differentiation, respectively (Buja *et al.*, 1993). It is frequently marked with atrophy (for example in the prostate following castration) and regression in adult disease (Trump *et al.*, 1997). Apoptosis is similar to proliferation and differentiation and is frequently started by specific receptor-ligand interactions (Huppertz *et al.*, 1999). Via an asynchronous way, apoptosis affect small groups of cells or single cells. Apoptosis is characterized by a distinct set of morphological, specific structural alterations and biochemical features as a consequence of theses complex mechanisms it leads to (or consist of the following characteristics): dehydration (loss of intracellular water and ions which leads to condensation of the cytoplasm), cell shrinkage, cytoplasm condense, formation of blebs in the cell surface or multiple cytoplasmic protrusions (typically containing organelles), nuclear/chromatin condensation/coalesce/clumping, proteolysis, nuclear fragmentation, internucleosomal deoxyribonucleic acid (DNA) cleavage, apoptotic body formation, on a background of resolute reliability of the plasma membrane (Huppertz *et al.*, 1999; Martin *et al.*, 1994; Edinger & Thompson, 2004; Schwartzman & Cidlowski, 1993; Buja *et al.*, 1993; Kerr *et al.*, 1993; Trump *et al.*, 1997).

During the process of apoptosis, DNA fragmentation (double-stranded DNA clevage) involves activation of endonuclease, which characteristically breaks the DNA between the clumps of chromatin (DNA cleavage at inker regions between nucleosomes) that are referred to as nucleosomes (Buja *et al.*, 1993), the consequence of this is that the DNA breaks into fragments of rather precise sizes that are multiples of about 200 DNA base pairs (bp) (180 bp and multiplicity of 180 bp) in length (Fang, 2006). The products of DNA degradation are nucleosomal and oligonucleosomal DNA fragments (Kerr *et al.*, 1993), which create a distinctive "ladder" pattern during agarose gel electrophoresis

(Darzynkiewicz, 1997). However, some cell types stop at the creation of 30 to 50 kilo-base pair (kb) sized DNA fragments (Darzynkiewicz *et al*, 1997; Darzynkiewicz, 1997). They do not continue with DNA fragmentation until nucleosomal sized fragments are formed. During the DNA cleavage process the nucleus start to break into fragments, similarly the cell splits into pieces, this was referred to as karyorrhexis (Fang, 2006). Sealed package fragments / membrane-enclosed (plasma membrane) (apoptotic bodies) with well-preserved organelles (Kerr *et al.*, 1993), are formed as a result of apoptosis and by this avoid inflammation that is caused by the uncontrolled release of intracellular contents and therefore apoptosis can be regarded as an injury-limiting mode of cell disposal. However, some exceptions do exist even though the inflammatory response is rare (Trump *et al.*, 1997). Thereafter, healthy neighbouring epithelial cells gradually digested or specialised migrating macrophages, engulf these fragments (Studzinski, 1995; Szende *et al.*, 1989, Kerr *et al.*, 1993; Buja *et al.*, 1993; Potten & Wilson, 2004) by phagocytosis that forms a phagosome. These apoptotic bodies are then degraded within the neighbouring epithelial cells by their lysosomes, which happen within hours.

There are numerous triggers well-known to be involved the activation of apoptosis that include the action of activators, effectors, and negative regulators (Huppertz *et al.*, 1999) such as removal of growth factors, DNA damage, Fas ligand (FasL) binding, use of chemotherapeutic agents, etc (Van Engeland *et al.*, 1998). During the apoptotic cell death process a complex cascade-like sequence of biological events can be observed (Huppertz *et al.*, 1999; Darzynkiewicz, 1997). These processes involves the activation of many diverse enzymes systems, regulatory pathways, maintenance and frequent modulation of transcriptional and translational activities, transformation of cell organelles activity, alteration of the cell plasma membrane structure and transport and of particular interest is changes in proteins whose function is to regulate the cells proclivity to apoptosis such as bcl-2, the interleukin converting enzyme (ICE) proteases family or caspase family of proteins (Darzynkiewicz, 1997). A relatively short launch of the alleged 'execution phase' of the apoptotic process is determined by the activation of the proteolytic cascade (Van Engeland *et al.*, 1998). Breakdown of cellular proteins (the nuclear matrix, cytoskeleton, and the poly-adenosine diphosphate (ADP)-ribose polymerase) is a consequence of either the direct activation of the caspase family proteases or through launching other cellular proteases (calpain or proteasomes) (Van Engeland *et al.*, 1998).

Among individual cell types differing in the steps of the apoptosis cascade may occur (Huppertz *et al.*, 1999). This cascade of molecular events leads to the cells' total disintegration. There is a close link between apoptosis and the of differentiation process that simultaneously create additional complexity in the difficulty of the apoptosis cascade (Huppertz *et al.*, 1999). Both pathways (apoptosis and the differentiation process) was found to partly use the same machinery (1) association of caspases (termed executioners through the apoptosis cascade) in lens fibre differentiation; (2) Cleavage of lamins as well as TUNEL (TdT-mediated dUTP nick end labeling) reactivity (DNA cleavage) in the deadly differentiation of erythroid cells; (3) in syncytial fusion apoptosis-induced phosphatidylserine flip result in the formation of placental syncytiotrophoblast (Huppertz *et al.*, 1999).

Different patterns of apoptosis were identified, according to Darzynkiewicz, 1997: early and delayed apoptosis, homo-phase, homo-cycle and post-mitotic apoptosis. On exposure (for a few hours) to a fairly high concentration of toxic agents, many cell types (especially "apoptosis primed cells") will rapidly undergo apoptosis (Darzynkiewicz, 1997). Here, apoptosis occurred in the same cell cycle, or the same phase when the injury was brought on the cell, it is an example of early apoptosis (Darzynkiewicz, 1997). Homo-phase apoptosis is the term used to define the apoptosis that happen in the same phase of the cell cycle wherein the cells were originally exposed to the apoptosis inducing agent. At a particular phase, during homo-phase apoptosis, the cells stay arrested (or traverse it slowly) and die without continuing into the next phase of the cell cycle (Darzynkiewicz, 1997). Homo-cycle apoptosis was used to define when apoptosis is taking place in the same cell cycle where the cells were exposed to the noxious agent (initially), excluding the specifying cell cycle phase, so the cells die before, or during the first mitosis following induction of the injury (Darzynkiewicz, 1997). The phrase post-mitotic apoptosis was used to describe the process of apoptosis happening in the cell cycle(s) after the one where the cells were originally exposed to the harmful agent, it is also indicative of a delayed apoptosis which frequently happen as the cells are pulse-exposed to a fairly low concentration of noxious agents and afterwards they are permitted to grow within drug free media (Darzynkiewicz, 1997). During post-mitotic apoptosis, apoptosis occurs due to damage to the genes which are vital for survival.

In recent years, an explosion of studies on apoptosis has clarified that it represents the mode of death that is a complex process actively driven by the cell (Fang, 2006). Currently there is an avalanche of interest, excitement and revelation in understanding of how cells undergo the process of apoptosis or evolutionary conserved process programmed cell death, and basic mechanism that triggers it (Kuan & Passaro, 1998; Van Engeland *et al.*, 1998). Solving the puzzle of apoptosis by knowing how, why and when cells are instructed to die will improve our understanding of many basic biological processes and may provide insights to the aging process, autoimmune syndromes, degenerative diseases and malignant transformations (Van Engeland *et al.*, 1998; Kuan & Passaro, 1998; Martin *et al.*, 1994). It will point to the development of potentially new targets for therapeutic treatment of diseases that show an imbalance between cell proliferation and cell loss and can result in major therapeutic implications for anticancer drugs (Martin *et al.*, 1994; Van Engeland *et al.*, 1998; Kuan & Passaro, 1998). One of the major obstacles in the successful treatment of cancer with drugs is failure to activate apoptosis (Cummings *et al.*, 2004).

It is however, surprising to see that in cancer cells the death programme never give the impression that it is completely inactivated, since cancer cells get progressively more malignant as they improve their ability to survive and proliferate through the accumulation of mutations (Raff, 1998). Cancer cells have evolved with random selection and mutation a process and therefore, mutations that inactivate apoptosis have to be beneficial to cancer cells (Raff, 1998).

Viability assays or colorimetric assays are commonly used when anticancer substances screenings are completed. This approach however has an inherent problem: all compounds which are toxic and growth inhibitory will give positive results, irrespective of the mechanism used to kill the cells (Hagg *et al.*, 2002). Therefore, a great need exist for an assay that is able to screen for compounds that specifically induce apoptosis.

2.1.2 Necrosis

Necrosis, an ancient word (Trump *et al.*, 1997), "accidental" cell death or degenerative cell death (Kerr *et al.*, 1993) is induced by a wide variety of external and noxious stimuli such

as lethal chemicals, biological or physical events, that include hyperthermia, complement attack, ischemia, metabolic poisons, hypoxia and direct cell trauma (Huppertz *et al.*, 1999; Schwartzman & Cidlowski, 1993). As a result, cells die by an uncontrolled, energy-independent process (Halestrap, 2005), and is the consequence of a passive (Kuan & Passaro, 1998), catabolic, degenerative process (Darzynkiewicz *et al.*, 1997), which is identified primarily by the loss of membrane integrity and their contents (Fang, 2006). Necrosis explains the alterations that cells and tissue endure following their death in a living organism (Trump *et al.*, 1997).

The alterations in the phase of necrosis are alike subsequent to either apoptosis or oncosis (Trump *et al.*, 1997). Necrosis exhibits distinctive morphological and biochemical characteristics. Early changes include swelling of the cytoplasm and organelles (endoplasmic reticulum, lysosomes, and other vesicles), especially mitochondria (intracellular organelles that produce ATP), with only minor changes in the nucleus (Schwartzman & Cidlowski, 1993). Pathologic in that injury is produced and the insult causes lyses/rupture of cell membranes after which the cytosol leak into the surroundings or extracellular space (Kuan & Passaro, 1998). These morphological alterations are cause by failure in the control of the plasma membrane's selective permeability and is in reaction to the early loss of membrane ion-pumping activities that are either directly caused by injury to the membrane or secondary to cellular energy exhaustion (Schwartzman & Cidlowski, 1993). Tremendous cellular swelling occurs as the outcome of fluid shifts associated with cations that move across the membrane along concentration gradients (Schwartzman & Cidlowski, 1993). Prior to membrane-bound phospholipases activation, free cytosolic calcium (Ca^{2+}) increases. Widespread disruption of membranes arises accordingly to the activation membrane-bound phospholipases that bring about disruption of membrane phospholipids (Schwartzman & Cidlowski, 1993). This then leads to an irritant effect on the nearby cells and therefore causes exacerbated injury which leads to consequent inflammation. Kinins are released from cytosol, of organelles without strong membranes (easily injured) such as the mitochondria, into the surrounding tissue to incite inflammation, edema (capillary dilation) and macrophage aggregation ensue (Kuan & Passaro, 1998). Strong membrane organelles (e.g. nucleus) will on the other hand remain intact. Thereafter, it can take hours or days for the inflammatory response to occur around the dying cell and eventually will subside and leaves traces of its presence by the formation

of a scar. Three stages occur during the pathophysiology of membrane injury: (1) distinct change in ionic transport systems of membranes, (2) non-specific increase in membrane permeability, and (3) physical membrane disruption and violation (Buja *et al.*, 1993).

Cells start to die by necrosis, for example, after a heart attack or stroke, when the blood supply to an area of the heart or brain is disrupted by a clot (Halestrap, 2005). Significant ATP depletion (level incompatible with cell survival) allegedly initiated primarily by cellular 'accidents' such as toxic insults or irreparable physical damage is the source of a bioenergetic disaster of which necrosis is the outcome (Edinger & Thompson, 2004). If a cell is deprived from a supply of ATP or if violation of the plasma membrane occurs every cell is entirely programmed for death, and this is however, what is frequently overlooked (Trump *et al.*, 1997).

2.1.3 Oncosis

Oncosis was derived from the Greek word "swelling", originally used by von Recklinghausen in 1910 (Trump *et al.*, 1997), hence, pathologists used the term to explain cell death related with cell swelling (Darzynkiewicz *et al.*, 1997). Early changes in oncosis include obvious alterations in the cell shape and volume. These marked adjustments occur with in seconds to minutes following application of injury (Trump *et al.*, 1997). During slow ischemia, for example in during bone formation the loss of osteocytes entombed in the bone, is when oncosis occur (Darzynkiewicz *et al.*, 1997). In a variety of systems it was found that the features of oncosis are identical to that observed in the early phase of accidental cell death (Darzynkiewicz *et al.*, 1997). Key features of oncosis viewed during *in vivo* (affects broad areas or zones) studies include displaying blebs (detaching later) along the luminal borders and vascular spaces and formation of casts in the lumens of the nephrons in the kidney (Trump *et al.*, 1997). The common characteristics of oncosis are plasma membrane transport violation, dissolution of remnants of chromatin (karyolysis, autolytic processes etc., early and reversible protein denaturation also occur and other commonly found through electron microscopy in these cells are: dilation of the endoplasmic reticulum and Golgi apparatus, mitochondrial and nuclear chromatin condensation and numerous cytoplasmic blebs (organell-free) (Darzynkiewicz *et al.*, 1997). Formerly, in

some situations oncosis was described to take part in programmed cell death (Trump *et al.*, 1997).

2.1.4 Autophagy

Non-apoptotic forms of programmed cell death have been described and classified as programmed necrosis or autophagic cell death (Edinger & Thompson, 2004). Cellular signal pathways are therefore used to set off necrosis in response to specific cues before accidental death occur. Autophagic cell death was formerly classified as separate type of non-apoptotic death although morphological similarities exist. It is separate from necrosis. The term autophagy means to eat oneself; as a suicide tactic the cells literally digest themselves to death and it is a tactic conserved across taxa. In times of famine (nutrient stress), autophagy plays a catabolic role, energy production is used for a survival mechanism, that is activated during the degradation of cellular constituents that arise when cells change to a catabolic metabolic program (Edinger & Thompson, 2004). In the cytosol a double membrane vesicle is formed to facilitate encapsulation of whole organelles and bulk cytoplasm, this is called an autophagosome, and thereafter fuses with the lysosome where degradation and recycling of the contents take place (Edinger & Thompson, 2004). Autophagy also provides a return for injured organelles and long-lived proteins via a turnover mechanism (Edinger & Thompson, 2004). In the end direct contradiction exists on the role of autophagy, it seems as if autophagy is more of a survival strategy than a programmed mechanism of cell kill. But further testing of this theory will go on, particularly with the aim of identifying the genes involved in these processes, so that autophagy could be separated from necrosis.

2.2 Cancer

2.2.1 What is cancer?

Cancer is generally considered to comprise more than 100 different diseases, each characterized by uncontrolled growth and spread of abnormal cells (Cancer facts & figures 2006), resulting from a chain of multiple genetic changes causing a loss of typical growth

controls, leading to unregulated growth, lack of differentiation, apoptosis, genomic instability, and metastasis (Baudino, 2004). The genetic alterations are caused by both external factors (tobacco, chemicals, radiation, and infectious organisms) and internal factors (inherited mutations, hormones, immune conditions and mutations that occur from metabolism). To initiate or promote carcinogenesis these factors can work jointly or in sequence. Cancer knows no boundaries and is able to develop in any tissue within any organ at any age and the frequency of cancers intensifies exponentially with age. According to Bagchi and Preuss (2005), the NCI (in the USA), two in five persons will be diagnosed with cancer some time in their life, and finally three out of every four families will be affected. A long latent period of ten or more years was found to be one of the hallmarks of tumour development. No obvious clinical evidence of disease can be seen during this latent period. Studies suggested that for the full development of cancer it is required that three to seven rate-limiting mutations should take place (Baudino, 2004). Both animal carcinogenesis models and human clinical data support the stepwise sequence of cancer (Figure 2.1).

Disruption of Normal Growth	Loss of Differentiation	Invasion

| Normal Epithelium | ⟷ | Hyperplasia | ⟷ | Carcinoma | ⟷ | Invasive Carcinoma |

Oncogene Activation

⟶

Tumor Suppressor Inactivation
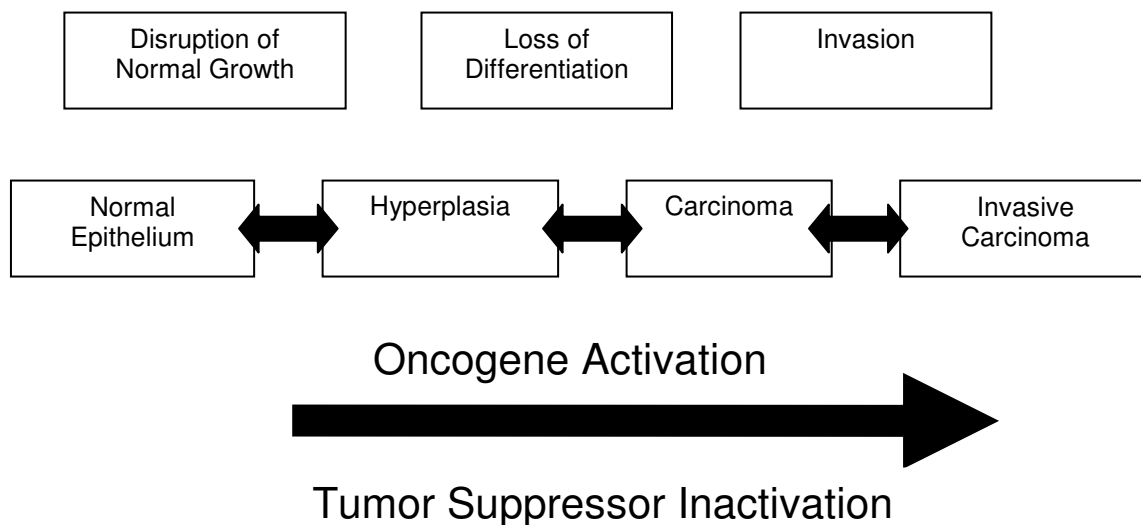
Figure 2.1 The upper row represents disturbances in growth, differentiation, and tissue integrity that lead to the phenotypes that characterize the different stages of cancer, shown in the lower row (Baudino, 2004).

Many genes that take part in normal cell processes and therefore, cancer cells can be damaged by any of these genes (Bagchi & Preuss, 2005). Multiple genetic modifications

and biochemical defects underlie cancer development, they are crucial for converting normal cells into a cancerous cell mass with atypical growth (Baudino, 2004). It is crucial for tumorigenesis that genetic modifications occur in the cells genes responsible for cell cycle progression and growth so that they can achieve the proliferative advantage (Baudino, 2004). Proteins are encoded via growth-regulating genes. Mutations in these genes will then alter levels or function of these proteins, equipped to alter cell division successfully (Baudino, 2004). Oncogenes and tumour suppressors (loss of function) are two main types of genes mutated in cancer.

2.2.2 Types of cancer

There are many different types of cells in the body and all the different cells can grow into cancer. Cells from different body parts do behave differently and some may grow faster or slower, some produce different symptoms, others respond differently to the same treatment and some might be more or less likely to spread to a specific part of the body. Cancers are divided into four main groups according to the body tissue from which they arise (Table 2.2) (Bagchi & Preuss, 2005).

Table 2.2 Cancer terminology (Bagchi & Preuss, 2005)

Cancer	**Developed from**	**Examples**
Carcinoma	Epithelial cells, such as the cells that line the digestive tract and make up organs such as the liver, kidney and pancreas.	Glandular, e.g., prostate: adenocarcinoma Squamous, e.g., cervix: carcinoma
Sarcoma	Cells that form things such as muscle, nerves, or blood vessels.	Smooth muscle: leiomyosarcoma; benign hyperproliferation is called a leiomyoma (fibroid) Bone: osteosarcoma Fat cells: liposarcoma; benign hyperproliferation is called a lipoma

Cancer	Developed from	Examples
Lymphoma	Cells in a lymph gland	solid tumour derived from B- or T-lymphocytes
Leukemias	Blood-forming tissue	Myeloid cells: myelocytic leukaemia Lymphocytes (white blood cells): lymphocytic leukaemia

2.2.3 Benign and malignant tumours

Bagchi & Preuss (2005) define a tumour as a mass of abnormal cells which are formed when a cells starts to divide uncontrollably. The tissue surrounding the cancer is firstly invaded and thereafter spread until a blood or lymph vessel is reached, then it is said that the cancer have metastasized. According to Bagchi and Preuss (2005), benign tumours contain cells that are not able to spread to a different site in the body, therefore they are most of the time not cancerous, and are contained within a covering of normal cells, they grow slowly and is most of the time harmless and therefore no treatment is required, whereas malignant tumours contain cells that are capable of spreading beyond the original tumour to another part of the body, this is dangerous because as the cells invade surrounding tissue they can damage them and stop them from working properly, and these tumours are cancerous.

2.2.4 Cancer stages

At the time of cancer diagnosis a staging is a process used to explain the degree or spread of the disease (Cancer facts & figures 2006). Staging is vital for determining the selection of therapy and measuring the prognosis. The size and location of the primary tumour determines a cancer's stage and also whether or not it has spread to other areas of the body. Different staging systems are used for all the different cancer types in order to assist

in describing the progress of that cancer (Bagchi & Preuss, 2005). The different types of staging used to classify tumours are:

1. TNM – this classification system measures tumours in three ways: extent of the primary tumour (T), regional lymph node involvement (N) absence or presence, and distant metastases (M) absence or presence (Cancer facts & figures 2006).

2. Once the TNM is assigned stages of I (early stage), II, III, and IV (most advanced stage) is assigned in addition.

3. Another system is used for descriptive and statistical analysis of tumours: in situ – if cancer cells do not invade deeper into the tissue and are present only in the layer of cells where they developed; invasive – if cells have spread to nearby tissue beyond the original layer; local – an invasive malignant cancer confined entirely to the organ of origin; regional – a malignant cancer that (1) has extended beyond the limits of the organ of origin directly into surrounding organs or tissues; (2) involves regional lymph nodes by way of lymphatic system; or (3) has both regional extension and involvement of regional lymph nodes; distant – a malignant cancer that has spread to parts of the body remote from the primary tumour either by direct extension or by discontinuous metastasis to distant organs, tissues, or via the lymphatic system to distant lymph nodes (Cancer facts & figures 2006; Bagchi & Preuss, 2005).

Selection of the most suitable type of treatment for a specific type of cancer can only be done when the stage of the cancer is known since it is important to decide on the right treatment for that specific stage of cancer. After a biopsy, grading is done in the lab where cancer cells are graded according to how much they look like normal cells and also their aggressiveness. Many different types of grading systems exist and depend on the type of cancer. The Gleason system is the most commonly used grading system which is based on a number from 0 to 10, the lower the number the lower the grade. According to Bagchi & Preuss (2005), grades under 4 mean that the cancer cells look similar to your normal cells and that the cancer is less likely to be aggressive, where grades 5 to 7 are the intermediates which means that these cancer cells do not look like normal cells and are more likely to be aggressive and more likely to grow faster and grades 8 to 10 means that the cancer is very aggressive in growth.

The staging system of the Gleason system according to Bagchi and Preuss (2005) is as follows:

Stage 0 (Carcinoma *in situ*.): Very early cancer. The abnormal cells are found only in the first layer of the primary site and do not invade deeper into the tissue.

Stage I: Cancer involves the primary site but did not spread to nearby tissue.

Stage IA: A very small amount of cancer was found to be visible under the microscope and is found deeper in the tissue.

Stage IB: Here a larger amount of cancer cells were found in the tissue.

Stage II: The cancer has spread to the nearby tissue but is still found inside the primary site.

Stage IIA: Cancer has spread beyond the primary site.

Stage IIB: Cancer has spread to other tissue around the primary site.

Stage III: Cancer has spread throughout the nearby area.

Stage IV: Cancer has spread to other parts of the body.

Stage IVA: Cancer has spread to organs close to the pelvic area.

Stage IVB: Cancer has spread to distant organs, such as the lungs.

Recurrent: cancer has recurred at the same location where the original tumour was or at a different location after it has been treated and supposedly eliminated.

2.2.5 Cancer globally

Each year cancer is newly diagnosed in 10 million people worldwide and account for 7.1 million deaths (12.5% of the global total). It is second to cardiovascular disease as a cause of death in developing countries, which causes overall 10% of all deaths in the world. People usually regard it as a problem of the developing world, but more than half of all cancers are seen in the three-quarters of the world's population who live in the developing countries (http://www.mrc.ac.za). According to the World Health Organization (WHO) global cancer rates could increase by 50% to 15 million by 2020 (WHO, 2003). This sharp and alarming increase in cancer rates both in developed and developing is due to steadily aging populations in countries, present trends in smoking prevalence along with the growing adoption of unhealthy lifestyles (WHO, 2003).

Malignant tumours were responsible for 12% of the nearly 56 million deaths worldwide, it developed in 5.3 million men and 4.7 million women and altogether 6.2 million died, in the year 2000 (WHO, 2003). In developing countries cancer has appeared as a main public health problem. However, the likelihood of being diagnosed with cancer in developed countries is twice as high as in developing countries. The highest over all cancer rates for industrial nations are: United States of America (USA), Italy, Australia, Germany, The Netherlands, Canada and France, and the lowest cancer rates for developing countries were Northern Africa, Southern and Eastern Asia (WHO, 2003).

Lung cancer is the most common cancer worldwide, accounting for 1.2 million new cases annually, of which the main cause is smoking and other causes include domestic and industrial pollution. A clear linear dose-response relationship exists between magnitude of cancer and the period of smoking as well as the amount smoked (http://www.doh.gov.za/.docs/research/vol5-4cancer.html). Cancer of the breast follow lung cancer by, just over 1 million cases; colorectal, 940 00; stomach, 870 000; liver, 560 000; cervical, 470 000; oesophageal, 410 000; head and neck, 390 000; bladder, 330 000; malignant non-Hodgkin lymphomas, 290 000; leukaemia, 250 000; prostate and testicular, 250 000; pancreatic, 216 000; ovarian, 190 000; kidney, 190 000; endometrial, 188 000; nervous system, 175 000; melanoma, 133 000; thyroid, 123 000; pharynx, 65 000; and Hodgkin disease, 62 000 cases (WHO, 2003). One should however note that the three leading killers differ from the three most common forms of cancer. Of all the cancer deaths in the world, according to the WHO (2003) the three leading killers are: lung cancer responsible for 17.8 %, then stomach cancer at 10.4 % and liver cancer at 8.8 %.

More than a quarter of deaths are attributed to cancer in many countries. As many as one third of cancers worldwide could be prevented by healthy lifestyles, and tobacco use the most preventable cause of cancer in the world.

Although, childhood cancers (aged one to 14) are rare and largely curable if detected early through modern molecular and imaging technology e.g. PET scanning for lymphomas, the treatment options is limited. Cancer causes the second most deaths, in children world wide. Three in five children (80 116 or 92%) still die in developing countries because of cancer and 133 931 (83%) cases are newly diagnosed each year (IARC, 2002). In

developed countries childhood cancers are newly diagnosed in 26 864 (17%) children and account for 6 863 deaths annually (IARC, 2002).

2.2.6 South African cancer statistics

Every year, about 80 000 cancer cases are reported in South Africa and of these approximately 60 000 are new cases. The incidence rates are expressed per 100 000 population and exclude basal cell carcinoma (BCC) and squamous cell carcinoma of skin (SCC of skin), which on average represents 18 % and 5 % of the total cancers reported in a year respectively. For females a total of 29 208 new cases and for males 29 499 new cases were reported by the National Cancer Registry (NCR), South Africa, in 1997 published only in October 2003 by the South African National Department of Health, Health Systems Research, Research Co-ordination and Epidemiology (http://www.doh.gov.za/.docs/research/vol5-4cancer.html). Thus, one in four males and one in five females, aged between 0 to 74 years, is at risk of developing cancer at some stage of their life their (http://www.doh.gov.za/.docs/research/vol5-4cancer.html).

Cancer Association of South Africa (CANSA) lists the five most common cancers for men, women and children in South Africa:

The five most common cancers in males are:
1. Prostate
2. Lung
3. Oesophagus
4. Bladder
5. Colorectal

The five most common cancers in females are:
1. Cervix
2. Breast
3. Colorectal
4. Oesophagus
5. Lung

The five most common cancers in children (0 to14 years) are:

1. Leukemia
2. Kidney
3. Brain
4. Non-Hodgkin's lymphoma
5. Bone

(http://www.cansa.co.za).

The following statistical results were obtained through a pathology-based registry.

One of the leading cancers in males all over the world is prostate cancer. It is also true for South Africa where the lifetime risk is 1 in 24 males (http://www.doh.gov.za/.docs/research/vol5-4cancer.html). For men the largest percentage of new cases (Figure 2.2) is for prostate cancer 15.2%. It is followed by lung cancer at 10.5%, thirdly is oesophagus cancer (9.0%) (Figure 2.2), which is also the third leading killer at 11.3% (Figure 2.3). Lung cancer is however the leading killer in males at 13.1% (Figure 2.3), followed by the second leading killer which is Kaposi sarcoma at 11.4% (Figure 2.3). The five year prevalence cases for males (age 15+) show that prostate will continue to increase up to 26.4% followed in the second place by oral cavity cancers to 8.6% and in the third place in increase will be colon and rectum cancers to 11.3% (Figure 2.4). Statistically oral cavity cancer is at the top with 1485 incidences and 843 mortalities, followed by nasopharynx cancers with 242 incidences and 159 mortalities. In the third place is other pharynx cancer with 233 incidences and 174 mortalities the list then follows further as oesophagus, stomach, colon and rectum, liver, pancreas, larynx, melanoma of skin, Kaposi sarcoma, prostate, testis and kidney etc (2002) (Table 2.3).
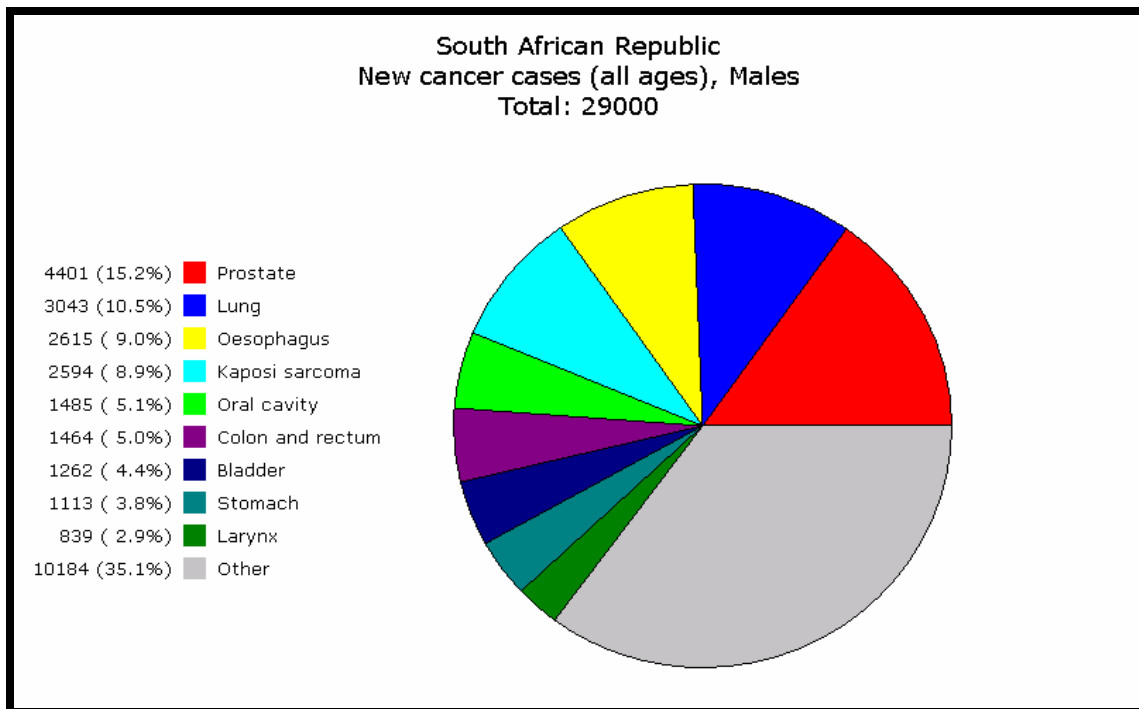
Figure 2.2 New cancer cases for males of all ages in South Africa (http://www-dep.iarc.fr/.).
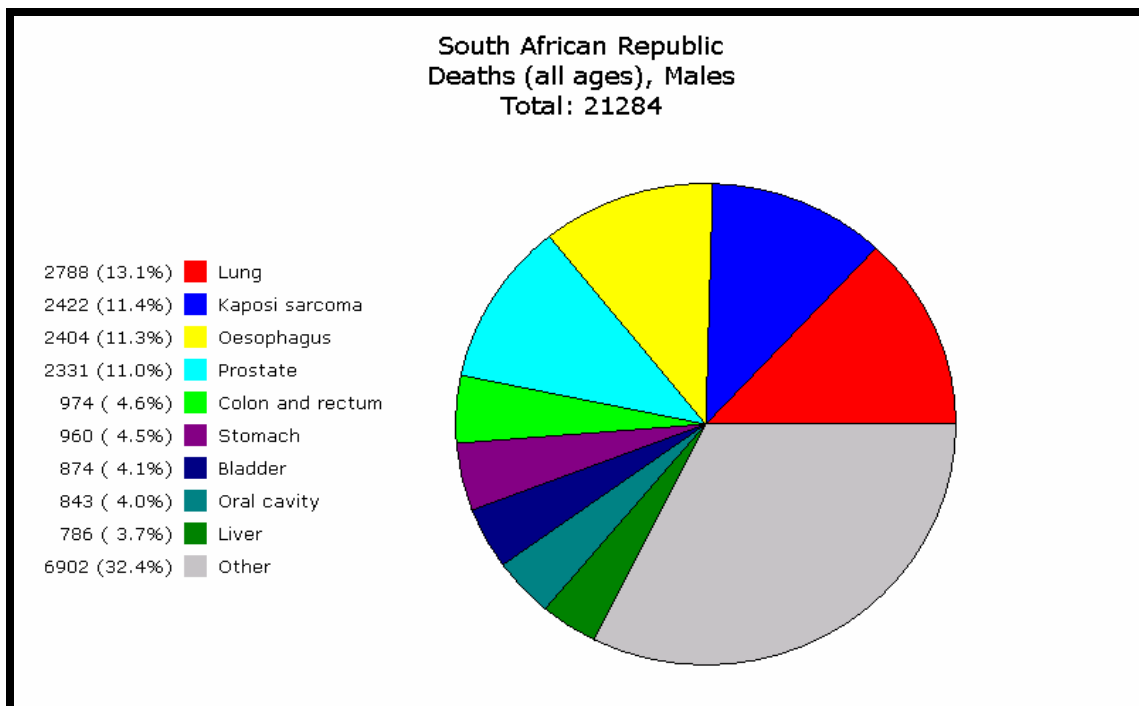
Figure 2.3 Cancer deaths for males of all ages in South Africa (http://www-dep.iarc.fr/.).

South African Republic
5-year prevalent cases (age 15+), Males
Total: 43886

11574 (26.4%)	Prostate
3759 (8.6%)	Oral cavity
2549 (5.8%)	Colon and rectum
2304 (5.2%)	Melanoma of skin
2289 (5.2%)	Oesophagus
1841 (4.2%)	Kaposi sarcoma
1696 (3.9%)	Larynx
1646 (3.8%)	Bladder
1562 (3.6%)	Lung
14666 (33.4%)	Other
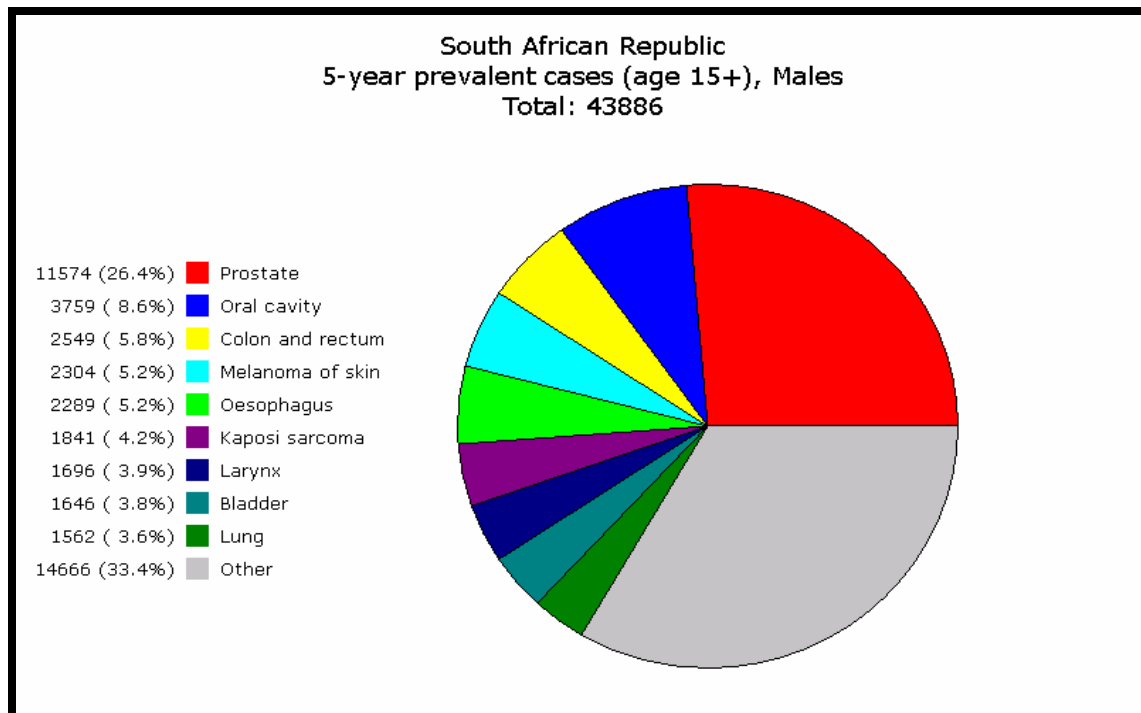
Figure 2.4 The 5-year prevalent cases for males 15 years and older in South Africa (http://www-dep.iarc.fr/.).

For females the situation is different. The leading cancer among South African women is cervical cancer and the largest percentage of new cases for females is cervix uteri cancers is at 23% (Figure 2.5) and it is the leading killer at 19.9% (Figure 2.6). Therefore one in every 29 women in South Africa will develop cervical cancer (http://www.doh.gov.za/.docs/research/vol5-4cancer.html). It is followed in the second place by breast cancer at 20.5% (Figure 2.5) which is also the second leading killer at 15.1% (Figure 2.6) (http://www-dep.iarc.fr/). One in every 31 South African females will develop breast cancer (http://www.doh.gov.za/.docs/research/vol5-4cancer.html). In the third place with percentage of new cases are colon and rectum cancers (5.4%) (Figure 2.5). The third leading killer is Kaposi sarcoma at 6.3% (Figure 2.6). For females the five year prevalence cases is breast cancer which increased up to 25.8%, followed by cervix uteri cancers (23.5%), which are followed by colon and rectum cancers in the third place increasing up to 5.7% (Figure 2.7). Statistically oral cavity cancer is at the top with 479 incidences and 261 mortalities (also at the top for males), followed by nasopharynx cancers with 70 incidences and 48 mortalities. In the third place is other pharynx cancer with 62 incidences and 46 mortalities the list then follows further as oesophagus, stomach,

colon and rectum, liver, pancreas, larynx, melanoma of skin, Kaposi sarcoma, breast, cervix uteri, corpus uteri, ovary and kidney etc (Table 2.4).
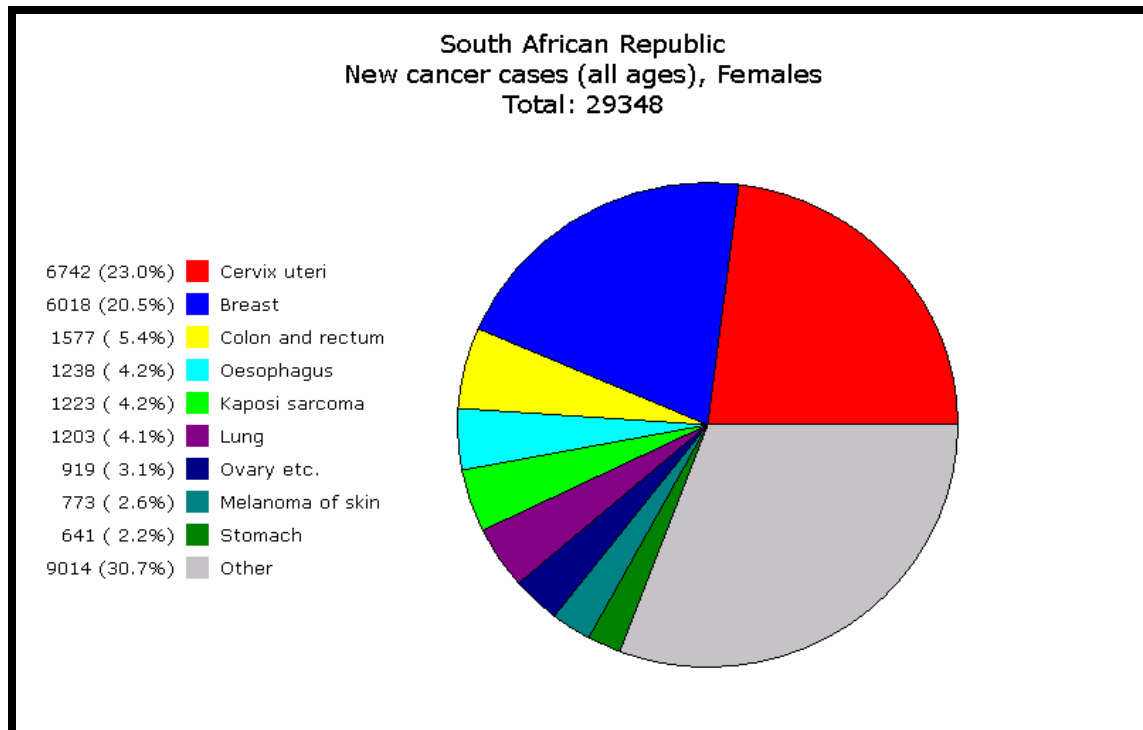
Figure 2.5 New cancer cases for females of all ages in South Africa (http://www-dep.iarc.fr/.).
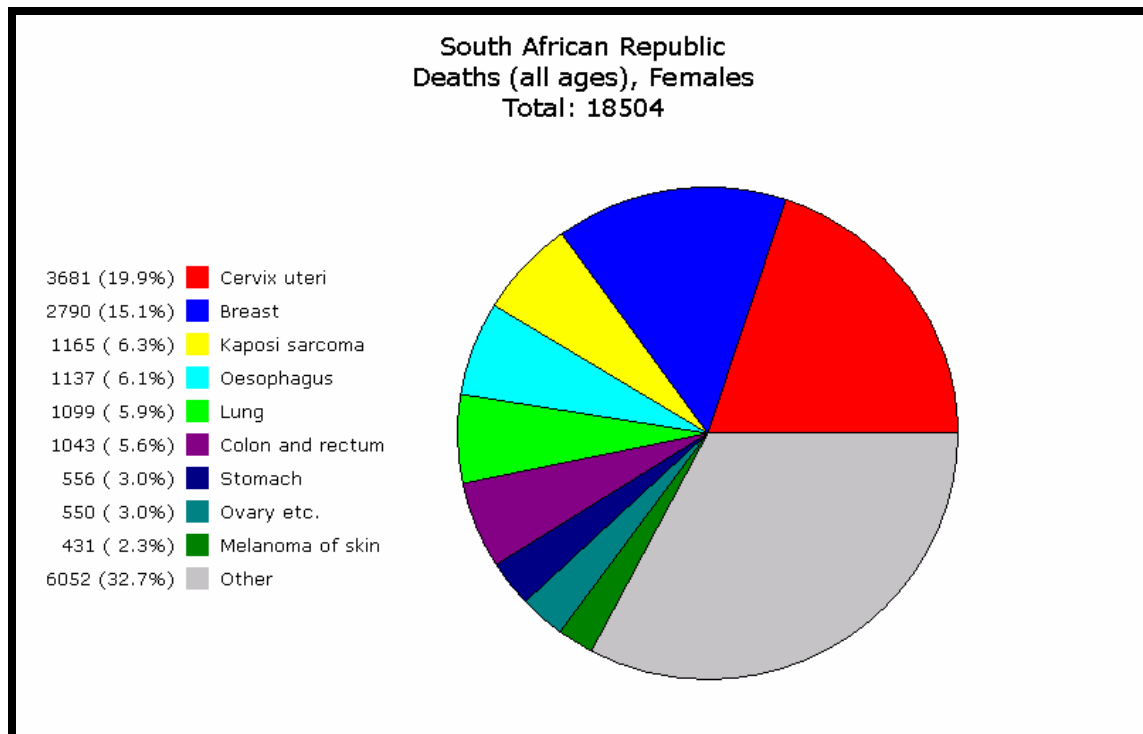
Figure 2.6 Cancer deaths for females of all ages in South Africa (http://www-dep.iarc.fr/.).

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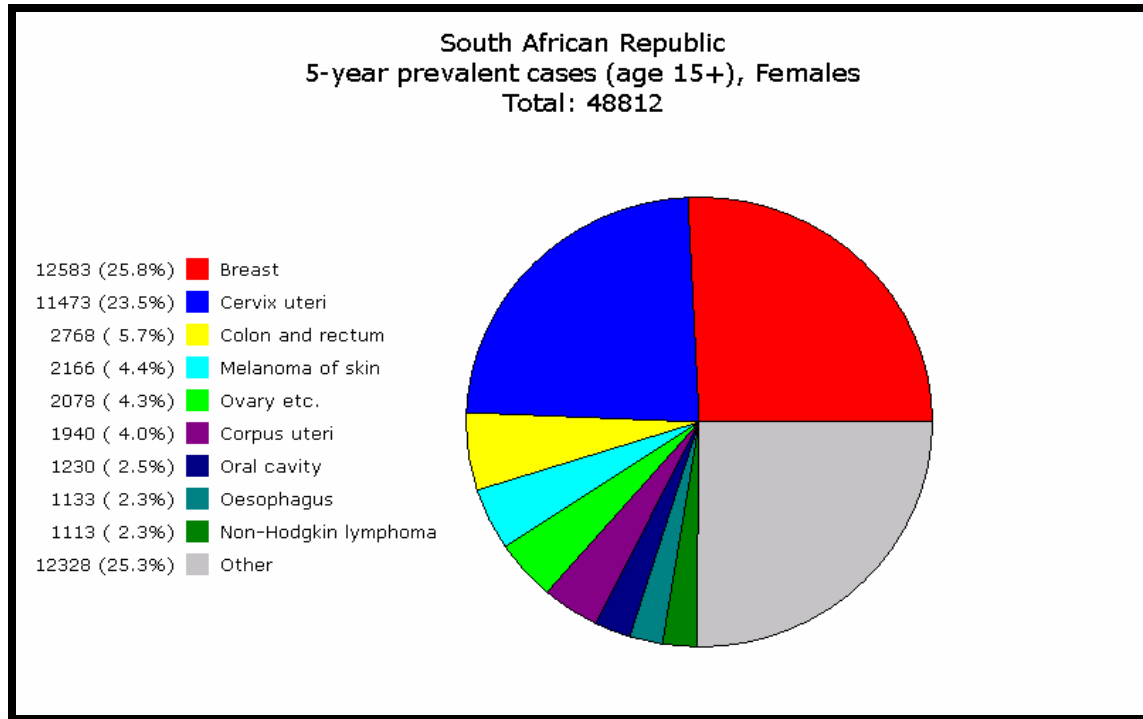
Figure 2.7 The 5-year prevalent cases for females 15 years and older in South Africa (http://www-dep.iarc.fr/.).

Table 2.3 Statistics for all cancer, males in South Africa (http://www-dep.iarc.fr/.).

South African Republic - Males

CANCER SITE	Incidence			Mortality			Prevalence		ICD-10
	Cases	Crude Rate	ASR (W)	Deaths	Crude Rate	ASR(W)	1-year	5-year	
Oral cavity	1485	6.8	11.2	843	3.9	6.3	1089	3759	*C00-C08*
Nasopharynx	242	1.1	1.7	159	0.7	1.1	172	550	*C11*
Other pharynx	233	1.1	1.7	174	0.8	1.3	154	464	*C09-C10,C12-C14*
Oesophagus	2615	12.1	20.8	2404	11.1	19.2	1224	2289	*C15*
Stomach	1113	5.1	8.8	960	4.4	7.6	555	1251	*C16*

CANCER SITE	Incidence			Mortality			Prevalence		ICD-10
	Cases	Crude Rate	ASR (W)	Deaths	Crude Rate	ASR(W)	1-year	5-year	
Colon and rectum	1464	6.8	12.3	974	4.5	7.9	1067	2549	C18-C21
Liver	827	3.8	6.1	786	3.6	5.8	308	591	C22
Pancreas	246	1.1	2.0	221	1.0	1.8	51	156	C25
Larynx	839	3.9	6.6	518	2.4	4.1	541	1696	C32
Lung	3043	14.0	25.0	2788	12.9	23.0	957	1562	C33-C34
Melanoma of skin	806	3.7	5.8	439	2.0	3.1	643	2304	C43
Kaposi sarcoma	2594	12.0	12.6	2422	11.2	11.8	937	1841	C46
Prostate	4401	20.3	42.9	2331	10.8	22.6	3471	11574	C61
Testis	190	0.9	0.9	62	0.3	0.3	133	614	C62
Kidney etc.	412	1.9	3.0	265	1.2	2.0	237	895	C64-C66,C68
Bladder	1262	5.8	11.3	874	4.0	8.1	547	1646	C67
Brain, nervous system	313	1.4	1.8	253	1.2	1.5	147	446	C70-C72
Thyroid	154	0.7	1.0	75	0.3	0.5	119	365	C73
Non-Hodgkin lymphoma	778	3.6	5.1	516	2.4	3.5	449	1524	C82-C85,C96
Hodgkin lymphoma	252	1.2	1.2	95	0.4	0.5	193	824	C81
Multiple myeloma	278	1.3	2.3	227	1.1	1.9	135	395	C90
Leukaemia	663	3.1	3.8	540	2.5	3.1	262	773	C91-C95

CANCER SITE	Incidence			Mortality			Prevalence		ICD-10
	Cases	Crude Rate	ASR (W)	Deaths	Crude Rate	ASR(W)	1-year	5-year	
All sites but non-melanoma skin	29000	133.8	223.5	21284	98.2	163.6	15029	43886	*C00-C96/C44*

Table 2.4 Statistics for all cancers, females in South Africa (http://www-dep.iarc.fr/.).

South African Republic - Females

CANCER SITE	Incidence			Mortality			Prevalence		ICD-10
	Cases	Crude Rate	ASR(W)	Deaths	Crude Rate	ASR(W)	1-year	5-year	
Oral cavity	479	2.1	2.9	261	1.2	1.6	353	1230	*C00-C08*
Nasopharynx	70	0.3	0.4	48	0.2	0.3	50	158	*C11*
Other pharynx	62	0.3	0.4	46	0.2	0.3	40	118	*C09-C10,C12-C14*
Oesophagus	1238	5.6	7.5	1137	5.1	6.9	596	1133	*C15*
Stomach	641	2.9	3.9	556	2.5	3.4	324	728	*C16*
Colon and rectum	1577	7.1	9.7	1043	4.7	6.3	1152	2768	*C18-C21*
Liver	388	1.7	2.3	370	1.7	2.2	144	277	*C22*
Pancreas	225	1.0	1.4	202	0.9	1.3	46	145	*C25*
Larynx	135	0.6	0.8	83	0.4	0.5	85	263	*C32*
Lung	1203	5.4	7.5	1099	4.9	6.9	374	608	*C33-C34*

CANCER SITE	Incidence			Mortality			Prevalence		ICD-10
	Cases	Crude Rate	ASR (W)	Deaths	Crude Rate	ASR(W)	1-year	5-year	
Kaposi sarcoma	1223	5.5	5.4	1165	5.2	5.2	442	866	*C46*
Breast	6018	27.0	35.0	2790	12.5	16.4	3802	12583	*C50*
Cervix uteri	6742	30.2	37.5	3681	16.5	21.0	3880	11473	*C53*
Corpus uteri	573	2.6	3.6	237	1.1	1.5	503	1940	*C54*
Ovary etc.	919	4.1	5.4	550	2.5	3.3	614	2078	*C56,C57.0-4*
Kidney etc.	285	1.3	1.6	178	0.8	1.0	161	623	*C64-C66,C68*
Bladder	601	2.7	3.7	407	1.8	2.5	260	791	*C67*
Brain, nervous system	248	1.1	1.3	204	0.9	1.1	115	340	*C70-C72*
Thyroid	426	1.9	2.2	209	0.9	1.1	328	976	*C73*
Non-Hodgkin lymphoma	569	2.5	3.2	380	1.7	2.1	330	1113	*C82-C85,C96*
Hodgkin lymphoma	173	0.8	0.8	66	0.3	0.3	137	574	*C81*
Multiple myeloma	236	1.1	1.5	193	0.9	1.2	115	336	*C90*
Leukaemia	478	2.1	2.5	393	1.8	2.1	181	550	*C91-C95*
All sites but non-melanoma	29348	131.6	168.3	18504	83.0	107.6	16034	48812	*C00-C96/C44*

2.2.7 Unproven methods or medical intervention for cancer treatment

Regardless of widespread positive research data from experimental and preclinical studies the existing importance and potential of botanical medicines used in cancer treatment remains largely untapped, but at the same time it is recognized with the rising of an integrative model. Multiple factors including, historical, political, and cultural factors in conjunction with confusion within the principles and practice of botanical medicine are invariably responsible for this (Treasure, 2005).

All over the world cancer patients include and used treatments, drugs and other unproven or questionable methods such as homeopathy, folk medicines, vitamins, healing "psychological" treatments, herbs, different dietary patterns rich in fruit, vegetables and herbs (Schraub, 2000). In German speaking countries a high frequency (52% – 65%) of complementary or conventional methods are used as curative or alternative treatment. Some products or medicine are country specific (in the Netherlands the Moerman diet, Ayurvedic medicine in India, Chinese medicine) and others are however used world wide: mistletoe and vitamins (Schraub, 2000). From countries in Asia (Schraub, 2000) and Africa (Nwoga, 1994), there is a lack of data although traditional/folk medicines are commonly used.

Complementary and/or alternative methods are ones that include diagnostic tests, methods of treatment or preventative treatments which are not scientifically tested or proven (Schraub, 2000). According to Angell and Kassirer (1998) medical intervention was defined as: a medicine, drug, herb etc "that has not been scientifically tested and whose advocates largely deny the need for such testing". Ernst and Cassileth (1998) give the definition as adapted by the Cochrane school of complementary medicine: "diagnosis, treatment and/or prevention which complements mainstream medicine by contributing to a common whole, by satisfying a demand not met by orthodox methods or by diversifying the conceptual framework of medicine" (Ernst & Cassileth, 1998). Most of the time these methods or treatments for cancer are either unique or extra treatments (complementary to classical ones) that can be given either according to classical concepts of cancer treatment or according to a new concept of the world and life. It was found that women and the members of the upper socioeconomic class (in the Western countries) are more frequently

using these unproven methods since they are sometimes expensive. Unproven methods or treatments, easily accessed world wide via the Internet, are mostly used by patients with a chronic or terminal disease and as soon as no more than 80% of patients having this disease might not be healed as previously with TB and now with cancer and acquired immunodeficiency syndrome (AIDS) (Schraub, 2000).

2.2.8 Cancer prevention

The WHO world cancer report (WHO, 2003) provides clear evidence that healthy lifestyles and public health action, governments and health practitioners could stem this trend, and prevent as many as one third of cancers world wide. If we all take action now today we can prevent one third of all cancers, cure another third and provide good palliative care to the remaining third. Cancer chemoprevention is defined as pharmacological intervention with synthetic or naturally occurring compounds that may prevent, inhibit, or reverse carcinogenesis, or prevent the development of invasive cancer (Park & Pezzuto, 2002).

Examples of where this immediate action can make differences:
- The reduction of tobacco (major preventable cause (WHO, 2003)) and alcohol consumption (WHO, 2006). Complete prevention is possible for all cancers due to cigarette smoking and excess alcohol consumption (Cancer facts & figures 2006).

- A healthy lifestyle and diet which consists of frequent high fruit and vegetable consumption (more than 400g/day) and physical activity is the second preventable cause of cancer (WHO, 2003; WHO, 2006; Cancer facts & figures 2006). An overall energy imbalance is the result of a Western lifestyle (highly caloric diet, rich in fat, refined carbohydrates and animal protein) together with low physical activity (WHO, 2003). In western countries, approximately 30% of cancers are caused by dietary factors and in developing countries about 20% (WHO, 2006). About 20% of all cancers could be prevented with a healthy diet (Park & Pezzuto, 2002). Daily consumption of fruit, vegetables and herbs such as broccoli, grapes, cabbage, sprouts, peanuts, ginko biloba, and garlic prove to reduce the incidence of cancer especially stomach cancer associated with *Helicobacter pylori* (UICC, 2005). It was also found that woman could reduce the risk of ovarian cancer by 60% with a dietary

supplement derived from ginko biloba and a daily sulphide supplement can reduce DNA damage in breast epithelial cells treated with carcinogens produced when protein-rich foods are cooked at high temperatures (UICC, 2005).

- Early detection through screening especially for breast and cervical, but also at early stages of colon, rectum, cervix, prostate, oral cavity, and skin. These screenings could allow prevention and successful cure for example: cervical cancer early cytological detection Papanicolaou test (PAP smear) led to impressive reduction and mortality in developed countries (Cancer facts & figures 2006), self-examination and mammography detection for breast, magnetic resonance (MR) and computed tomography. Today more than 80 percent of all cervical cancer deaths occur in developing countries because they do not have excellent public health infrastructure. Other good examples are that of breast cancer which is detected by mammography, it may reduce breast cancer mortality by 25 to 30 percent and in nation-wide screening programmes a reduction of 20 percent appears feasible. Lower mortality rates for prostate cancer due to screening by assessment of serum prostate specific antigen (PSA) levels, but early lesion management is still extremely invasive (WHO, 2003). A colonoscopy for colon cancer is considered the gold standard although extensive medical resources are needed for use in population-based screening programmes (WHO, 2003).

- Curb infections which cause cancer, because up to 23 % of malignancies in developing countries are caused by infectious agents. These agents include hepatitis B and C virus (liver cancer), helicobacter pylori (stomach cancer) and human papillomaviruses (cervical and ano-genital cancers). Roughly eight percent of all malignancies at some stage in cancers are caused by chronic infections in developed countries (WHO, 2003). The solution designed for preventing these cancers could be vaccinations. In high-incidence countries it was shown that liver cancer could be prevented by Hepatitis B virus (HBV) vaccination and currently there is a vaccine marketed and used for human papillomavirus (HPV).

2.2.9 Cancer treatment

Cancer is treated by:

2.2.9.1   Surgery

Surgery is the oldest and most frequently used cancer therapy utilized to eradicate cancer. However, a very drastic measurement, but is the most efficacious in the treatment of local disease in the region of the primary tumour and in regional lymphatics. Although surgery is no longer believed to be the sole therapy for several neoplasms it is still preferred in a high percentage of cases (Fang, 2006). In the management of the disease especially with multiple metastasis, once a neoplasm has spread from the primary site to a distant organ surgery should have little role since many types of cancer are currently managed by the use of chemotherapy and radiation therapy in combination with surgery (Fang, 2006). The extent of surgery procedure required has therefore reduced considerably.

2.2.9.2   Radiation

Today, a wide range of malignancies are treated with radiation and it has become a standard treatment option.   Radiation treatment is frequently included in primary oncological treatment as revealed by the data from the Surveillance, Epidemiology and End Results (SEER) program. More than half of all cancer patients receive radiotherapy in their care if subsequent palliative interventions are included (Fang, 2006). Small to moderate amounts of radiation can enhance apoptosis in certain tissue without producing necrosis. Radiation therapy/radiotherapy act mainly by inducing apoptosis and the degree differ extremely from one tumour to another (Westphal & Kalthoff, 2003). Cancer cell resistance can occur as a result of defects in the apoptotic pathway (Westphal & Kalthoff, 2003). Cells that are mostly susceptible are differentiating spermatogonia, gut crypts, rapidly proliferating cells in the fetus and lymphocytes. The way in which radiation triggers the apoptotic cascade in normal and neoplastic cells seems to involve the p53 tumour suppressor gene (Kerr *et al.*, 1994).

2.2.9.3   Chemotherapy

Cancer treatment via chemotherapy was introduced more than 50 years ago into the clinic (Johnstone *et al.*, 2002).   Toxic drugs are needed in most cases of chemotherapy treatment in patients which often result in unpleasant side effects, but the beneficial effects of these toxic drugs outweigh their adverse reactions. Bagchi & Preuss (2005), therefore define chemopreventive agents as any or all natural or synthetic compounds that can suppress, inhibit or reverse the development and progression of cancer.

The focus during cancer therapy is on strategies to trigger the apoptotic program so that tumour growth can be suppressed in the cell (Lee *et al.*, 2003). Earlier, various cancer chemotherapy agents were proved to affect tumour cell killing by launching apoptosis (Lee *et al.*, 2003).

Secondary metabolites from natural products such as plants and microbes contribute towards chemotherapy and play an important role in the amelioration of cancers (Kinghorn *et al*, 2003). Plant materials (edible and nonedible) have phytochemicals present which may function as chemopreventative or chemotherapeutic agents (Bagchi & Preuss, 2005). Newman and his co-workers did a study on the analysis of the antineoplastic drugs that are available in western countries and Japan, out of the 140 compounds a majority of 54% are from natural products (14%), natural product derivatives (26%), or compounds made by total synthesis, but modelled on natural product leads (14 %) (Newman *et al*, 2003). Three major types of chemopreventive agents derived from their activities have been identified to be of plant origin i.e. inhibitors of carcinogen configuration (mainly by formation of nitrosoamines from secondary amines), blocking agents (to prevent carcinogens from reaching or reacting to target sites) and repressing agents or anti-progression agents (Mukherjee *et al.*, 2001).

With carcinogens there are a sequence in which things happen – initiation, promotion and progression.   Chemotherapy begins where chemoprevention ends, at the stage of progression. The stage where promotion ends and progression begins (Figure 2.8), is not very clear. Chemopreventive agents can conceptually reach a point in time where they can be effectively made use of for blocking cancer progression (Bagchi & Preuss, 2005).
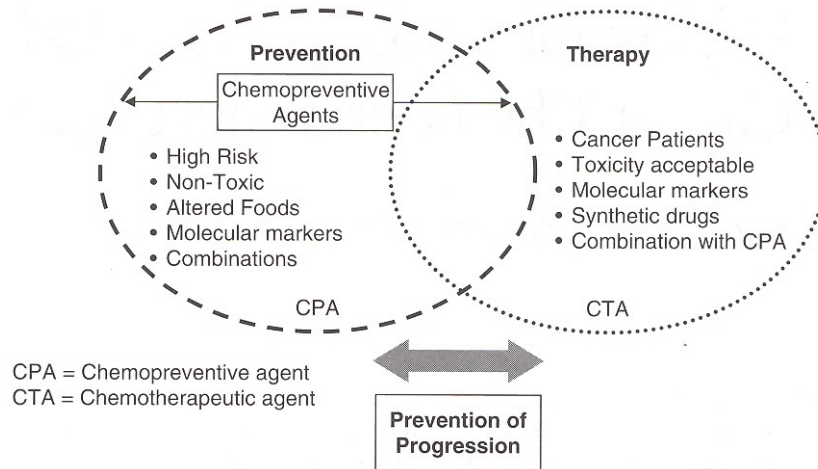
Figure 2.8 Schematic diagram showing the range of efficacy of chemopreventative agents (Bagchi and Preuss, 2005).

2.2.9.4   Hormones

According to Kerr and his co-workers (1994), "aopotosis is involved in the atrophy of endocrine-dependent organs, such as prostate and adrenal cortex, that follows withdrawal of trophic hormonal stimulation, and as might be expected, it also is enhanced in hormone-dependant tumours after successful ablation therapy". In contrast to this they also found that, apoptosis of thymocytes is induced by increased levels of glucocorticoid, and many lymphocytic leukaemias and malignant lymphomas demonstrated a similar effect (Kerr *et al.*, 1994). The *bcl-2* proto-oncogene is involved in the resistance to hormone therapy. In some lymphoid cell lines its expression has been revealed to be related with resistance to induction of apoptosis via glucorticoids. No effective cure exists particularly for hormone-independent cancer and advanced breast cancer that is highly resistant to chemotherapy (Hsu *et al.*, 2005).

Cancer is a global problem since intrinsic and acquired drug resistance occurs due to the adaptability of tumour cells and therefore, the need for new anticancer agents is urgent. Most of the cell killing anticancer drugs used today also affect normal cells; therefore the challenge remains to find way to kill cancer cells specifically.

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CHAPTER 3

Anticancer activity of traditionally used plant extracts

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3.1 Introduction

South Africa has a wealthy supply of plants (about 23 500 species of higher plants) (Taylor *et al.,* 2001) together with a high degree of endemicity (36.6%) in the indigenous South African flora (Scott *et al.*, 2004), of which 4000 plant taxa are ethnomedicinally used (Fennell *et al.*, 2004) and approximately 500 species are used in traditional medicine by an estimated 70% South Africans on a regular basis (Scott *et al.*, 2004). These plants are used either separately or in combination. Few data and scientific information exist for ethnomedicinally or traditionally medicinal plants used in South Africa. Nowadays, extensive interest is given to natural products especially plant derived natural products that show various pharmacological properties (including cytotoxic) and cancer chemo-preventative effects (Babu *et.al.*, 2002). Therefore, South Africa has huge potential in identifying novel compounds to treat many diseases.

Seven plants belonging to the Asteraceae, Apiaceae, Ebenaceae, Euphorbiaceae, Hypoxidaceae, and Alliaceae families were selected for the present study. These plants (*Artemisia afra*, *Centella asiatica*, *Euclea natalensis*, *Euphorbia ingens*, *Foeniculum vulgare*, *Hypoxis hemerocallidea*, and *Tulbaghia violacea*) were selected because they are

used by a traditional healer, in Cape Town, as a mixture which he gives to his cancer patients. A detailed description of the plant-family and the selected plants is as follows:

3.2. Asteraceae

The Asteraceae is the largest angiosperm family. It is rich in secondary chemicals (alkaloids of the pyrrolizidine, pyridine, quinoline, and diterpenoid groups) which are of particular medicinal interest such as: the great variety of sesquiterpene lactones as well as acetylenic compounds (Scott *et al.*, 2004). Other secondary metabolites in the Asteraceae are the prevalent flavonoids and saponins and tannins which are less prevalent in the lower taxa. Plant species belonging to the subfamily Tubuliflorae, also have been reported to have antimalarial, anticancer and immunostimulant properties which support the medicinal uses of South African Asteraceae plants (Scott *et al.*, 2004).

3.2.1 Ehhnobotanical use of *Artemisia*

It is mainly the leaves that are used medicinally as infusions, decoctions, inserted directly into nostrils, fumes are also inhaled when boiled in water (e.g. clear a blocked nasal passage) but sometimes the roots are also used to treat fever and colds (Van Wyk *et al.*, 1997). *Artemisia afra* Jacq. ex Wild. var. *afra* is used to treat many ailments such as stomachic, colds, influenza, fever, coughs, infection, an anthelmintic, colic, intestinal worms, headache, earache, loss of appetite, cancer, and malaria. *Artemisia absinthium* has been reported to have anthelmintic, stomachic and febrifuge properties. The antimalarial sesquiterpene lactone artemisinin was isolated from *A. annua*, which has led to the search for similar antimalarial compounds in *A. afra* (Scott *et al.*, 2004). Antihistaminic and narcotic analgesic effects have been reported for *A. afra* following preliminary tests which may underpin traditional uses to treat headache and upper respiratory tract congestion, and also have antimicrobial activity against an assortment of fungi and bacteria, which supports its use to treat infection (Mukinda, 2005).

3.2.2 *Artemisia afra*

A. afra is commonly known as African wormwood (Zulu, Xhosa: umhlonyane) and belongs to the Asteraceae family (Figure 3.1 a). This is a very prevalent species in South Africa (Figure 3.1 b) distributed over a large area. Its natural distribution expands northwards into tropical east Africa (Van Wyk *et al.*, 1997). This is an upright multi-stemmed perennial shrub that can rise up to two meters and has feathery leaves that are extremely aromatic and finely divided with a greyish-green colour. The inconspicuous pail yellow flowers are borne alongside the branch ends (Van Wyk *et al.*, 1997).

(a)   (b)
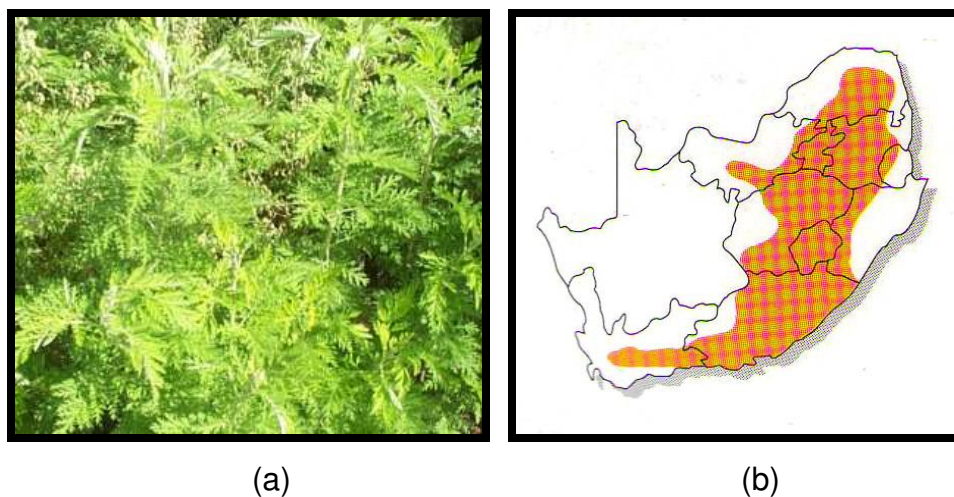
Figure 3.1 (a) *A. afra* (b) The distribution of *A. afra* in South Africa (www.plantzafrica.com/plantab/artemisafra.htm) (Van Wyk *et al.*, 1997).

3.2.3 Phytochemicals in the *Artemisia* genus

From the leaves of the South African species the triterpenes 'α- and β-amyrin' and 'freiedelin' have been identified (Scott *et al.*, 2004). The existence of two luteolin methyl ethers was discovered from the leaf exudate flavonoids (Scott *et al.*, 2004). In the above ground parts of *A. afra* 10 guaianolids and 5 glaucolids were found when the sesuiterpene lactones were analysed (Jakupovic *et al.*, 1988).

Essential oils acquire from a number of South African populations of *A. afra* leaves were examined and extensive variation in the oil composition have been verified (Scott *et al.*,

2004). α- and β-thujone (toxicity of α-thujone (LD_{50} s.c. in mice: 87.5 mg/kg) greater than β-thujone (LD_{50} s.c. in mice: 442.2 mg/kg) and thujone has low solubility in water), 1,8-cineole, camphor, and α-pinene was identified as the main constituents of the oil (Scott *et al.*, 2004). In rabbit volatile oils of *A. afra* have revealed to produce degenerative changes in the liver, hemorrhagic nephritis as well as pulmonary edema (Watt & Breyer-Brandwijk, 1962).

3.3   Apiaceae

3.3.1 Ehhnobotanical use of *Centella asiatica*

Centella asiatica (L.) Urban (Umbelliferae/Araliaceae) is frequently used for the treatment of various diseases in the Ayurvedic medicine system (Babu *et al.*, 1995). The dried leaves (aboveground plant parts consist primarily of leaves) are mainly used medicinally (Van Wyk *et al.*, 1997). It has been used to treat leprosy, wound healing, inflammation, diuretic, fever, skin complaints, rheumatoid arthritis, acne, circulatory problems, purgative, asthma, bronchitis, epilepsy, immune system deficiencies, syphilis, pulmonary tuberculosis, anxiety, eczema, anti-viral activity, fungal infections, anti-hepatoma activity, cognition-enhancement and anti-tumour activity. *C. asiatica* has been subjected to extensive experimental and clinical investigations (Punturee *et al.*, 2005).

3.3.2 *Centella asiatica*

C. asiatica (L.) Urb. is commonly known as pennywort, gotu kola, hydrocotyle, Indian pennywort, marsh penny, thick-leaved pennywort and white rot, and belongs to the Apiaceae family (Figure 3.2 a). In China, Southeast Asia, India, Sri Lanka, Africa, and Oceanic countries it has been widely cultivated as a vegetable or spice (Yoshida *et al.*, 2005). This plant has a pantropical distribution, growing predominantly in the southern hemisphere. *C. asiatica* is a creeping plant often found in moist places. It has an extensive distribution within South Africa, from the Cape Peninsula northwards along the moist eastern parts (Figure 3.2 b) (Van Wyk *et al.*, 1997). It is a perennial weed that forms a thin stem. The leaves are characteristically round or kidney-shaped on, elongated

slender stalks and tiny inconspicuous flowers are borne in groups of three (Van Wyk *et al.*, 1997).

<div align="center">(a)   (b)</div>
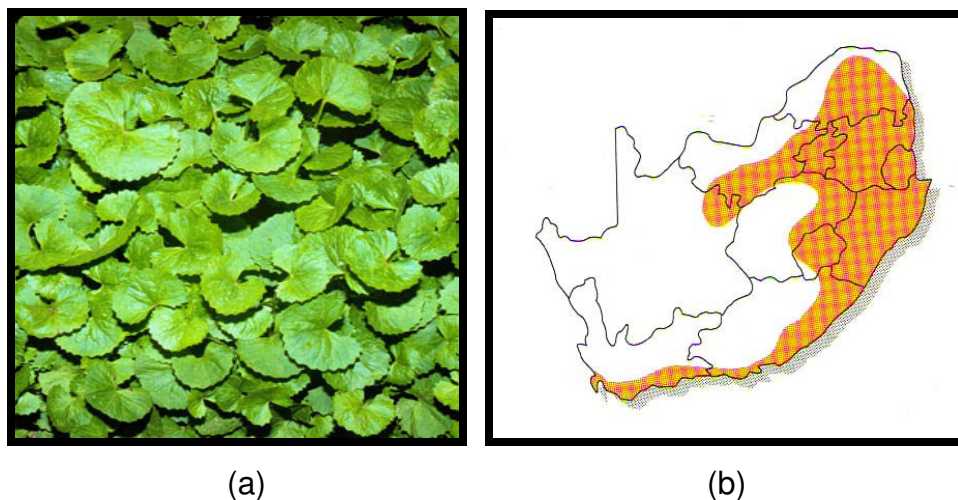
Figure 3.2 (a) *C. asiatica* round or kidney-shaped leaves. (b) The distribution of *C. asiatica* in South Africa (http://www.naturalcosmeticsupplies.com/centella-extracts.html) (Van Wyk *et al.*, 1997).

3.3.3 Phytochemicals in the *Centella* genus

Yoshida *et al.* (2005), isolated 10 compounds from the Methanol (MeOH) and chloroform (CHCl$_3$) extracts: 11,12-dehydroursolic acid lactone(1) ursolic acid (2), pomolic acid (3), 2α,3 α-dihydroxyurs-12-en-28-oic acid (4), 3-epimaslinic acid (5), asiatic acid (6), corosolic acid (7), 8-acetoxy-1,9-pentadecadiene-4,6-diyn-3-ol (8), β-sitosterol 3-*O*-β-glucopyranoside (9), and rosmarinic acid (10) which they tested for antiproliferative activity (cytotoxicity) on human gastric adenocarcinoma (MK-1), cervical epithelial carcinoma (HeLa), and murine melanoma (B16F10) cells. The antiproliferative activity of these compounds ranged from 8 – 200 μM. Asiaticoside was isolated from *C. asiatica* and was reported to possess an IC$_{50}$ of 1.58 ± 0.15 mg/ml in MCF-7 cells (Steenkamp & Gouws, 2006).

Previously it was reported by Babu and co workers (1995) that a methanolic extract of *C. asiatica* and potentially purified fractions inhibit proliferation of transformed cell lines it had an IC$_{50}$ of 62 μg/ml for mouse Ehrlich ascites carcinoma (EAC) and 75 μg/ml for Dalton's

lymphoma ascitic (DLA) cells (Babu *et al.,* 1995). The methanol extract and potentially purified fractions were also non-toxic to normal human lymphocytes (Steenkamp & Gouws, 2006). 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 and that it could possibly be accounted mainly by ursolic acid.

3.4   Ebenaceae

The Ebenaceae or ebony family is a medium sized plant family of suffrutices, shrubs and medium sized trees. It is a woody family with about 35 species native to southern Africa. These plants occur mainly in the tropics and subtropics throughout the world but are most abundant in Africa and South-East Asia (Schmidt *et al.*, 2002). They are vegetatively rather indistinct with simple, entire leaves without stipules (Van Wyk & Van Wyk, 1997). In Southern Africa, there are two native genera: *Diospyros* and *Euclea* which consist of 37 species, 10 subspecies and 7 varieties. The two native genera are much easier to recognize: *Diospyros* has alternate leaves and fruit that is subtended or enclosed by the persistent and enlarged calyx. *Euclea* has hard, leathery leaves which tend to be opposite and with undulate margins (Van Wyk & Van Wyk, 1997).

Euclea occurs in the tropics - subtropics throughout the world and about 20 species are found in South Africa (Schmidt *et al.*, 2002). The genus is characteristic of the Cape flora and very few specimens are widespread in South Africa (Dyer *et al.*, 1963). Sexes are separate on different trees. The fruits are spherical and one seeded berries (Palgrave, 1991). The fruits are small, thinly fleshy, edible but not very palatable.

3.4.1 Ehhnobotanical use of *Euclea*

In South Africa native people use the *Euclea* genus extensively for various purposes. *Euclea pseudebenus* fruit are fed to chickens to harden their eggshells (Van Wyk & Van Wyk, 1997). The twigs of *E. pseudebenus*, *E. crispa*, *E. divinorum* and *E. natalensis* are used as toothbrushes. Roots of *E. crispa*, *E. divinorum*, and *E. natalensis* are used for dyes in basket weaving because of the dark brown or black dyes when pounded and boiled

(Palgrave, 1991; Van Wyk & Gericke, 2000). The source of the dye can be linked to the presence of a few compounds such as diospyrin and 7-methyljuglone as well as other quinones (Van Wyk & Gericke, 2000). The ebony tree, *E. pseudebenus* has pitch black wood and is valuable as general timber for building and carving (Van Wyk & Gericke, 2000). The wood of *E. undulata* is used for firewood in the little karoo in South Africa (Van Wyk & Gericke, 2000).

Euclea species have many uses in traditional medicine, including as a treatment for chest complaints, bronchitis, pleurisy, chronic asthma, urinary tract infections, and venereal diseases (Pujol, 1990). *E. undulate* is used for toothache and headache (Van Wyk *et al.*, 1997). The powdered roots of *E. natalensis* are also used for toothache and headache. The Zulus use it as a remedy for scrofula. The infusions are used for abnormal pains, while charred powdered root is applied by the Shangaans to treat skin lesions caused due to leprosy (Schiafella *et al.*, 1975). The roots are also burned and the smoke inhaled as a hypnotic (Van Wyk & Gericke 2000; Van Wyk & Van Wyk, 1997). An infusion of the roots of *E. crispa* is taken orally for epilepsy (Van Wyk & Gericke, 2000).

3.4.2 *Euclea natalensis*

E. natalensis can be a shrub to a medium sized tree up to 12 meter in height with a spreading crown (Palgrave, 1991) (Figure 3.3 a). This tree occurs in coastal dune bush in a variety of habitats from dry arid areas to open woodland, riverine fringes, among koppies and rocks. The bark is grey to dark grey in colour. The tree has alternate leaves, elliptic to obovate-oblong with a glossy dark green above and a paler under surface, which is covered with dense pail rusty woolly hairs (Palgrave, 1991). The hairy leaves are a distinguishing characteristic of all *Euclea natalensis* subspecies. The flowers are small, greenish-white to cream, that are sweetly or rather unpleasantly scented in dense branched axillary heads (Palgrave, 1991). The fruits are about 10 mm in diameter and turn black when mature (Figure 3.3 b).

(a)   (b)
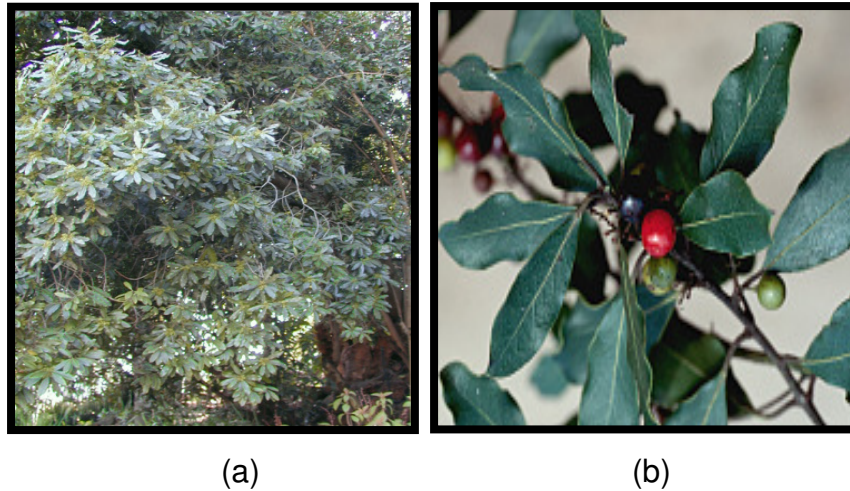
Figure 3.3 *Euclea natalensis*: (a) Tree (b) Fruit.

It commonly occurs on the eastern coast of southern Africa and also grows widely in eastern Mozambique. According to the South African National Botanical Institute (SANBI) distribution list, *E. natalensis* consists of six subspecies. As can be seen from figure 3.4, the distribution of *E. natalensis* is restricted to KwaZulu-Natal and the Cape coastal parts as well as to the upper parts of South Africa towards the boarder of Mozambique.
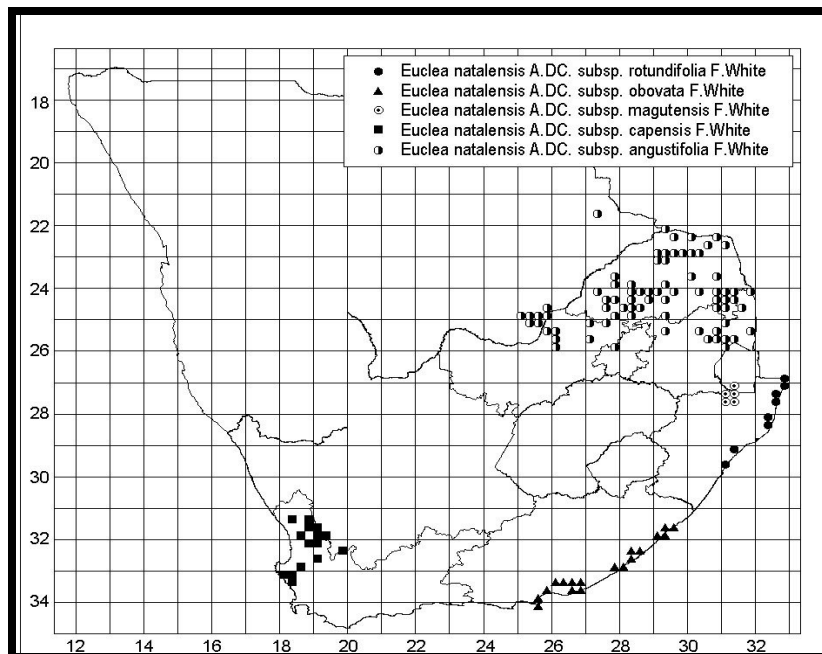
Figure 3.4 The distribution of the subspecies of *E. natalensis* in South Africa.

3.4.3 Phytochemicals in the *Euclea* genus

There is a diverse range of phytochemicals (secondary compounds) found in the different species of *Euclea*. *E. divinorum* contains compounds such as mamegakinone, a rare compound, diosindigo A, 2-methylnaphthazarin, lupeol and terpinoids like betulin (Áurea Cruze Costa *et al.* 1976, Van der Vijver & Gerritsma, 1974). *E. natalensis, E. cripsa* and *E. schimperi* have very common compounds mamegakinone and bn-quinones (8,8' – dihydroxy - 4.4 'dimethoxy – 6 ,6' – dimethyl - 2,2 – binaphtyl - 1,1 -quinone). Schiafella *et al.* (1975) reported in *E. natalensis* and *E. kellau* a host of pentacyclic triterpenoids. Lupeol, ursolic acid and betulin were the most common compounds found in these two species. *Euclea pseudebenus* showed the presence of naphthoquinones such as 2-methylnaphthazarin, 2,2,-binaphthyl-1,1'-quinones, mamegakinone and diospyrin (Ferreira *et al.*, 1973 and Ferreira *et al.*, 1974). Khan (1985) isolated 4,8-dihydroxy-6-methyl-1-tetralone from the root bark of *E. natalensis* and this was the first time that this substance was found in another genus other than that of *Diospyros.*

Some of the most frequently used anticancer drugs have been derived from quinonoid natural products (Sanyal *et al.*, 2003). Experimental evidence exists for lapachol and other naphthoquinone based drugs to be too toxic for human use as antitumour drugs (O'Brien, 1991). Clinical use have been found for some naphthoquinone based drugs e.g. 2-methyl-1,4-naphthoquinone and menadione, in combination radiation they can act as radiosensitizers or can be used combination with other chemotherapeutic agents (O'Brien, 1991). Lower redox potential naphthoquinones are less toxic than the higher redox potential naphthoquinones, which are much more toxic. Toxicity of naphthoquinones was found to be higher in naphthoquinones with a hydroxyl groups (mono- or dihydroxy substitution) at the 5- and 8-positions which makes it a high redox potential naphthoquinones. Cytotoxicity induced by most other naphthoquinones probably also involves both oxidative stress and alkylation, because alkylation of enzymes involved in the metabolism of hydrogen peroxide could make the cell highly susceptible to oxidative stress (O'Brien, 1991).

3.5   Euphorbiaceae

About 250 of the approximate 2000 species of Euphorbiaceae are indigenous to South Africa, of which 14 species grow as succulents. Spurges (*Euphorbia spp.*) are well documented in the medical literature of Greek and Rome for their use to treat tumours. Even today in recent studies and many areas of the world in traditional medicine (ethnobotanical use) these plants have shown that they are still used to treat cancerous conditions although paradoxical tumour-promotion activities also exist (Blanco-Molina *et al.*, 2001).

3.5.1 Ethnobotanical use of *Euphorbia*

The flowers of *Euphorbia* produce quantities of nectar and honey ('noos honey') when added to drinking water it cause a burning in the mouth. The toxic latex most frequently cause severe irritation and blistering to the skin. It can also cause temporary or even permanent blindness if it does come in contact with the eyes. A fish poison is prepared from the *Euphorbia ingens* E. Mey. ex Boiss. by Africans in the Limpopo valley (in South Africa). A bundle of grass is soaked in the latex, tied down to a stone and thrown into a pool with fish (Palgrave, 1981). According to Palgrave (1981) the fish (paralysed but still breathing) will rise within 15 minutes. Others use the latex of *Euphorbia ingens* as a drastic purgative (only a small dose), an antidote for dipsomania and a cancer treatment (Palgrave, 1981). Due to its toxicity several deaths have been reported form an over-dose. The latex also has several side effects such as extreme, intractable purging, fierce abdominal pain and vomiting.

3.5.2 *Euphorbia ingens*

E. ingens (Figure 3.5 a) is commonly known as candelabra tree or in Afrikaans the 'gewone naboom', and is part of the Euphorbiaceae. It can become an enormously large branched tree that can reach a height of up to 10 meters (m). *E. ingens* is often predominantly found on rocky koppies and occur at low to medium altitudes in a wide range of deciduous woodland types (Figure 3.5 b) (Palgrave, 1981). They are often connected with termite

mounds. This tree makes heavy branches from rather low down and therefore, these braches makes the individual crown and candelabriform shape, not as clearly obvious as with all the other species of *Euphorbia*. With *E. ingens* it however forms a typically enormous, branched and rounded crown. With the other *Euphorbia* species the lower branches shed each year and new branches form at the top to give the characteristic increasingly long stem. This then gives rise to the characteristic crown of branchlets. The branches are irregularly constricted and usually four- to five-winged (Palgrave, 1981). Spines are sometimes completely absent. Paired spines are most common, frequently reduced and up to 2mm long. Obsolescent spine shields are generally found which become corky and senescent. In April yellowish-green inflorescence are cyathia of the normal pattern (Figure 3.5 a). Three lobed capsules (fruits) become conspicuous in August and are up to 10 mm in diameter.
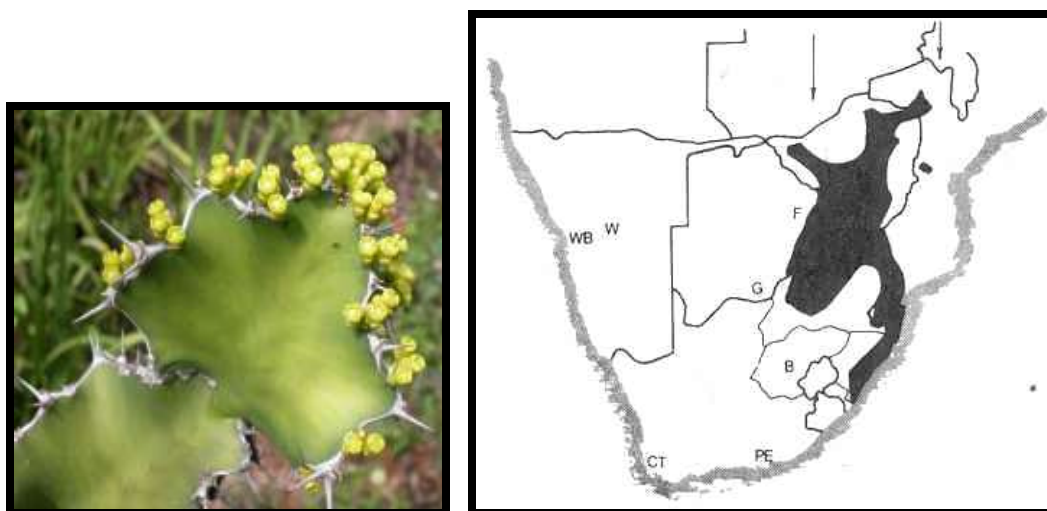
Figure 3.5 (a) The yellowish-green inflorescence of *E. ingens*. (b) The distribution of *E. ingens* in Southern Africa (www.csdl.tamu.edu/FLORA1/imaxxeup1.htm) (Palgrave, 1981).

3.5.3 Phytochemicals in the *Euphorbia* genus

During the investigation of antioxidant activity of *Euphorbia thymifolia* L. it was found that MeOH, CHCl$_3$, ethyl acetate (EtOAc), n-butanol and water fractions (except one) and 3-*O*-galloy-4,6-(*S*)-HHDP-*D*-glucose, rugosin B and 1,3,4,6-tetra-*O*-galloyl-K-β-*D*-glucose, pure compounds possessed antioxidant activities (Lin *et al*., 2002). Antiviral activity was also found during this study for a EtOAc fraction and 3-*O*-galloy-4,6-(*S*)-HHDP-*D*-glucose.

From *E. ingens* various esters of the macrocyclic diterpene ingol were isolated, as well as from the dried latex ('Euphorbium' drug) of *Euphorbia resinifera* Berg. (Upadhyay & Hecker, 1975).   In 1970, *E. ingens* latex and *E. lathyris* seed oil isolation and characterization led to the reporting of a new irritant and cocarcinogenic hexadecanoic acid monoester (tetracyclic diterpene ingenol-triacetate) (Zechmeister *et al.*, 1970).   From the latex of *E. lactea*, methanol and acetone extracts led to the isolation of a new ingol ester and a diterpene parent alcohol: 3,12-di-*O*-acetylingol 8-tigliate and 16-hydroxy-ingol-3,5,16,20-tetraacetate (Upadhyay & Hecker, 1975).   Several other compounds isolated from *E. ingens* include the diterpene ingenol and 3,7,12-triacetate-8-nicotinate (Opferkuch & Hecker, 1973) as well as the Euphorbia factors I1, I5, and I6 which are esters of ingenane-type poly-functional diterpene alcohols of which Euphorbia factors I1 was characterized as 3-hexadecanoate of the polyfunctional parent alcohol ingenol, I6 as the 3-deca-2,4,6-trienoic acid ester of ingenol and I5 the 16-angelate-3-deca-2,4,6-trienoate of the macrocyclic lathyrane-type polyfunctional diterpene alcohol ingol, (Opferkuch & Hecker, 1982).   Ingenol 3,20-dibenzoate (IDB), and certain ingenoids (semi-synthetic) have potent antineoplastic activity with some of the most potent cytotoxic agents known (Blanco-Molina *et al.*, 2001).   Their IC_{50} values are in the sub-nanomolar range.   It was documented that ingenoids have important properties such as tumour promotion, induce apoptosis in Jurkart cells through an AP-1 and NF-κB independent pathway, skin irritancy, protein kinase C activation, vascular cell adhesion molecule-1 (VCAM-1) inhibition, nerve growth factor promotion, pro-inflammatory, molluscicide, and antiviral activities (Blanco-Molina *et al.*, 2001).

3.6   Apiaceae

3.6.1 Ethnobotanical use of *Foeniculum*

Foeniculum vulgare Mill. var. *vulgare* (Fennel), has a long history of medicinal use, it has been used since antiquity to reduce the gripping effect of laxatives and also to treat flatulence especially in infants (Van Wyk *et al.*, 1997).   Apparently it has been known to increase milk secretion, promote menstruation, facilitate birth and increase libido (Javidnia *et al.*, 2003).   Chronic coughs have been treated with syrup made from the juice, and to

enhance the renal excretion of water where the roots are used as a diuretic. Commonly (in the Western Cape, South Africa) it is also used for a poor appetite and indigestion (Van Wyk *et al.*, 1997).

For centuries fennel was exported from country to country due to its therapeutic effects and large culinary utilisation (Puelo, 1980). Fennel seeds are used for savoury formulations, sauces, liqueurs, confectionery, etc, and the swollen base are freshly consumed in salad or cooked as vegetable (Oktay *et al.*, 2003). It is also used to flavour breads, fishes, salads and cheeses. The oil is used as an ingredient of cosmetic and pharmaceutical products for its balsamic, cardiotonic, digestive, lactogogue and tonic properties (Damjanović *et al.*, 2005).

3.6.2 *Foeniculum vulgare*

F. vulgare (Mediterranean origin) is an aromatic edible plant commonly known as fennel (Afrikaans: vinkel, Zulu: imboziso) and belongs to the Apiaceae family (Umbelliferae) (Figure 3.6 a). Because of fennels flavour every country surrounding the Mediterranean Sea cultivated it (Oktay *et al.*, 2003). Today it is cultivated worldwide. It is a familiar roadside weed in South Africa that was introduced from Europe (Figure 3.6 b). *F. vulgare* is an erect multi-branched robust annual, biennial or perennial (depending on the variety) herb of up to 1,5 metres in height (Van Wyk *et al.*, 1997; Van Wyk, 2005). Sheaths are formed by the leaf stalks around the thick stems and the leaves are finely divided into several needle-shaped segments with a feathery look (Van Wyk *et al.*, 1997; Van Wyk, 2005). The small yellow flowers are borne in a distinctive umbel with the flower stalks almost equal in length (Figure 3.6 a), and the small yellowish-brown fruits are divided into two segments (mericarps). It is the fruits that are mainly used for their medicinal properties, it has cancer activity.

Figure 3.6 (a) The small yellow flowers and leaves are numerous needle-shaped giving *F. vulgare* a feathery appearance. (b) The distribution of *F. vulgare* in South Africa (Van Wyk *et al*., 1997).

3.6.3 Phytochemicals in the *Foeniculum* genus

The main constituents of the essential oil of *F. vulgare*, trans-anethol, di-anethol, limonene (which are used as essence in cosmetics and perfumes) and further oligomers with estrogenic effect are described to be the actual pharmacological active ingredients of the plant (Peulo, 1980; Oktay *et al*., 2003). *F. vulgare* extracts added to creams (2 % better than 1 %) showed to reduce the hair diameter and the growth in women with idiopathic harsutism (Javidnia *et al*., 2003). The seeds have been used in Turkish folk medicine as tranquilliser, tonic and soporific drug (Oktay *et al*., 2003). Formerly *F. vulgare* was also reputed to enhance milk secretion, encourage menstruation, facilitate birth and increase libido (Javidnia *et al*., 2003). Problems such as mild dyspeptic, spasmodic gastrointestinal complaints, bloating and flatulence are effectively treated with fennel and its herbal drug preparations (Parejo *et al*,. 2004). *F. vulgare* fruit were found to have antioxidant activity and it was also established to be an active diuretic, analgesic and antipyretic (Parejo *et al*., 2004),.

Nine components (accounting for 68.9% of the total amount) were revealed to be present during analysis of a *F. vulgare* acetone extract of which the main components in the extract were linoleic acid (54.9%), palmitic acid (5.4%) and oleic acid (5.4%) (Singh *et al*., 2006). In natural oils of star anise and fennel the *trans* isomer of anethole is much more abundant

(>99%) than the *cis* isomer (Nakagawa & Suzuki, 2003). Anethole has been used for many years as a popular aniseed flavouring agent and for thousands of years as a vital component of herbal medicine. Due to its various toxicological properties it was widely studied *in vivo*, *in vitro* and in it was also found to be not potently toxic in dietary, genotoxic, immunotoxic, and mutagenic studies. In rats and mice, the target of the anethole-induced toxicity is the liver and dose-related increases in liver weights accompanied by hepatocellular hypertrophy or hydropic hepatocytes was established by oral administration of anethole (30-900 mg/kg/day) it is well absorbed undergoes extensive metabolism via ω-side-chain oxidation, side-chain epoxidation and O-demethylation and is finally excreted in the urine of mice and humans (Nakagawa & Suzuki, 2003).

From the stems of *F. vulgare* a phenyl propanoid derivative was isolated, dillapional with antimicrobial activity (MIC values of 125, 250 and 125 against Bacillus *subtilis, Aspergillus niger* and *Cladosporium* cladosporioides respectively) and a scopoletin a coumarin derivative, (marginally antimicrobial), along with dillapiol, bergapten, imperatorin and psolaren (inactive compounds) (Yong *et al.* 2002). Waste from a *F. vulgare* (aqueous extract) bioassay guided isolation (phenolic acids and flavonoids only some aglycones, flavonoid glycosides) directed the isolation of 12 key phenolic compounds, eight compounds was isolated for the first time: 3-caffeoylquinic acid, 4-caffeoylquinic acid, 1,5-*O*-dicaffeoylquinic acid, rosmarinic acid, eriodictyol-7-*O*-rutinoside, quercetin-3-*O*-galactoside, kaempferol-3-*O*-rutinoside, and kaempferol-3-*O*-glucoside, and their antioxidant activity were reported (Parejo *et al.*, 2004). Strong antiradical scavenging activity was revealed by these compounds.

In Chinese medicine the essential oils extracted from *F. vulgare* are used since they have strong skin whitening effects when the trans-anethole has condensed with monoterpenoids (Motoki *et al.*, 2003). It was also previously confirmed that estragole (also isolated from *F. vulgare*) has skin whitening effects. A number of new cyclic acetals of estragole were synthesized. Some of these new derivatives inhibited the activity of tyrosinase (*in vitro*) and proved to be more potent than arbutin, ellagic acid and kojic acid, which are currently skin whitening agents on the market (Motoki *et al.*, 2003).

Kitajima *et al.* (1998a) exhaustively investigated the methanolic extract of *F. vulgare* isolated many compounds from the water soluble portion: ethyl β-D-glucopyranoside, isopropyl β-D-glucopyranoside, propane-1,2-diol 1-O-β-D-glucopyranoside, butane-2,3-diol 2-O-β-D-glucopyranoside, 3-methylbutan-1-ol β-D-glucopyranoside, (2*S*)-2-methylbutan-1-ol β-D-glucopyranoside, (2*E*)-2-methyl-2-buten-1-ol β-D-glucopyranoside, 3-methyl-2-buten-1-ol β-D-glucopyranoside, butane-2,3-diol 2-O- β-D-apiofuranosyl-(1→ 6)-β-D-glucopyranoside. That same year, they also isolated four erythro-anethole glycol monoglucosides (also from a methanolic extract of the fruit of *F. vulgare*) which were characterised as (1'S, 2'R)- erythro-anethole glycol 1'-O-β-D-glucopyranoside, (1'R, 2'S)- erythro-anethole glycol 1'-O-β-D-glucopyranoside, (1'S, 2'R)- erythro-anethole glycol 2'-O-β-D-glucopyranoside, (1'R, 2'S)- erythro-anethole glycol 2'-O-β-D-glucopyranoside and two new glycosides of p-hydroxyphenylpropylene glycol which were characterized as threo-1'-(4-hydroxyphenyl)propane-1', 2'-diol 4-O- β-D-glucopyranoside (a mixture of two stereoisomeric forms) and 1'-(4- hydroxypenyl)propane-2',3'-diol 4-O- β-D-glucopyranoside (an epimeric mixture at C-2') (Kitajima *et al.* 1998b), as well as sixteen glycosides of which four were new phenylpropanoid glycosides, three were new benzyl alcohol derivative glycosides, a new phenylethanoid and its glycoside (Kitajima *et al.* 1998c).

In 1999, Kitajima *et al.* continued with their study on the water soluble portion of the methanolic extract of *F. vulgare* and continued to isolate many compounds: alkyl glycosides, aromatic compound glycosides, various types of monoterpenoid glycosides, glucides and nucleosides (Kitajima *et al.*, 1999a). Commercial fennel led to the isolation and structure elucidation of six glycosides: 6-carboxyethyl-7-hydroxy-2,2-dimethylchromanone 7-O-*β*-D-glucopyranoside (1), cnidioside A (2), (1'*R*)-1'-(3,4-dimethoxyphenyl)ethane-1',2'-diol 1'-O-*β*-D- glucopyranoside (3), 1'-(3,4-dimethoxyphenyl)ethane-1', 2'-diol 2'- O-*β*-D-glucopyranoside (4), *β*-sitosteryl *β*-D-glucopyranoside (5) and stigmasteryl *β*-D-glucopyranoside (6). Some anethole related compounds were isolated from the ether-soluble portion: *threo*-epoxyanethole, *p*-anisic acid, erythro- and *threo*-anethole. Other compounds include *β*-sitosterol, stigmasterol and oleanolic acid. *F. vulgare* were therefore found to contain 3-8% essential oil of which 57-82% is anethole and 6-27% is *p*-ansaldehyde (Kitajima *et al.*, 1999a). While the *F. vulgare* is preserved, an auto-oxidation product of anethole is produced in the form of *p*-

ansaldehyde (Kitajima *et al.,* 1999a). Seven new sugar alcohols were also isolated together with seven known glucosides, a sugar lactone and four nucleosides also from the water-soluble portion of the methanolic extract (Kitajima *et al.,* 1999b).

3.7 Hypoxidaceae

3.7.1 Ethnobotanical use of *Hypoxis*

Linnaeus, in 1759, coined from the Greek words *hypo* ('below) and *oxy* ('sharp), the epithet *Hypoxis,* which refers to the fruit that are pointed at the base (Drewes & Khan, 2004).

Hypoxis is currently used as an immunostimulant by the South African primary health care community for patients with human immunodeficiency virus (HIV) or acquired immunodeficiency syndrome (AIDS), 2400 mg (raw plant) was purported to be a successful therapeutically as a daily dose (Mills *et al.*, 2005). Teas and tinctures are prepared from the two species, *Hypoxis hemerocallidea* Fisch. Mey. & Ave-Lall. and *H. colchicifolia*. For centuries Zulu traditional healers and other Southern African traditional healers have used the rootstock of *H. hemerocallidea* for the treatment of urinary and uterus infections, heart weakness, internal tumours, inflammation of the joints, gout, skin ailments, menstrual pain, blood pressure problems, psoriasis, prostate problems, nervous disorders and cancer (Singh, 1999; Mills *et al.*, 2005; Vincent *et al.,* 2006). Other unproven uses for this herb include benign prostatic hypertrophy, cancer and hyperglycemia (Wilt *et al.*, 2000; Smit *et al.*, 1995; Mahomed & Ojewole, 2003; Mills *et al.*, 2005). Immune related illnesses (such as the common cold, flu, arthritis, cancer and HIV/AIDS) are treated with the corms of *H. hemerocallidea* (Mills *et al.*, 2005). A study conducted by Dold and Cocks, on medicinal plants and their trade in the Eastern Cape province, and results was acquire from various respondents from all the stakeholder groups, the plant species *H. hemerocallidea* was listed among the top 10 most frequently sold plant species and therefore their study revealed that *H. hemerocallidea* with a frequency figure of 98 tops the list of the 60 most common trade species (Drewes & Khan, 2004).

3.7.2 *Hypoxis hemerocallidea*

H. hemerocallidea of the Hypoxidaceae family, most probably the most well known muthi plant, is also well known as 'African potato' (Eng.), Afrika patat (Afr.), magic muthi, gofbol, sterblom, lotsane, molikharatsa, inkomfe, yellow stars and star lily. The underground part however does not resemble a potato, as consistently referred to as 'African potato', and is in reality a corm, which is an enlarged stem of several nodes and nodules (Drewes & Khan, 2004).

This perennial plant has easily recognizable star shaped flowers and long leaves resembling a strap (Figure 3.7 a). In South Africa, it is widely distributed within the grassland areas (Figure 3.7 b) (Van Wyk *et al*., 1997). On the African continent it has a long history of medicinal use.

<center>(a)   (b)</center>
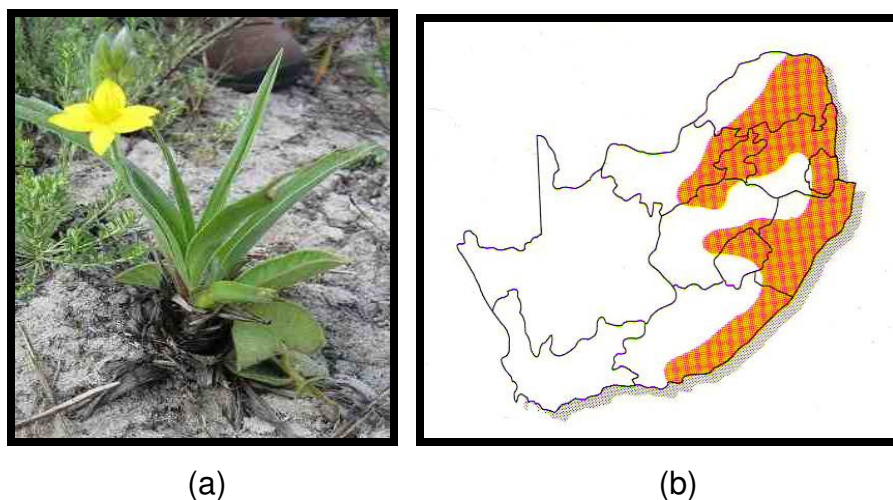
Figure 3.7 (a) The star shaped flowers and long strap like leaves of *H. hemerocallidea*. (b) The distribution of *H. hemerocallidea* is extensively in the grassland areas of South Africa (Van Wyk *et al*., 1997).

3.7.3 Phytochemicals in the *Hypoxis* genus

Important constituents of this plant are the non-lignan glycoside called hypoxoside as well as various sterols (β-sitosterol, stigmasterol) and their glycosides (sterolins) such as β-sitosterol glycoside, stanols such as sitostanol (stigmastanol), dicatechols and other

bioactive agents include flavonoids. Rooperol and stigmastanol are purported to have medicinal properties including cytotoxic to cancer cells, antimutagenic stimulators of the immune system and inhibitors or activators of gene expression. There is some indirect evidence showing that sterols and sterolins have the potential to enhance the immunity (Mills *et al.*, 2005). Once hypoxoside reach the gut it is readily converted to the aglycone, rooperol. In Europe and the USA isolated or synthetic β-sitosterols are extensively used for the treatment of benign prostatic hypertrophy (BPH), limited clinical trials was done but more widespread work is necessary to measure long term effects mainly on unconjugated β-sitosterols (Van Wyk *et al.*, 1997; Vincent *et al.,* 2006). In prostate cells, the translocation of PKC-α, the expression of TGF-β and the expression of plasminogen activator is induced by β-sitosterols (Vincent *et al.,* 2006). Cytokine and leukotriene biosynthesis can be inhibited by the dicatechols and might be effective probably other metabolic processes, cellular proliferation of cancer cells *in vitro*, against prostate, lung and other cancers *in vivo*, these compounds can also possibly alter cellular metabolism and cooperate in providing the complex benefits of traditional phytotherapies (Vincent *et al.,* 2006).

3.8   Alliaceae

3.8.1 Ethnobotanical use of *Tulbaghia*

For traditional medicinal purposes the leaves and bulbs of *Tulbaghia violacea* Harv. are used against fever and colds, oral infections also for asthma and tuberculosis (Watt & Breyer-Brandwijk, 1962; Van Wyk *et al.*, 1997). Oesophagus cancer is treated with the leaves of *T. violacea* and the freshly harvest bulbs used for stomach problems and decoctions are administered as enemas.

3.8.2 *Tulbaghia violacea*

T. violacea is commonly known as wild garlic (Zulu: isihaqa) and belongs to the Alliaceae family (Figure 3.8 a). In South Africa it occurs in the Eastern Cape and southern KwaZulu-Natal (Figure 3.8 b). Wild garlic is a bulbous plant with elongated, slender, bald leaves that

develop from several white, fleshy bases (Van Wyk *et al*., 1997). A strong smell of garlic is found in all the plant parts. At the tips of slender stalks groups of about ten or more beautiful purple flowers arise (Figure 3.8 a).

Figure 3.8 (a) The purple flowers occur in groups at the tip of slender stalks of *T. violacea.* (b) The distribution of *T. violacea* is predominantly in the Eastern Cape and southern KwaZulu-Natal (http://arboretum.sfasu.edu/plants/perennials/perennialgallery.htm) (Van Wyk *et al*., 1997).

3.8.3 Phytochemicals in the *Tulbaghia* genus

Preliminary evidence showed that this plant species may have the same/similar medicinal properties as garlic, such as antibacterial and antifungal activities. *T. violacea* could be a promising and important indigenous phytotherapy for inhibiting *Candida albicans* the causative agent for candidiasis, which is the fourth leading source of nosocomial infections (Vincent *et al.,* 2006). Mortality rates from systemic candidiasis are currently reaching 50% (Vincent *et al.,* 2006). The active ingredients/compounds are sulphur-containing which gives the characteristic smell of garlic. The main sulphur-containing substance is alliin (Van Wyk *et al*., 1997). It is said to have similar activities as of garlic (*Allium sativum)* since both belong to the Alliaceae family (Van Wyk *et al*., 1997).

3.9   Positive controls used for cytotoxicity

Doxorubicin and zearalenone were included as positive controls in the present study. The detailed descriptions of these two drugs are as follows:

3.9.1 Doxorubicin a quinonoid anticancer drug

In 1969 the antibiotic 'doxorubicin' (Figure 3.9) was isolated from *Streptomyces peucetius* subsp *caesius* (ATCC 27952) (Hutchinson & Colombo, 1999) and was found to contain more enhanced efficacy than the existing clinically important anticancer - drug 'daunorubicin' against human solid tumours as well as those of the breast, lung, ovary, head and neck, bladder, endometrium and prostate (O'Brien, 1991). *Streptomyces peucetius* subsp *caesius* is the only organism reported to produce doxorubicin. Currently more than 225 kilograms (kg) of doxorubicin is manufactured annually by semi-synthesis from daunorubicin. Due to doxorubicin extensive use in clinical cancer treatment current research is aimed at improving it and therefore acts as the source for synthesizing various analogs and derivatives (Hutchinson & Colombo, 1999). Doxorubicin gained rapid acceptance as a major therapeutic agent in the treatment of cancer as a part of a combination chemotherapy regimen (Powis, 1987). Since then many millions of patients have received doxorubicin and it is still used today. It is used for the treatment of acute non-lymphocytic leukaemia, Hodgkin's and non-Hodgkin's lymphomas, breast cancer and sarcomas, alone or in combination with other chemotherapy administrations (Young *et al*., 1981). An extensive variety of activities in human solid tumours were found for doxorubicin including those of the breast, lung, ovary, head and neck, bladder, endometrium and prostate (Powis, 1987). In adults, doxorubicin as single agent is the most effective against soft-tissue sarcomas, even though it is rarely curative in the advanced disease (Powis, 1987).

High doses of doxorubicin can lead to meylosuppression, pathological changes in the heart with swelling of the sarcoplasmic reticulum, mayofibrillar dropout and cardiomayopathy is the dose-limiting toxicity. Doxorubicin cause extensive cardiac damage that cannot endure

considerable repair. A grave prognosis is at hand once the cardiomyopathy is clinically evident (Powis, 1987). Due to the cardiotoxicity of doxorubicin it has led to the synthesis of many analogs, with the hope of those being less cardiotoxic.
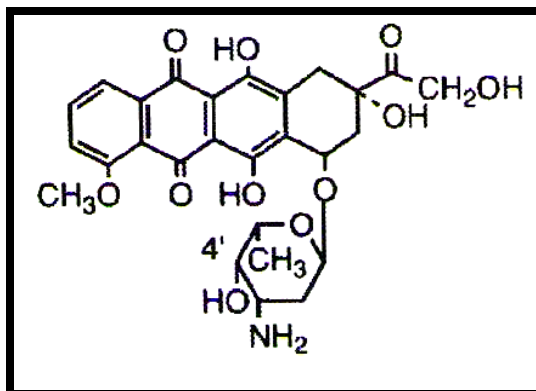
Figure 3.9 Quinonoid doxorubicin (Hutchinson & Colombo, 1999).

Doxorubicin hydrochloride belongs to a group of chemotherapy drugs known as anthracycline antibiotics and is used in the treatment of non-Hodgkin's lymphoma, multiple melynoma, acute leukemias, and cancers of the breast, adrenal cortex, endometrium, lung, and ovary (http://www.cancer.org/docroot/CDG/content/CDG_doxorubicin_hydrochloride).

3.9.2 Zearalenone a phytoestrogen

Phytohormones are extremely popular for the treatment of various diseases as an alternative medicine and practically all phytoestrogens exhibit pro-apoptotic effects in some cell systems, genotoxicity and some estrogenic activity (Stopper *et al.*, 2005). Phytoestrogens belong predominantly to the flavonoids which are characterized structurally via a $C_6C_3C_6$ carbon skeleton (Figure 3.10) (Stopper *et al.*, 2005). These phyoestrogens are found in many diets which consist of high amounts of leguminoseae and soy as well as in fruits (citrus fruits and berries) and vegetables. Dietary phytoestrogens has been proposed to be involved in the prevention of estrogen-related cancers for example breast cancer, prostate cancer as well as endometrial and testicular cancer, but to a smaller degree (Stopper *et al.*, 2005).

The non-steroidal estrogenic mycotoxins, zearalenone is also a phytoestrogens, which are produced by fungi of the genus *Fusarium* as secondary metabolites. It was confirmed that zearalenone (at concentrations of 10-40 µM) can cause DNA fragmentation or ladder pattern, apoptotic bodies formation, induce apoptosis, cell cycle perturbation, inhibit protein and DNA syntheses, increase MDA formation in Vero, Caco-2 and DOK cells through oxidative damage and cytotoxicty mechanisms, as well as strong estrogenic activity, moreover it is genotoxic, hepatotoxic, immunotoxic and haematoxic (Abid-Essefi *et al.*, 2004). Zearalenone was capable of revealing tunnel labelling and DNA ladder formation in rat sperm cells which were given a single intraperitoneal (i.p.) dose of 5 mg/kg, thus inducing apoptosis (Kim *et al.*, 2003).
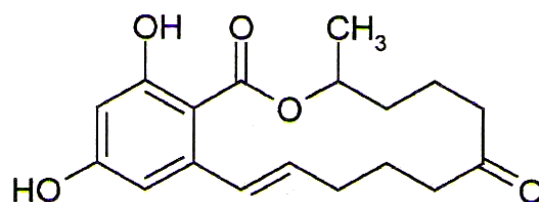
Figure 3.10 Zearalenone a non-steroidal estrogenic mycotoxin and phytoestrogen (Stopper *et al.*, 2005).

3.10 XTT assay

The cell viability assay is one of the most commonly used assays for anticancer screening (Hagg *et al.*, 2002) especially with the help of tetrazolium salts such as 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and sodium 3'-[1-(phenyl amino-carbonyl)-3,4-tetrazolium]-bis-[4-methoxy-6-nitro (XTT) which are able to assay the quantification of cell proliferation and viability (Roche, 2005). The XTT assay cell viability assay was done with the XTT proliferation kit II (Roche Diagnostics GmbH, SA). Cleavage of the XTT occur in order that an orange formazan dye form (which is soluble in aqueous solutions), by using a scanning multiwell spectrophotometer (ELISA reader by metabolic active cells) it is able to be directly quantified (Figure 3.11). A high degree of accuracy is

ensured. Samples of a large amount can be handled quickly and conveniently and it also permits on-line data processing (Roche, 2005).
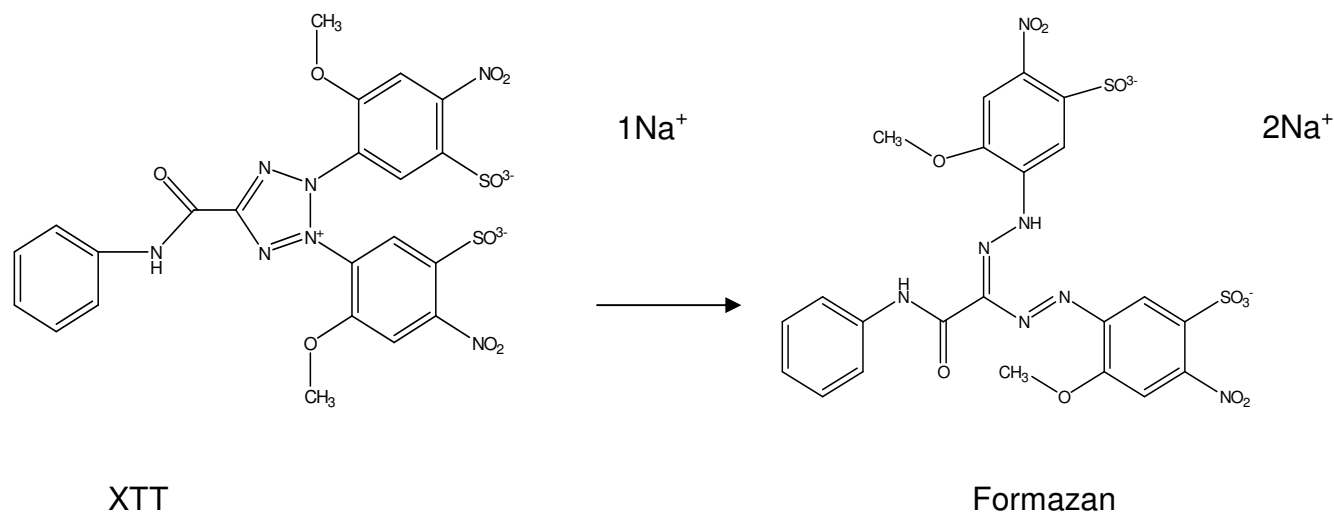
Figure 3.11 Metabolization of XTT to water soluble formazan salt by viable cells (Roche, 2004).

Unacceptable levels of toxicity are exhibited by a large proportion of prospective anticancer drugs against normal tissue and cells, and that's why it is crucial to pre-screen these forthcoming compounds (Montoya *et al.*, 2005).

3.11 Materials and Methods

3.11.1 Collection of plant material

The leaves of *Artemisia afra*, *Centella asiatica* and *Tulbaghia violacea* as well as the stems of *Euphorbia ingens* and the corms of *Hypoxis hemerocallidea*, were collected from the botanical garden at the University of Pretoria during February and March 2006. Fennel seeds were bought from a local shop and *Euclea natalensis* were collected in Mozambique. Plants were identified at the H.G.W.J. Schwelckerdt Herbarium (PRU) of the University of Pretoria to which voucher specimens were submitted (Table 3.6).

Table 3.1 Plant samples collected for the present study:

Name of plant	Plant parts	Voucher herbarium specimen number
Artemisia afra	Leaves	PRU 112085
Centella asiatica	Leaves	PRU 112086
Euclea natalensis	Roots	NL 22
Euphorbia ingens	Stem	PRU 112087
Foeniculum vulgare	Seeds	PRU 112089
Hypoxis hemerocallidea	Corms	PRU 112088
Tulbaghia violacea	Leaves	PRU 095452

3.11.2   Extraction of plant materials

Solvent extraction is usually used when the material to be extracted is in solid form and where the material used comes into contact with a solvent. There are quite a few different solvent extraction methods all with their own advantages and disadvantages. For infusion the principle is the same, but in this case the solvent is poured directly onto the plant material and left to "sleep". The extraction of the plant material will only happen until saturation is achieved. After the process the solid plant material is filtered off to get a clean extract. To achieve total extraction the extraction method must be repeated a few times with fresh solvent each time, therefore large amounts of solvent are needed.

Selecting an appropriate extractant is difficult when the chemical nature of the active constituents is unknown and the first step would be to release them from the matrix by means of extraction (Momtaz, 2007). The method of extraction is of great importance because some of these bioactive compounds are found only as minute amounts. It is important to consider the physical properties of solvents such as availability, detector compatibility, solvent reactivity, boiling point, viscosity, miscibility and safety for extractions and fractions (Momtaz, 2007; Rabie, 2005). One should also be careful when considering the solvent to ensure that the desired compounds are extracted/separated (Rabie, 2005).

There are many different extraction methods and each of them can be used differently depending on the properties of the solvents and amount and type of material to be extracted. Solvent extraction is usually used when the material to be extracted is in solid form and where the material used comes into contact with a solvent. There are quite a few different solvent extraction methods all with their own advantages and disadvantages.

For the present study, 30g of shade dried plant material (as mentioned in table 3.1) were ground using a small Junke and Kunkel grinder. Only ethanol was used as a solvent to extract the compounds from the plant material.

All the different plant material was extracted with 200ml of ethanol and left for 24 hours at room temperature while constantly stirring. The extracts were then vacuum filtered through filter paper (Whatman number 2 filter paper 15 cm) after which the plant material were collected from the filter paper and again the filtered off solvent were replaced with an equal amount of solvent. This procedure was repeated three times. When the three times repeat were finished the extracted solvent that were colleted after filtration were removed from the extract under vacuum using a rotavapor (BUCHI, Rotavapor, R-200) to yield dry extracts.

3.11.3   Cell lines

Five human cancer cell lines: breast adenocarcinoma (MCF-7), cervical epithelial carcinoma (HeLa), oesophageal carcinoma (SNO), prostate epithelial carcinoma (DU145), and African green monkey kidney cells (Vero) were maintained in culture flasks in complete Minimum Essential Medium, Eagle supplemented with 10% fetal bovine serum (Highveld biological, SA), in a humidified 5% CO_2 incubator at 37℃. Subculture was done every 2-3 days after it had formed a confluent monolayer. During subculture, cells that attached to the culture flask were trypsinized (0.25% trypsin containing 0.01% EDTA) for 10 min at 37℃ then stopped by the addition of complete medium. About 1×10^5 of the viable cells were then re-suspended in complete medium (Figure 3.13).

These cancer cell lines that were selected for this study were selected because of:

1. SNO - Oesophageal cancer. In the Eastern Cape Province, Transkei region (South Africa), amongst the Xhosa-speaking people the incidence rates for males with oesophageal cancer (an important public health problem) are among the highest in the world (Somdyala *et al.*, 2003).

2. DU145 - Prostate cancer is the leading most common cancer in males in South Africa.

3. HeLa - Cervix cancer is the most common cancer in females in South Africa.

4. MCF-7 - because after Lung cancer (most common cancer worldwide), accounting for 1.2 million new cases annually; is breast cancer, which accounts for just over 1 million cases.

3.11.4   Cytotoxicity assay

Cytotoxicity of the adherent cells was measured by the XTT method using the Cell Proliferation Kit II (Roche Diagnostics GmbH). The cancer cells (100 µl) were seeded at 1×10^5 per ml onto a microtiter plate and incubated for 24 hours to allow the cells to attach to the bottom of the plate (Figure 3.12). A dilution series were made of the extracts as well as the positive controls (0.1-100 µg/ml) and complete medium for the negative control were added to the microtiter plate and incubated for 48 hours (Figure 3.13). The XTT reagent was added to a final concentration of 0.3 mg/ml and incubated for 1-2 hrs. After incubation the absorbance of the colour complex was quantified at 490 nm using an ELISA plate reader with a reference wavelength set at 690 nm (Figure 3.14). Fifty percent inhibitory concentration (IC_{50}) was defined as the concentration of the compounds at which absorbance was reduced by 50%.

3.11.5 Statistical analysis with GraphPad Prism4

All the results of the extracts were statistically analysed with the GraphPad Prism 4 (version 4 Graph Pad Software, San Diego, Ca, USA) statistical programme. During analysis a 95% confidence interval was chosen and is represented by the doted line. A sigmoidal dose-response (variable slope) curve fit was done and the IC_{50} values of the extracts were determined from the concentration-effect relationship.

Figure 3.12 Schematic representation of the preparation of the cells and 96-well plates for the experiment.

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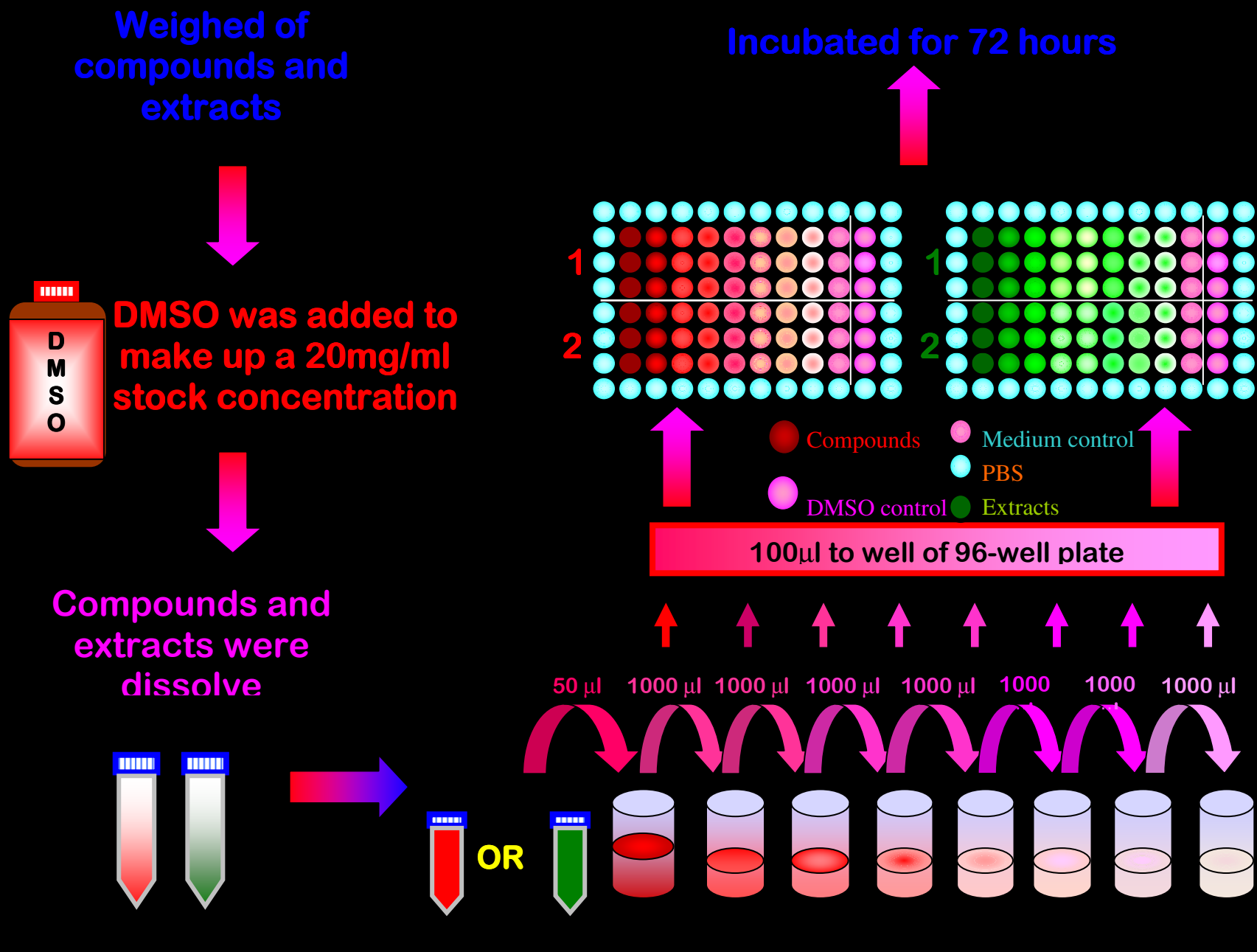
Figure 3.13 Schematic representation of the preparation of extracts/compounds for addition to the 96-well plates which contain the cells

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Figure 3.14 Schematic representation of the XTT assay
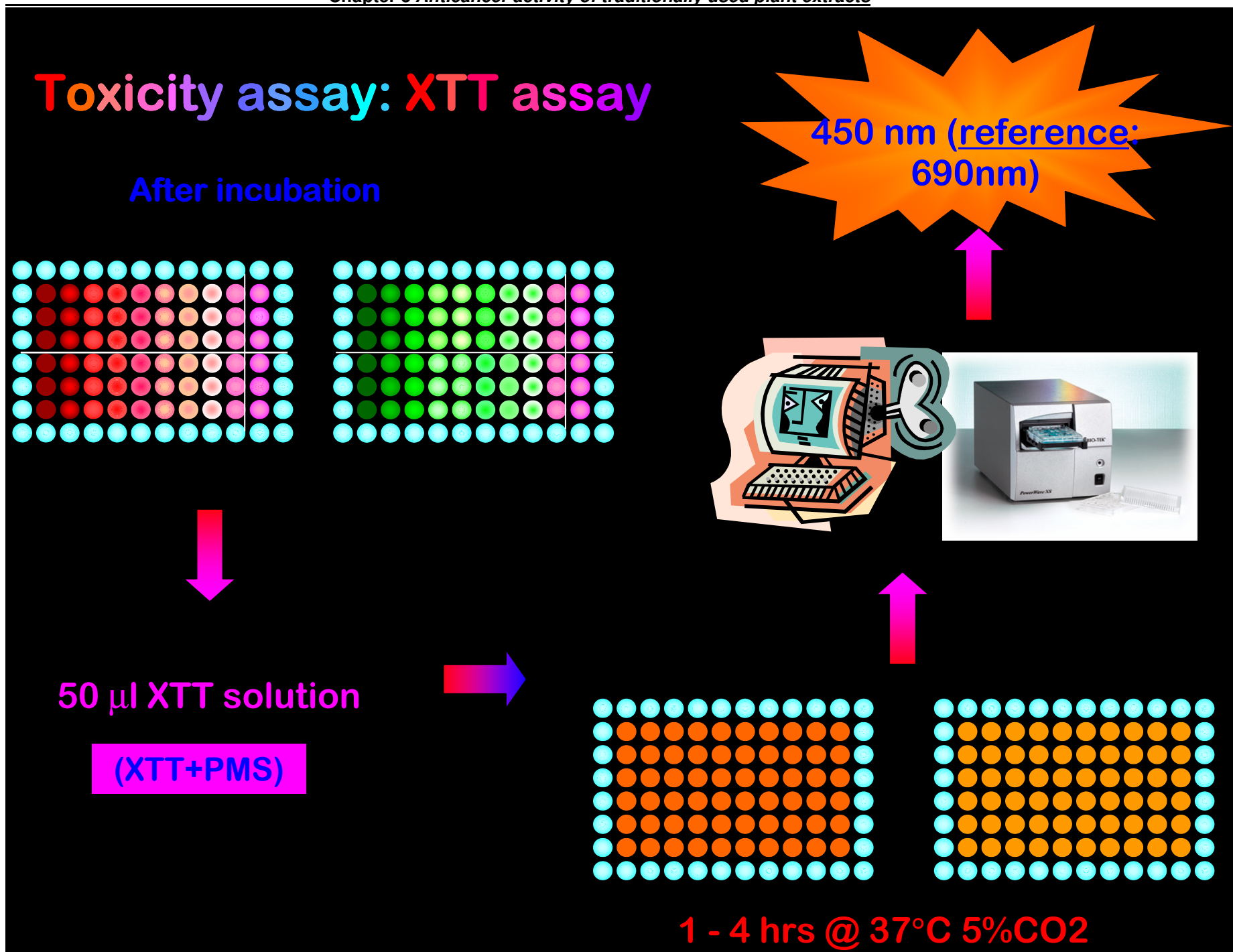
3.12 Results

Table 3.2 Summary of the cytotoxicity results towards the cancer cell lines as well as Vero cells.

Plant extracts	MCF-7 IC_{50} (µg/ml) ± SD	HeLa IC_{50} (µg/ml) ± SD	SNO IC_{50} (µg/ml) ± SD	DU145 IC_{50} (µg/ml) ± SD	Vero IC_{50} (µg/ml) ± SD
Artemisia afra	64.59 ± 3.55	22.12 ± 1.55	29.95 ± 0.04	20.90 ± 0.11	14.49 ± 0.12
Centella asiatica	>100	83.24 ± 3.10	>100	66.58 ± 0.16	13.55 ± 0.19
Euphorbia ingens	>100	>100	>100	85.31 ± 0.05	14.45 ± 0.18
Euclea natalensis	25.27 ± 1.40	29.49 ± 0.34	Not tested cells died due to load-shedding	6.82 ± 0.39	35.16 ± 0.18
Foeniculum vulgare	>100	19.97± 0.048	>100	56.41 ± 0.28	>100
Hypoxis hemerocallidea	>100	52.63 ± 2.02	>100	>100	27.89 ± 0.09
Tulbaghia violacea	30.83 ± 2.71	20.35 ± 0.39	>100	22.29 ± 1.35	70.28 ± 0.06

3.13 Discussion

From the results it is clear that *A. afra* (Table 3.2) is the least specific towards the cell lines tested of all the extracts tested. *A. afra* has its lowest cytotoxicity towards DU145 with an IC_{50} = 20.90 ± 0.111 (µg/ml) followed by its cytotoxicity towards HeLa cells with an IC_{50} = 22.12 ± 1.550 (µg/ml). On SNO and MCF-7 it had higher cytotoxicity with its IC_{50} = 29.95 ± 0.04 (µg/ml) and IC_{50} = 64.59 ± 3.55 (µg/ml) respectively. On Vero cells it however has more specificity with an IC_{50} lower than on all the other cell lines at 14.49 ± 0.12 (µg/ml), indicating that this extract is more toxic towards normal cells. Previous investigation of the antitumour activity in the mouse of fresh leave extracts (50% ethanol) of South African *A. afra* collections showed no activity against Leul-L-1210 and Sarcoma-WM256 (IM) cell lines (Scott *et al.*, 2004). The aqueous extract of Treurnicht (2000) was found to be cytotoxic at higher concentrations used in the assay to HeLa, Vero, Jurkat E6.1, AA-2 and CEM-SS cells (Scott *et al.*, 2004) similar to the cytotoxic results observed on HeLa and Vero cells in the present study.

T. violacea (Table 3.2) has greater specificity than *A. afra* with IC_{50} values on MCF-7, HeLa and DU145 ranging between 30.83 ± 2.71 µg/ml and 20.35 ± 0.39 µg/ml, and less cytotoxic activity (less toxic toward normal cells) on Vero cells with an IC_{50} = 70.28 ± 0.062 (µg/ml). It however did not have any toxic activity (less cytotoxicity) at the highest concentration tested (100 µg/ml) on SNO cells and is therefore more specific in its anticancer activity to the other cell lines. Methanol extracts of *T. violacea* leaves and bulbs inhibited the growth of MCF-7, WHCO3, HT29 and HeLa cell lines (Bungu *et al.*, 2006). Ethanol extract cytotoxicity on MCF-7 cells (30.83 ± 2.71 µg/ml) observed in the present study was found to be less than that of the methanol extract of Bungu *et al*, (2006) (MCF-7 cells 43.9 ± 1.8%). Similarly the IC_{50} value of the ethanol extract on HeLa cells was found to be 20.35 ± 0.385 µg/ml as compared to the one observed by Bungu *et al*. where they found that methanol extract exhibited an IC_{50} value of 45.7 ± 5.9% µg/ml. The leaf extract was more active in squamous oesophageal carcinoma (WHCO3) (30.3 ± 1.8%). HeLa and MCF-7 cells treated with bulb extract had higher apoptotic indices than the other two cell lines (HeLa, 25.80 ± 3.90%; MCF-7, 19.0 ± 4.30%) (Bungu *et al.*, 2006).

With regard to anticancer specificity *C. asiatica* (Table 3.2) didn't have any cytotoxic activity towards MCF-7 and SNO cells. *C. asiatica* was however the most toxic towards the Vero cells. In an earlier study it was found that an aqueous extract of *C. asiatica* stimulated the growth of DU-145, MDA-MB-231 and MCF-7 cells (Steenkamp & Gouws, 2006).

H. hemerocallidea (Table 3.2) has more anticancer specificity, it didn't have any activity at the highest concentration tested (100 µg/ml) towards MCF-7, SNO and DU145 cells, all >100 (µg/ml). It did however, have considerable toxicity towards Vero cells. In a previous study, an aqueous extract of *H. hemerocallidea* stimulated DU-145 cell growth and inhibited the cell growth of MCF-7 cells (Steenkamp & Gouws, 2006), which confirm the activity *H. hemerocallidea* had in this study towards the DU145 cells. However, the ethanol extract also had no inhibitory effect on the cell growth of MCF-7 cells. It was also reported by Ojewole (2002) that the methanolic extracts of *H. hemerocallidea* corm displayed anti-inflammatory activity which is an activity related to cancer.

E. natalensis had IC$_{50}$ values less than the American National Cancer Institute guidelines for crude extracts (30 µg/ml after an exposure time of 72 hours) on all the tested cancer cell lines, MCF-7 (25.27 ± 1.40 µg/ml), HeLa (29.49 ± 0.34 µg/ml) and its highest cytotoxicity was found on DU145 (6.82 ± 0.39 µg/ml). Due to its previously isolated 7-methyljuglone which were too toxic to peripheral blood mononuclear cells it was decided to synthesize several derivatives for further investigation.

E. ingens (Table 3.2) had less cytotoxicity than the tested concentration (>100 µg/ml) on all four cancer cell lines and were considerably toxic towards Vero cells with an IC$_{50}$ = 14.45 ± 0.18 (µg/ml).

F. vulgare (Table 3.2) was the only extract which did not have any toxicity towards the Vero cells and had the highest cytotoxicity of all the extracts towards HeLa cells, 19.97± 0.048 µg/ml. It also has some cytotoxic activity towards DU145, 56.41 ± 0.28 µg/ml. The American National Cancer Institute guidelines set the limit of activity for crude extracts at 50% inhibition of proliferation of less than 30 µg/ml after an exposure time of 72 hours (Steenkamp & Gouws, 2006). Because of its cytotoxic activity towards HeLa (the most toxic, lower than the 30 µg/ml limit) and DU145 cells and not towards the other human cancer cell lines and also Vero cells it was further selected for isolation of the bioactive compound/s.

3.14 References

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CHAPTER 4

Cytotoxicity of 7-methyljuglone and its derivatives on cancer cells and on non cancerous cells

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Cytotoxicity of 7-methyljuglone and its derivatives on cancer cells and on non cancerous cells

4.1 Introduction

An essential route to new pharmaceuticals emerged with the modern medicine and single pure drugs era, which were greatly provided by plant-derived active principles and their semi-synthetic and synthetic analogs (Lee, 1999). During the rational drug design modification and development stage of new anticancer drugs, the aim is to create new synthetic analogs with better activity, enhanced pharmacological profiles and reduced toxicity (Lee, 1999).

In the US bioactivity directed fractionation and isolation led to the acceptance of nine plant-derived compounds (vinblastine (Velban), vincristine (Oncovin), etoposide (VP-16), teniposide (VM-26), taxol (Paclitaxel), navelbine (Vinorelbine) for use as anticancer drugs since 1961. Newer accepted (in 1996) anticancer drugs include taxotere (docetaxel), topotecan (Hycamtin), and irinotecan (Camptosar) (Lee, 1999). In China, 11 other anticancer agents (d-tetrandrine, colchicinamide, 10-hydroxycamptothecin, homoharringtonine, monocrotaline, gossypol, (-)-sophocarpine, curcumol, lycobetaine, curdione and indirubin) are currently also used.

4.1.1 Quinonoids

Quinoid compounds are widely distributed in nature and they are found in all respiring animal and plant cells as well as in fungi and bacteria, primarily as secondary metabolites (Chakrabarty, 2002). Naturally occurring quinones are divided in three main groups: benzoquinones, naphthoquinones and anthraquinones, in order of increasing size (Powis, 1987). A number of natural quinoids such as anthracyclines, mitoxantrones and saintopin as well as many other quinoids and their synthetic analogues (simple and more complex) have been found to possess significant antitumour activity, selected throughout the random screening of an enormous number of compounds (Powis, 1987), by virtue of their facile redox capacity (Chakrabarty, 2002; Chung *et al.*, 2004; Kim *et al.*, 2006). By the year 1974 the National cancer Institute's Drug Research and Development Programme in the USA tested more than 1500 quinones. Quinonoids were initially studied in the late 1950s in soil fungi, i.e. *Streptomyces* strains, formed as pigmented antibiotics (O'Brien, 1991). In 1963 the anthraquinones glycosidic antibiotic (daunorubicin) was the first to be isolated and its antileukemic activity was revealed. The first synthetic anticancer drugs, rationally designed and synthesized in 1954, which were able to cross the blood brain barrier was the aziridinylbenzoquinones (O'Brien, 1991).

The quinoids comprise also the second largest class of antitumour agents currently in use. Many traditional oriental herbal preparations include several commonly used medicinal plants from which numerous quinonoid compounds were isolated recently, which are currently being studied for their anticancer properties and mechanisms of action associated with these properties (Hazra *et al.*, 2005). Most antitumour quinoid compounds have complex structures. The chemical reactivity contributions are therefore often tricky to separate from the different metabolic pathways and from the overall biological activity (Powis, 1987). Antitumour activity flaunted by many naphthoquinone derivatives (e.g. menadione) has been used as antitumour drugs (Chen *et al.*, 2004). However, the prospective anticancer activity of quinonoid compounds and their derivatives has remained unexplored at large. Furthermore studies confirmed the powerful pharmacologic effects of several 1,4-naphthoquinone derivatives and also their connection with distinct antimicrobial and antitumour activities (Chung *et al.*, 2004; Kim *et al.*, 2006). Naphthoquinones are capable of eliciting and inducing both apoptotic as well as necrotic cell death (Montoya *et*

al., 2005; Stasiauskaite *et al.*, 2006). Strong corresponding relationships occur primarily between apoptosis frequency and growth inhibition potency (Stasiauskaite *et al.*, 2006). Semiquinone radicals' formation can also be induced by quinone analogues. Superoxide is formed by a process catalyzed by flavoenzymes (e.g. nicotinamide adenine dinucleotide phosphate (NADPH)-cytochrome-P-450 reductase) as soon as semiquinone radicals are able to transfer an electron to oxygen (Powis, 1987; Kim *et al.*, 2006). The semiquinone radical anions as well as the superoxide of the naphthoquinone analogues can produce the hydroxyl radical which can cause DNA strands to break (Kim *et al.*, 2006). It was recently confirmed that a number of 1,4-naphthoquinones were capable of inhibiting Cdc25, including vitamin K_3 (Ham & Lee, 2004).

Studies conducted by Orrenius and his co-workers with 2-methyl-1,4-naphthoquinone known as menadione and isolated hepatocytes provided significant insights into the manner in which simple quinones metabolism could possibly lead to damage and death of cells (Powis, 1987). Depletion of the protein thiol groups and intracellular glutathione can take place with no a modification in the redox state of cellular pyridine nucleotides and lead to toxicity, as observed with menadione-induced hepatocyte toxicity (Powis, 1987). It has been established that the alkylation of vital protein thiol or amine groups and/or the oxidation of necessary protein thiols by triggered oxygen species and/or glutathione (oxidized form) (GSSG) in resting or non-dividing cells is the molecular basis used for launching the quinone cytotoxicity (O'Brien, 1991). When reductases reduce a quinone to a semiquinone radical, oxidative stress occurs. Eventually the quinone is reformed after superoxide radicals were produced once the semiquinone radical reduces oxygen. Cytotoxic levels of hydrogen peroxide and GSSG is retained by the cell due to this futile redox cycling and oxygen activation and furthermore causes cytotoxic mixed protein disulfide formation (O'Brien, 1991). Glutathione (reduced form) GSH conjugates are formed by most quinones. These GSH conjugates also go through futile redox cycling and oxygen activation (O'Brien, 1991). Naphthoquinones and benzoquinones are very cytotoxic since they have higher electrophilicity and thiol reactivity and also because these quinines or GSH conjugates are easily reduced to semiquinones which will in turn activate oxygen (O'Brien, 1991). A wide range of toxic effects are exerted by quinonoids and when administered cause side effects such as: nausea, vomiting, hair loss and tissue necrosis (Young *et al*., 1981).

4.1.2 Bioactivity, cytotoxicity of 7-methyljuglone

The hydroxynaphthoquinonoids are known to have antiprotozoal activities since the 1930's and have attracted renewed attention during the past three decades, when some synthetic compounds of this class were identified as potential drugs against several protozoan parasitic diseases (Croft *et al.*, 1992). Marked improvement were showed from *in vitro* studies in the antileishmanial, antitrypanosomal activities of diospyrin and its derivatives which was attributable to minor changes in the functional groups of the natural product (Yardley *et al.*, 1996). Similar observations were recorded in the studies on antitumour and antiplasmodial effects of these compounds (Hazra *et al.*, 1994, 1995).

Plant extracts should have the ability to trigger/induce a cleavable complex with purified mammalian topoisomerases in order for them to be recognizing new plant metabolites with antitumour activity (Fujii *et al.*, 1992). It was then found by Fujii and co-workers, in 1992, that 'plumbagin' and 'shikonin', two plant naphthoquinones, are effective inducers, *in vitro*, of the cleavable complex formation by means of topoisomerase II. Therefore, these naphthoquinones induced topoisomerases II-mediated DNA cleavage by creating a cleavable complex. This is also seen with antitumour agents such as 'Lawson', 'demethylepipodophyllotoxin', ethylidene-β-glucoside' and 'lapacol'. These antitumour plant metabolites are structurally related to naphthoquinone moieties, but plumbagin and shikonin proved to have a parallel DNA cleavage pattern with topoisomerase II which was nothing like the cleavage patterns induced by other prominent topoisomerase II-active drugs (Fujii *et al.*, 1992).

Diospyrin derivatives were synthesized with the objective of improving the therapeutic effects, and reduce toxicity towards normal cell lines as well as opting to enhancing their efficacy to tumour cells. Diospyrin and its synthetic derivatives have been shown to induce apoptosis in different human tumour cells (Chakrabarty *et al.*, 2002). Tazi *et al.* (2005) found that even though diospyrin and its derivatives inhibit kinase activity of topoisomerase I and interfere with camptothecin-independent topoisomerase I mediated DNA cleavage, on the other hand, on DNA no reactions of topoisomerase I were block. Diospyrin derivatives therefore mediate the topoisomerase I conformational modification. They also

showed that diospyrin derivatives change several steps, either the first or the second catalytic step, of splicing *in vitro*, but not spliceosome assembly and that they are able to stall the assembly (dynamic) of the spliceosome (Tazi *et al.*, 2005). Full spliceosome formation was however prevented by diospyrin. The antitumour activity of diospyrin was enhanced through liposomal encapsulation, *in vivo* (Hazra *et al.*, 2005). Ting *et al.* (2003) found that isodiospyrin showed cytotoxicity against colon carcinoma (COLO-205) as well as lymphocytic leukaemia (P-388) and also a novel human DNA topoisomerase I inhibitor. Isodiospyrin has been examined for its inhibition of human DNA topoisomerase I, antibacterial and anti-inflammatory activities (Gafner *et al.*, 1987).

7-Methyljuglone, a monomer of diospyrin has been proved to have potent anti-tuberculosis and anticancer activity. It has been reported to possess among other activities antibacterial and cytotoxic properties against human colon carcinoma cells. There are several reports on the anticancer activity of naphthoquinones on a panel of cancer cell lines. 7-Methyljuglone, isolated from *E. divinorum,* showed marked cytotoxicity against murine lymphocytic leukaemia (P-388) and human prostate cancer (LNCaP) (Mebe *et al.*, 1998). Considering reports of previous researchers on various naphthoquinones, including 7-methyljuglone, it was decided to investigate the anticancer/cytotoxic activity of the synthetic derivatives on various cancer cell lines.

4.2 Materials and Methods

4.2.1 Synthesis of 7-methyljuglone and its derivatives

The parent compound 7-methyljuglone and its derivatives were successfully synthesized by Dr A. Mahapatra (Mahapatra, *et al*, 2007) (Table 4.1, Figure 4.1).

Table 4.1 List of naphthoquinones studied for anticancer activity modified from (Mahapatra, *et al*, 2007).

Naphthoquinones	X	R1	R2	R3
1) 8-Chloro-5-hydroxy-7-methyl-1, 4-naphthoquinone	Cl	Me	H	OH
2) 8-Bromo-5-hydroxy-7-methyl-1, 4-naphthoquinone	Br	Me	H	OH
3) 8-Fluoro-5-hydroxy-7-methyl-1, 4-naphthoquinone	F	Me	H	OH
4) 8-Chloro-5-hydroxy-6-methyl-1,4-naphthoquinone	Cl	H	Me	OH
5) 5-Hydroxy-6-methyl-1,4-naphthoquinone	H	H	Me	OH
6) 5-Hydroxy-7-methyl-1,4-naphthoquinone (7-MJ)	H	Me	H	OH
7) 8-Chloro-5-methoxy-7-methyl-1,4-naphthoquinone	Cl	Me	H	OMe
8) 5-Ethoxy-8-chloro-7-methyl-1,4-naphthoquinone	Cl	Me	H	OEt
9) 5-Methoxy-7-methyl-1,4-naphthoquinone	H	Me	H	OMe
10) 5-Ethoxy-7-methyl-1,4-naphthoquinone	H	Me	H	OEt
11) 8-Chloro-5-methoxy-6-methyl-1,4-naphthoquinone	Cl	H	Me	OMe
12) 5-Ethoxy-8-chloro-6-methyl-1,4-naphthoquinone	Cl	H	Me	OEt
13) 5-Acetoxy-8-chloro-7-methyl-1,4-naphthoquinone	Cl	Me	H	OAc
14) 5-Acetoxy-7-methyl-1,4-naphthoquinone	H	Me	H	OAc
15) 5-Acetoxy-8-chloro-6-methyl-1,4-naphthoquinone	Cl	H	Me	OAc
16) 8-Chloro-7-methylnaphthalene-1,2,4,5-tetra-O-acetate	Cl	Me	H	
17) 7-Methylnaphthalene-1,2,4,5-tetra-O-acetate	H	Me	H	
18) 8-Chloro-6-methylnaphthalene-1,2,4,5-tetra-O-acetate	Cl	H	Me	
19) 2,5-dihydroxy-7-methyl-1,4-naphthoquinone		CH$_3$		OH
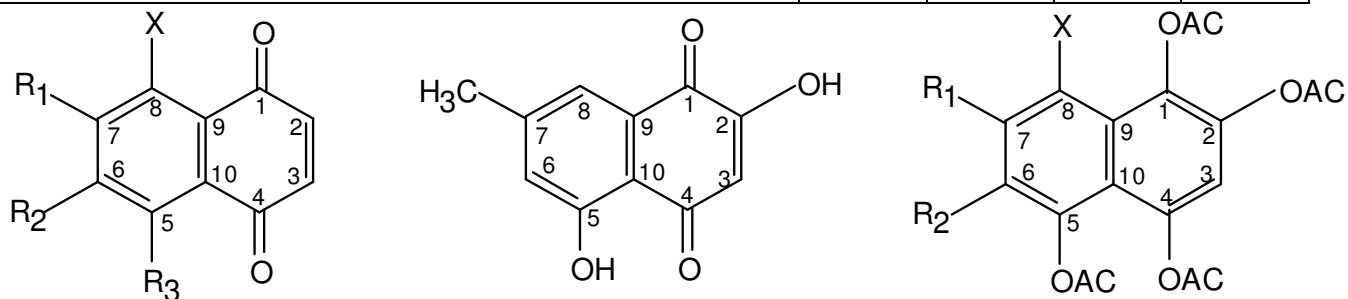
Figure 4.1 (A) Compounds 1-15 (B) 19 (C) 16-18.

4.2.2 Culture of cancer cells

U937 cells were maintained in culture flasks in complete RPMI1640 medium (Sigma, Germany) supplemented with 10% fetal bovine serum (Delta Bioproducts, SA), containing 25mM HEPES and 2mM glutamine, in a humidified 5% CO_2 incubator at 37°C.

All the cell culture procedures were the same, as for the extracts (see chapter 3) the naphthoquinone derivatives were tested on the same cancer cell lines and the same concentrations (0.781 µg/ml – 100 µg/ml) as for the extracts.

4.2.3 Cytotoxicity in peripheral blood mononuclear cells (PBMCs)

Blood was obtained from healthy adult volunteers. PBMCs were separated with BD Vacutainer^{TM} CPT^{TM} cell preparation tubes containing sodium heparin. Blood was drawn into the tubes and immediately the tubes were inverted 10 times to mix the anticoagulant additive with the blood. The tubes were centrifuged within 30 minutes after the blood collection at room temperature for 30 minutes at 1800xg. After centrifugation different layers were observed in the BD Vacutainer^{TM} CPT^{TM} cell preparation tubes. The plasma, mononuclear cells and platelets were transferred to other sterile 15 ml tubes and were inverted gently 10 times. The tubes were centrifuged for 15 minutes at 300xg at room temperature, the supernatants were aspirated and the pellets were resuspended in complete RPMI1640 medium (Figure 4.2). The centrifugation step was repeated, the supernatants were aspirated and this time the pellets were resuspended in complete RPMI1640 medium, after which the PBMCs were counted with trypan blue and a hemacytometer. Thereafter, the PBMCs were quite densely resuspended, $2x10^6$ cells per millilitre and were seeded in 96-well plates at 200 µl/well (or $4x10^5$ cells per well). The plates were incubated at 37°C in a humidified atmosphere until they were used.

The test compounds were weighed and dissolved in DMSO to give a 40 µg/µl stock solution. The stock solution of each of the compounds was diluted with RPMI1640 complete medium to give the different final concentrations tested (1, 10 and 100 µg/ml)

which were added to the 96-well plate. Control wells were prepared in which only complete RPMI1640 medium was added. The 96-well plates were then incubated for 48 hours at 37°C in a humidified atmosphere with 5% CO_2.

After 48 hours, 40 µl of the signal reagent CellTiter-Blue® (Promega) was added, to all the wells. The plates were incubated at 37°C in a humidified atmosphere with 5 % CO_2 in aluminium foil for four hours. After the incubation period, 200 µl from each well was transferred to a black 96-well plate (Nunc). Data were recorded using a ThermoLabsystem Fluoroskan AscentFL fluorescence microplate reader at excitation and emission wavelengths of 560 and 590 nm, respectively.

(a)   (b)   (c)
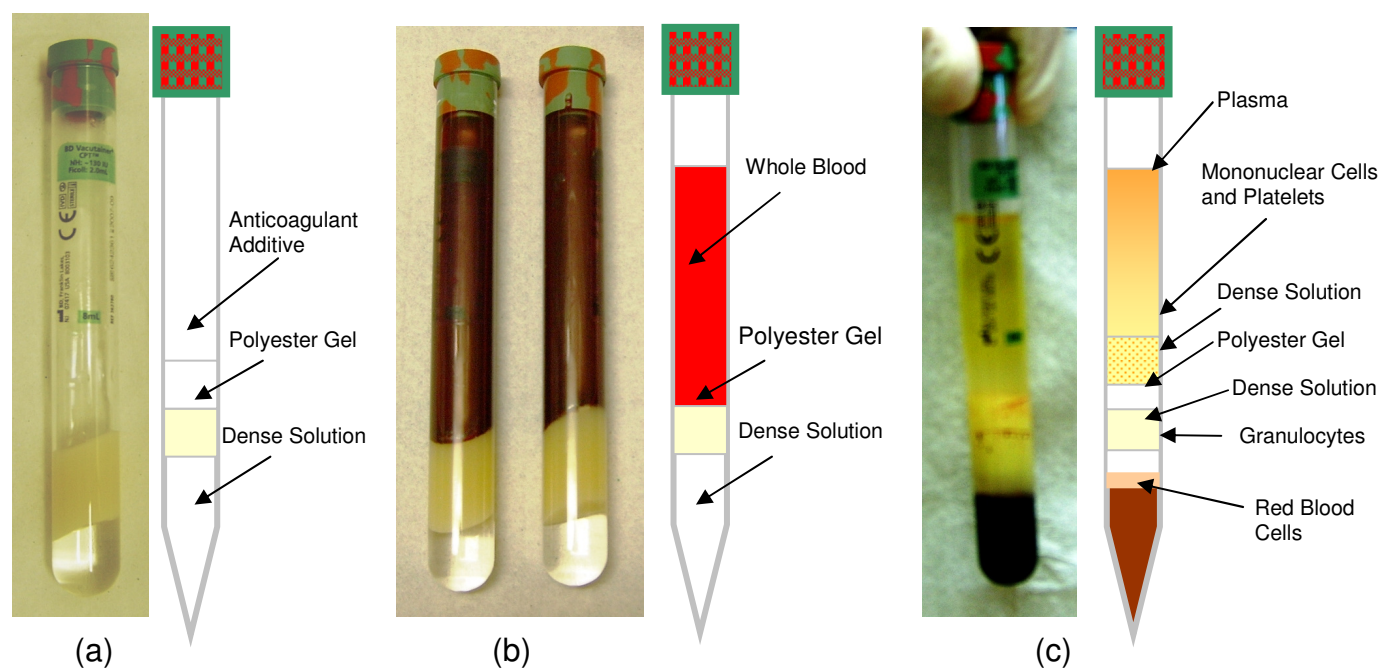
Figure 4.2 (a) A tube before use. (b) After blood collection. (c) After centrifugation

4.3 Results

4.3.1 Cytotoxicity on four human cancer cell lines

The growth inhibition results on the four adherent cancer cell lines (Figure 4.3) are examples of the dose response curves (Table 4.2) showed that all the naphthoquinone derivatives showed activity against these cell lines. With GraphPad Prism 4 a sigmoidal

dose-response (variable slope) curve fit was done and the IC$_{50}$ values of the extracts were determined from the concentration-effect relationship (Figure 4.4). However, the positive control doxorubicin was more effective, by at least an order of magnitude, than any of the synthesized derivatives. It is clearly evident, however, that most of these display considerable cytotoxicity against the cancer cell lines.
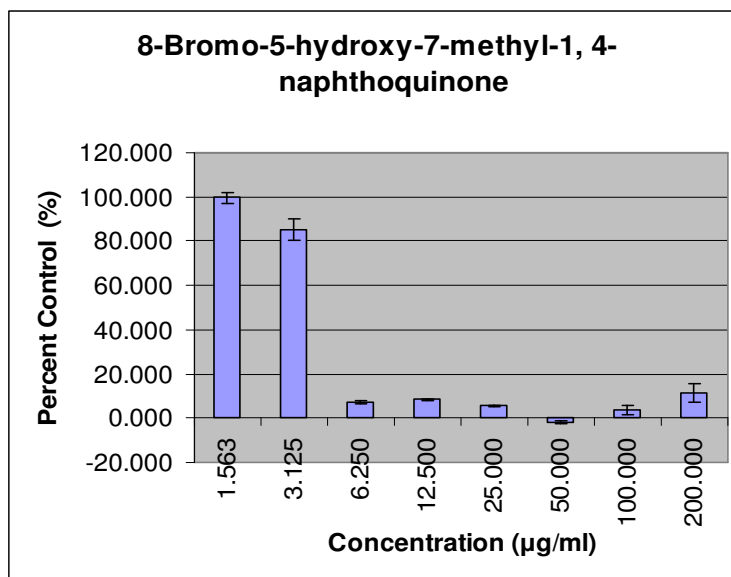
Figure 4.3 Dose response of 8-Bromo-5-hydroxy-7-methyl-1, 4-naphthoquinone on MCF-7 cell viability.
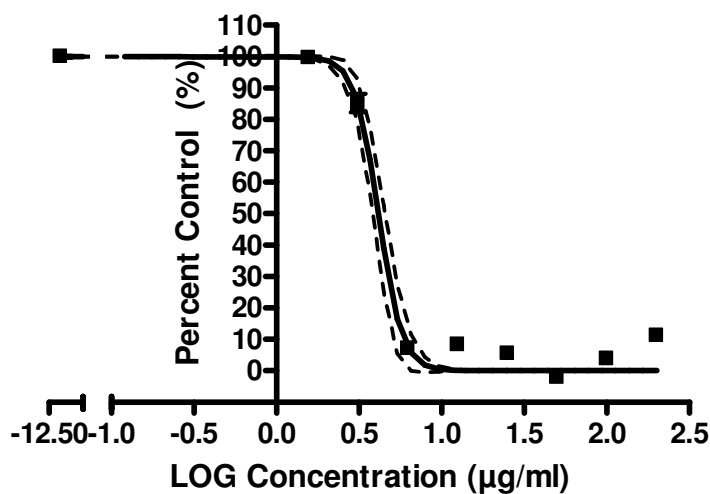
Figure 4.4 Dose response curve fit of 8-Bromo-5-hydroxy-7-methyl-1, 4-naphthoquinone on MCF-7 cells.

Table 4.2 Summary of all the IC_{50} results on all four the human cancer cell lines of all the derivatives of 7-methyljuglone and the positive controls.

Compound	MCF-7	HeLa	SNO	DU145
	IC_{50} (µg/ml) ± SD	IC_{50} (µg/ml) ± SD	IC_{50} (µg/ml) ± SD	IC_{50} (µg/ml) ± SD
1. 8-Chloro-5-hydroxy-7-methyl-1, 4-naphthoquinone	3.50 ± 0.16	3.02 ± 0.87	3.85 ± 2.01	6.57 ± 0.88
2. 8-Bromo-5-hydroxy-7-methyl-1, 4-naphthoquinone	4.15 ± 0.28	3.04 ± 0.92	3.98 ± 1.10	12.37 ± 1.25
3. 8-Fluoro-5-hydroxy-7-methyl-1, 4-naphthoquinone	4.19 ± 0.35	2.11 ± 0.67	3.91 ± 2.20	3.18 ± 1.91
4. 8-Chloro-5-hydroxy-6-methyl-1,4-naphthoquinone	2.23 ± 0.18	2.24 ± 0.90	2.11 ± 0.23	2.07 ± 2.11
5. 5-Hydroxy-6-methyl-1,4-naphthoquinone	2.89 ± 0.21	3.09 ± 1.24	3.59 ± 2.17	2.91 ± 2.14
6. 5-Hydroxy-7-methyl-1,4-naphthoquinone (7-MJ)	5.11 ± 0.62	12. 54 ± 2.45	15.33 ± 4.32	2.24 ± 0.27
7. 8-Chloro-5-methoxy-7-methyl-1,4-naphthoquinone	4.88 ± 0.20	4.54 ± 1.03	5.83 ± 1.60	6.31 ± 2.35
8. 5-Ethoxy-8-chloro-7-methyl-1,4-naphthoquinone	10.05 ± 1.54	4.78 ± 1.81	11.10 ± 2.00	6.37 ± 1.43
9. 5-Methoxy-7-methyl-1,4-naphthoquinone	3.10 ± 0.76	4.27 ± 0.82	4.56 ± 1.56	6.22 ± 1.53
10. 5-Ethoxy-7-methyl-1,4-naphthoquinone	5.14 ± 1.13	6.43 ± 2.69	5.48 ± 1.63	3.25 ± 1.24

Compound	MCF-7	HeLa	SNO	DU145
	IC$_{50}$ (µg/ml) ± SD	IC$_{50}$ (µg/ml) ± SD	IC$_{50}$ (µg/ml) ± SD	IC$_{50}$ (µg/ml) ± SD
11. 8-Chloro-5-methoxy-6-methyl-1,4-naphthoquinone	3.84 ± 0.65	9.83 ± 3.41	10.63 ± 1.56	8.66 ± 0.96
12. 5-Ethoxy-8-chloro-6-methyl-1,4-naphthoquinone	8.09 ± 1.81	8.33 ± 7.68	9.37 ± 1.83	6.59 ± 1.31
13. 5-Acetoxy-8-chloro-7-methyl-1,4-naphthoquinone	10.04 ± 1.71	6.96 ± 1.32	7.64 ± 1.35	6.76 ± 1.73
14. 5-Acetoxy-7-methyl-1,4-naphthoquinone	3.39 ± 0.60	3.98 ± 3.76	6.49 ± 1.62	5.57 ± 1.66
15. 5-Acetoxy-8-chloro-6-methyl-1,4-naphthoquinone	4.95 ± 1.01	5.77 ± 1.57	7.58 ± 1.63	3.32 ± 2.17
16. 8-Chloro-7-methylnaphthalene-1,2,4,5-tetra-O-acetate	20.90 ± 3.08	21.64 ± 5.75	23.29 ± 0.03	24.37 ± 1.37
17. 7-Methylnaphthalene-1,2,4,5-tetra-O-acetate	30.70 ± 4.86	8.39 ± 4.51	9.39 ± 3.52	24.21 ± 1.38
18. 8-Chloro-6-methylnaphthalene-1,2,4,5-tetra-O-acetate	23.52 ± 4.22	11.42 ± 1.80	21.91 ± 1.41	33.97 ± 1.31
19. 2,5-dihydroxy-7-methyl-1,4-naphthoquinone	2.99 ± 0.52	1.09 ± 0.22	4.74 ± 1.35	1.39 ± 0.75
20. Doxorubicin	0.36 ± 0.18	0.0086 ± 0.0011	0.0085 ± 0.0045	0.0088
21. Zearalenone	2.62 ± 1.15	1.63 ± 0.30	1.68 ± 0.10	2.43 ± 0.23

4.3.2 Cytotoxicity on U937 cells

The comparative growth inhibition results on U937 cells (Figure 4.5) suggest that compounds 1-4 were the least cytotoxic, whilst the other compounds showed much greater cytotoxicity. Compound 5 had the highest cytotoxicity followed by compound 6 and 19.
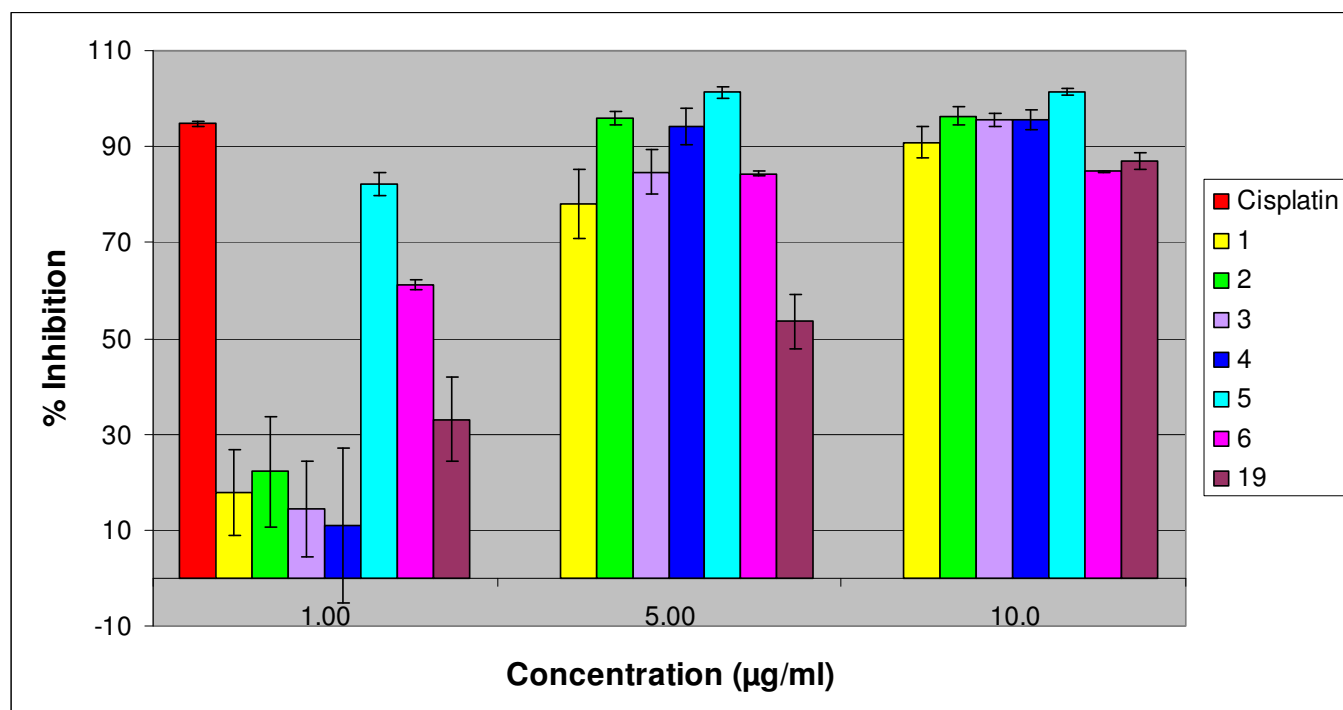
Figure 4.5 Percentage inhibition of selected compounds on U937 cells. Bars and error bars indicate mean ± SD of quadruplicates U937

4.3.3 Cytotoxicity on PBMCs

The comparative growth inhibition results on PBMCs (Figure 4.6) suggest that compounds 3 and 19 were the least cytotoxic, whilst the other compounds showed much greater cytotoxicity at the lower concentrations tested (Table 4.3).
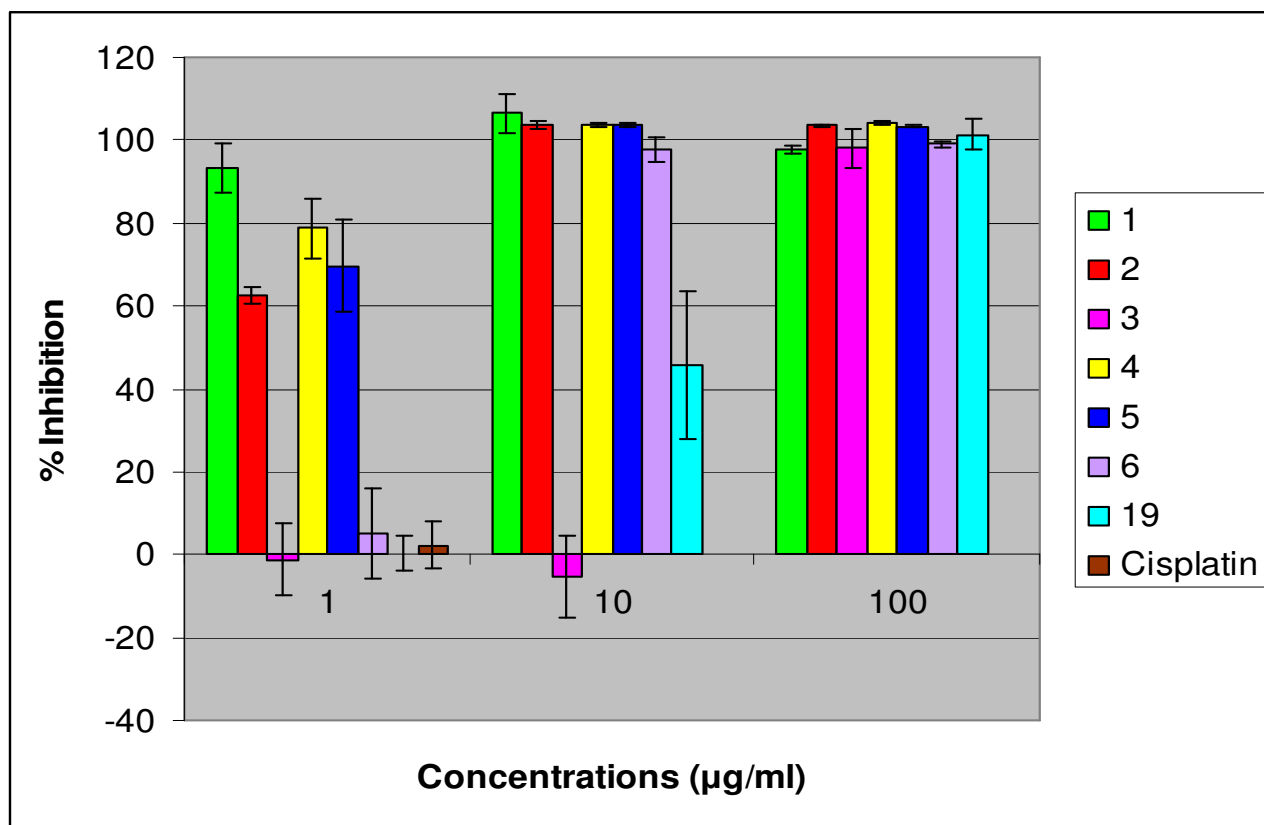
Figure 4.6 Percentage inhibition of selected compounds on peripheral blood mononuclear cells. Bars and error bars indicate mean ± SD of quadruplicates.

Table 4.3 Cytotoxic activity of 8-fluoro-5-hydroxy-7-methyl-1, 4-naphthoquinone, 5-hydroxy-7-methyl-1,4-naphthoquinone   (7-MJ)   and   2,5-dihydroxy-7-methyl-1,4-naphthoquinone on peripheral blood mononuclear cells.

Treatment	(Peripheral blood mononuclear cells) Lymphocytes IC$_{50}$ (µM)
Compound 3	188.70
Compound 6	18.40
Compound 19	53.97

4.4 Discussion

4.4.1 Cytotoxicity on four human cancer cell lines

From the synthesized compounds the 1,2,4,5-tetra-*O*-acetate derivatives: compounds 17, 16 and 18 gave the least anticancer or cytotoxic activity, mostly with activity above 20 μg/ml, of all the synthesized compounds on all the cancer cell lines except compound 17 (without the Cl at the 8 position) which had higher cytotoxic activity on the HeLa and SNO cancer cell lines, but was also the least cytotoxic on the MCF-7, IC_{50} = 30.70 ± 4.86 μg/ml. Of the 1,2,4,5-tetra-*O*-acetate derivatives it is also has the best cytotoxic activity and specificity towards the DU145 cells with an IC_{50} = 24.21 ± 1.38 μg/ml. Therefore, substituting compound 6 (the parent) with 1,2,4,5-tetra-*O*-acetate groups did give less cytotoxicity, but also the least for anticancer activity and were not worth further investigating. The 5-ethoxy derivatives, compounds 8, 12 and 10 had anticancer activity of 11.10 ± 2.00 μg/ml and less on all four cancer cell lines, with compound 10 (without the Cl at the 8 position) the best anticancer activity of the 5-ethoxy derivatives with a value as low as 3.25 ± 1.24 μg/ml on DU145 cells. Substituting with 5-ethoxy led to enhanced cytotoxic activity than the 1,2,4,5-tetra-*O*-acetate derivatives, and as a result thereof to greater anticancer activity. The novel compound 11 and the other two 5-methoxy derivatives, compounds 9 and 7, had activity of 10.63 ± 1.56 μg/ml and less on the four cell lines. Compound 9 had the best cytotoxic activity of the on MCF-7 cells with an IC_{50} = 3.84 ± 0.65 μg/ml. But of all the 5-methoxy derivatives, compound 9 (without the Cl at the 8 position) had the least cytotoxic activity of IC_{50} = 3.10 ± 0.76 μg/ml on MCF-7 cells. Subsequently it were concluded that compounds without a Cl functional group at the 8 position had less cytotoxic activity and consequently more anticancer activity.

Compound 3 had the best cytotoxic activity on all the cell lines with an IC_{50} value on HeLa cells IC_{50} =2.11 ± 0.67 μg/ml and had activity of 4.19 ± 0.35 μg/ml on MCF-7 cells and less than that on all four the other cell lines. The introduction of a fluorine atom into antibiotic quinolones has been shown to enhance their activity (Domagala *et al.*, 1986; Miyamoto *et al.*, 1990), and now the same was found with its cytotoxic activity. The other 5-hydroxy (8-Halogen substituted) derivatives, compounds 1, 4 and 2 also had cytotoxic activity on all

the cell lines with 6.57 ± 0.88 µg/ml and less on all four the tested cell lines, except compound 2 which had less cytotoxic activity on DU145 cells with an IC_{50} value of 12.37 ± 1.25 µg/ml.

Previous studies have shown that mono- or dihydroxy substitution (at C5 or C5 & C8 positions in the aromatic ring) of naphthoquinones results in higher toxicity as compared to the parent 1,4-naphthoquinone due to increased efficiency of redox cycling (Ollinger & Brunmark, 1991). The 2,5-dihydroxy substitution of compound 19 consequently led to the greatest toxicity of all the derivatives on HeLa cells with an IC_{50}= 1.09 ± 0.22 µg/ml (utmost cytotoxic) while the parent compound 6 was less cytotoxic than several of these derivatives. Compound 6 had its highest cytotoxic activity and specificity towards DU145 cells, IC_{50}= 2.24 ± 0.27 µg/ml, but it ranged between 2.24 ± 0.27 and 15.33 ± 4.32 µg/ml, on the four cell lines. In this study higher cytotoxicity were found in most of the substituted compounds, except compounds 16-18 and others on some of the selected cell lines, than was found in compound 6 (the parent) except on DU145 cells where compound 6 had an IC_{50} = 2.243 ± 0.271µg/ml. However, the positive controls: doxorubicin was much more effective than any of the synthesized derivatives with its IC_{50} values between 0.0085 ± 0.0045 and 0.36 ± 0.18 µg/ml. Zearalenone had less cytotoxic activity than doxorubicin, IC_{50} values ranging between 2.62 ± 1.15 and 1.63 ± 0.30 µg/ml on MCF-7 and HeLa respectively.

Some naphthoquinones and naphthoquinone derivatives that were proved to have antitumour activity, by one mechanism of action, is the stabilization of the cleavable complexes which were formed by topoisomerase II (topo II) along with DNA leading to apoptosis (Chen *et al.*, 2004). Therefore several compounds with the lowest IC_{50} values were selected for further investigation for the mechanism of action used by these compounds.

4.4.2 Cytotoxicity on U937 cells

Compounds 1-6 and 19 were selected based on their toxicity, and were tested on U937 cells (Figure 4.5). All these compounds were toxic at 5 and 10 µg/ml. Cisplatin, the

positive control, had the highest toxicity/percentage of inhibition even at 1 µg/ml. Compound 5 was the most toxic of all the selected compounds, and the other compounds had IC_{50} values between 1 and 5 µg/ml and were therefore less toxic. Compound 4 were the least toxic of the selected compounds.

4.3.3 Cytotoxicity on PBMCs

Compounds 3, 6 and 19 were the least cytotoxic on the PBMCs, whilst the other compounds showed much greater cytotoxicity. Compound 6 had the lowest IC_{50} at 18.40 µM and was followed by compound 19 IC_{50} at 53.97 µM and these compounds (3 and 6) was therefore more toxic towards PBMCs than compound 3 which had an IC_{50} of 188.70 µM (Table 4.3). The IC_{50} values of the other compounds (compounds 1, 2, 4 and 5) were less than 1 µM, the lowest concentration tested, and therefore very cytotoxic towards PBMCs (Figure 4.6).

4.5 References

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CHAPTER 5

Isolation of the bioactive compounds from

Foeniculum vulgare

Chapter 5

Isolation of the bioactive compounds from *Foeniculum vulgare*

5.1   Introduction

The skilful application of fractionation methods exploits the fact that an extract can be separated into groups of compounds sharing similar physico-chemical characteristics and this process is called fractionation and can be carried out in various ways, each, of which group compounds according to one or more particular feature such as solubility, size, shape, electrical charge and several other features which may influence grouping (Houghton & Raman, 1998). By the application of fractionation methods many different fractions of a single extract can be obtained which will contain different components.

5.1.1 Chromatography

Chromatography is one of the most widely used techniques in the fractioning of techniques. The major uses of chromatography are: as an analytical method to quantify a certain compound, and in the isolation of compounds for identifying.   All chromatographic techniques are based on a method that uses stationary and mobile phase. The compound has different properties for each of them making the separation of compounds with different

properties easy. Compounds that move slowly through the column have a high affinity for the stationary phase, and a compound that moves fast or at a rapid pace have high affinity for the mobile phase. There are quit a few principles involved in the separation of compounds from each other in a mixture. These are adsorption, partitioning, reversed-phase partitioning, ion exchange and affinity.

5.1.4 Steps for isolation

There are sequences of events that need to be followed to get a usable drug from any sample. According to Houghton & Raman (1998) the steps involved are as follows:

- Step 1 Preparation of sample in form suitable for tests.
- Step 2 Testing of sample, preferably in a range of conditions.
- Step 3 If sample gives a positive reaction to test, fractionation of the sample is carried out.
- Step 4 Testing of fractions.
- Step 5 Fractions showing activity are further fractionated, usually by a different method than that first employed.
- Step 6 Testing of each new fraction.
  (NB steps 5 and 6 may be repeated several times until one or more pure compounds are obtained which display activity.)
- Step 7 Elucidation of the molecule structure of the active molecules.
- Step 8 Determination of the concentration of the active compound necessary for a defined biological activity to be observed.
- Step 9 Determination of the amount of active compound present in an active amount of the original extract and comparison with the value found in step 8.
- Step 10 Isolation, synthesis and testing of related compounds.
- Step 11 Investigation of mode of action and metabolism of the active component.

The time-tested method, bioassay-guided fractionation, has enhanced success in the natural product discovery field. It however remains subjective even though it is the 'best' bioassay system for examining cancer chemoprevention (Park & Pezzuto, 2002). A suitable number of lead starting materials can be identified by means of *in vitro* assays and

it also enable the procurement of active agents through bioassay directed isolation in a fixed period of time from a realistic number of sources (Park & Pezzuto, 2002). Numerous classes of cytotoxic compounds with novel antitumour activities were isolated from medicinal herbs/plants via bioactivity-directed fractionation; these include polyphenolic compounds, sesquiterpene lactones, ligans, triterpene glucosides, flavonoids, colchicines derivatives, and quinine derivatives (Lee, 1999).

5.3   Materials and Methods

5.3.1 Collection of plant material

Fennel seeds were bought from a local shop, and were identified at the H.G.W.J. Schwelckerdt Herbarium (PRU) of the University of Pretoria.

5.3.2 Isolation of bioactive compounds

5.3.2.1   Preparation of extract

Two kilogram (kg) of *Foeniculum vulgare* seeds were ground with a kitchen grinder until and was extracted with 1 litre (l) of ethanol, then left for 24 hours at room temperature while constantly stirring. The extracts were then filtered after which the rest of the plant material was extracted with equal amount of fresh solvent. This procedure was repeated three times. The filtered extracts were collected after filtration and concentrated under reduced pressure to yield 70 g of total extract.

5.3.2.2   Column chromatography

The total extract of 70 g was applied to silica gel column chromatography using hexane and ethyl acetate in different ratios ((9:1); (8:2), (7.3), (0.100)) as eluent. Similar fractions were colleted together according to the thin layer chromatography (TLC) profiles developed with 0.34% vanillin in 3.5% sulphuric acid in methanol.

Twelve final fractions from column were obtained and tested for their anticancer activity on HeLa cells. Based on the results fraction eight (443 mg) which exhibited the highest cytotoxicity was fractionated on silica gel column with hexane: ethyl acetate at ratios of (8:2), (7.3) and (0.100) again as eluent (Figure 5.1). A series of columns silica gel (1 column) with hexane: ethyl acetate as eluent and shephadex columns (2 columns) with 100% ethanol as eluent.
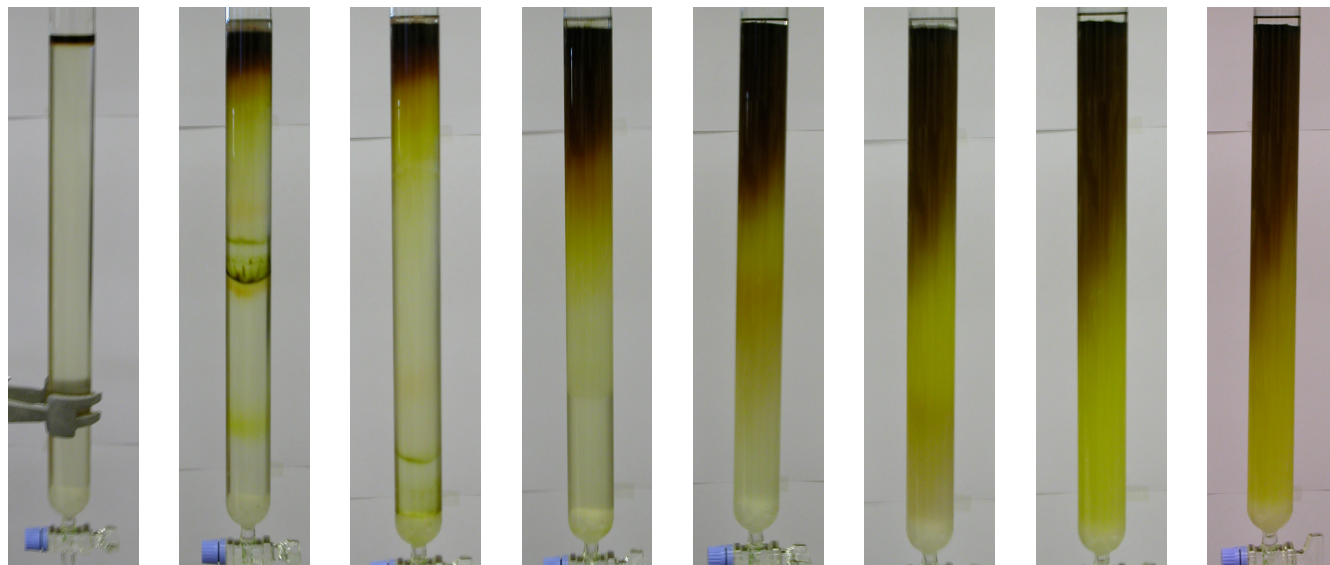
Figure 5.1 Silica gel column of the *F. vulgare* ethanol extract.

Figure 5.2 TLC plates (hexane: ethyl acetate (6:4) as eluent) after treatment with Vanillin in sulphuric acid (H_2SO_4),
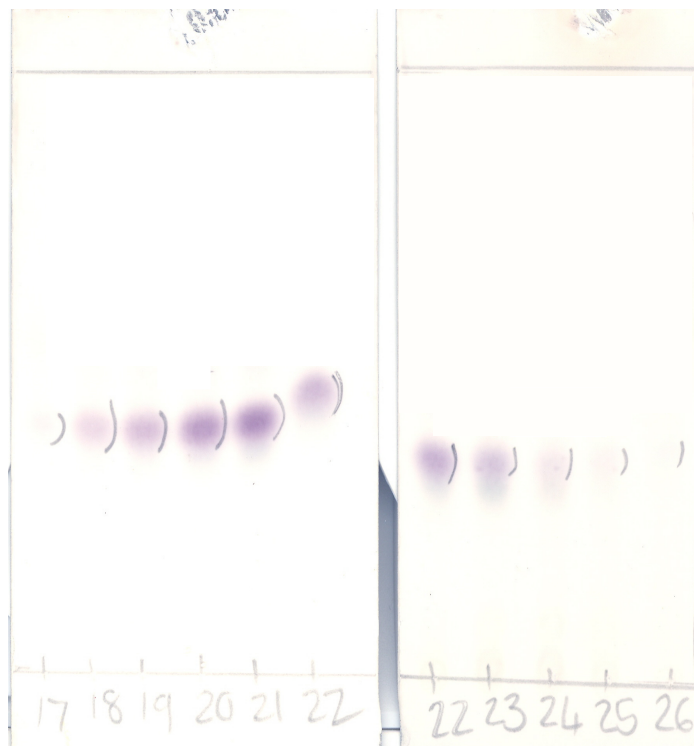
The second total ethanol extract of 249.645g was also applied to silica gel column chromatography using hexane and ethyl acetate in different ratios ((9:1); (8:2), (7.3), (0.100)) as eluent. Twenty-one (1 litre) fractions were collected. All the fractions were spotted five times on separate TLC plates and developed with different solvents as mobile phase; hexane: ethyl acetate (7:3), (2:8) and (1:1) and dichloromethane: methanol (95:5) and (90:10). Fraction 18 was selected for further isolation and was subjected to a sephadex column (LH x 20, Sigma-Aldrich, South Africa) with 100% ethanol as eluent. Thereafter another two sephadex columns and a silica gel column followed after which the selected fraction was subjected to HPLC.

5.3.2.3 Identification of the isolated compounds

The first compound (syringin) was isolated as a amorphous powder, the ^{1}HNMR spectrum exhibited singlet integrated for 6H (2xOMe) at 3.85, multiple's signals at 3.00-3.50 (4H, H-2", 3", 4", 5"), two protons at 3.64, 3.77 (dd each H-6"), another two protons at 4.21 (*dd, J,* = 5.4, 1.2 Hz, H-1), H-1'' anomeric proton at 4.87, two proton signals at 6.30 (1H, *dt, J,* =

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16.2, 5.0 Hz, H-2), 6.54 (1H, *d*, *J*, = 16.2 Hz, H-3) and 6.7s (2H, *s*, H-2', 6'). The ^{13}CNMR (50 MHz, MeOH-*d*$_4$): δ 56.1, 61.6 (2 x OMe), 61.6 (C-6''), 62.6 (C-3'), 70.4 (C-4''), 74.7 (C-3''), 76.8 (C-2''), 77.4 (C-5''), 104.39 (C-1''), 104.52 (C-2, C-6), 129.1 (C-2'), 130.0 (C-1'), 132.0 (C-1), 135.1 (C-4) and 153.4 (C-3, C-5) (Wazir *et al.*, 1995).
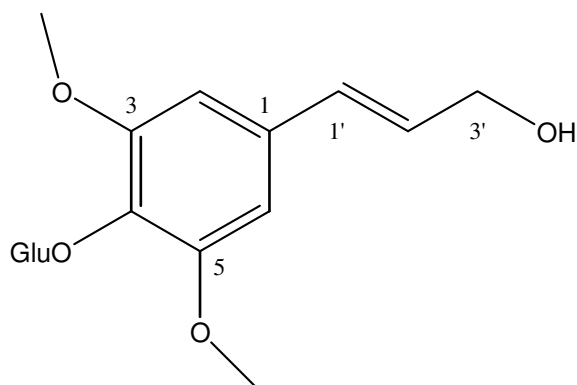
Figure 5.3 Syringin the first isolated compound.

The second compound was (4-methoxycinnamyl alcohol or 3-(4-methoxyphenyl)-2-propen-1-ol) was islolated as colorless crystals mp 130–132 ◦C; IR (KBr): 3345, 2924, 1604, 1243, 1018 cm–1; the ^{1}HNMR(CD$_3$OD) showed signals at *δ* 3.76 (3H, s, OMe), 4.18 (2H, d, *J* = 5.9 Hz, CH$_2$-1), 6.20 (1H, dt, *J* = 5.9, 16.0 Hz, CH-2), 6.48 (1H, d, *J* = 16.0 Hz, CH-3), 6.85 (2H, d, *J* = 8.8 Hz, CH-5,6), 7.31 (2H, d, *J* = 8.8 Hz, CH-7,8). According to the data demonstrated by the ^{1}H NMR we concluded that the compound 2 is 4-methoxycinnamyl alcohol (Akita *et al.*, 2006).
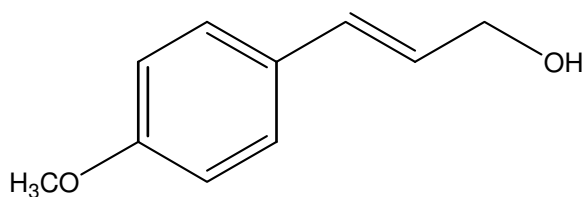
Figure 5.4 Second isolated compound 4-methoxycinnamyl alcohol.

5.3.2.4   Culture of cancer cells

The procedures are all the same as for the naphthoquinone derivatives that were tested on the same cancer cell lines, and the same concentrations were tested as for the naphthoquinone derivatives.

5.3.2.5   Cytotoxicity of various fractions from *F. vulgare*

The cytotoxic activity of the first 12 fractions of the ethanol extract was determined on HeLa cells (Table 5.1). Two fractions (1 and 2) had cytotoxic activity of larger than100 µg/ml. All the other fractions had various cytotoxic activities.

Table 5.1 The IC_{50} values of various fractions from column on the HeLa cell line.

Fractions of the ethanol extract of *F. vulgare*	HeLa
	IC_{50} (µg/ml) $\pm$ SD
1	>100
2	>100
3	38.55 ± 3.235
4	50.04 ± 2.646
5	52.43 ± 3.574
6	31.26 ± 3.965
7	31.13 ± 3.670
8	20.98 ± 4.480
9	36.91 ± 7.150
10	53.24 ± 7.955
11	56.04 ± 13.130
12	48.46 ± 9.500

5.3.2.6   Cytotoxicity of the compounds Isolated *F. vulgare*

The compounds which were isolated from the ethanol extract of *F. vulgare* were tested for their cytotoxicity on MCF-7, HeLa and DU145 (Table 5.2).

Table 5.2 The IC_{50} values of the compounds tested on the selected human cancer cell lines

Isolated compounds	MCF-7 IC50 (µg/ml) ± SD	HeLa IC50 (µg/ml) ± SD	DU145 IC50 (µg/ml) ± SD
Siringin	21.88 ± 0.13	10.26 ± 0.18	>100
4-methoxycinnamyl alcohol	14.21 ± 0.16	7.82 ± 0.28	22.10 ± 0.14

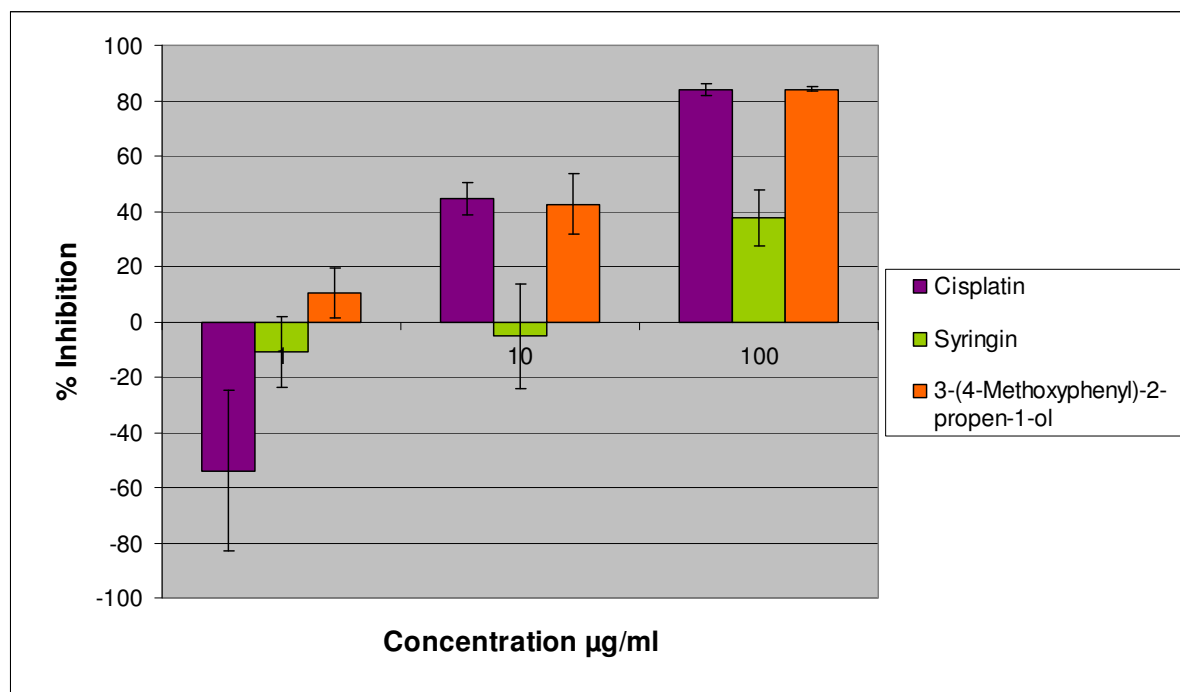### 5.3.2.7 Cytotoxicity of the compounds Isolated *F. vulgare* on U937 cells

The compounds which were isolated from the ethanol extract of *F. vulgare* were tested for their cytotoxicity on U937 cells, and on PBMCs following the methods as described previously.

Figure 5.5 Percentage inhibition of selected compounds on U937 cells. Bars and error bars indicate mean ± SD of quadruplicates.

5.3.2.8   Cytotoxicity of the compounds Isolated *F. vulgare* on PBMCs
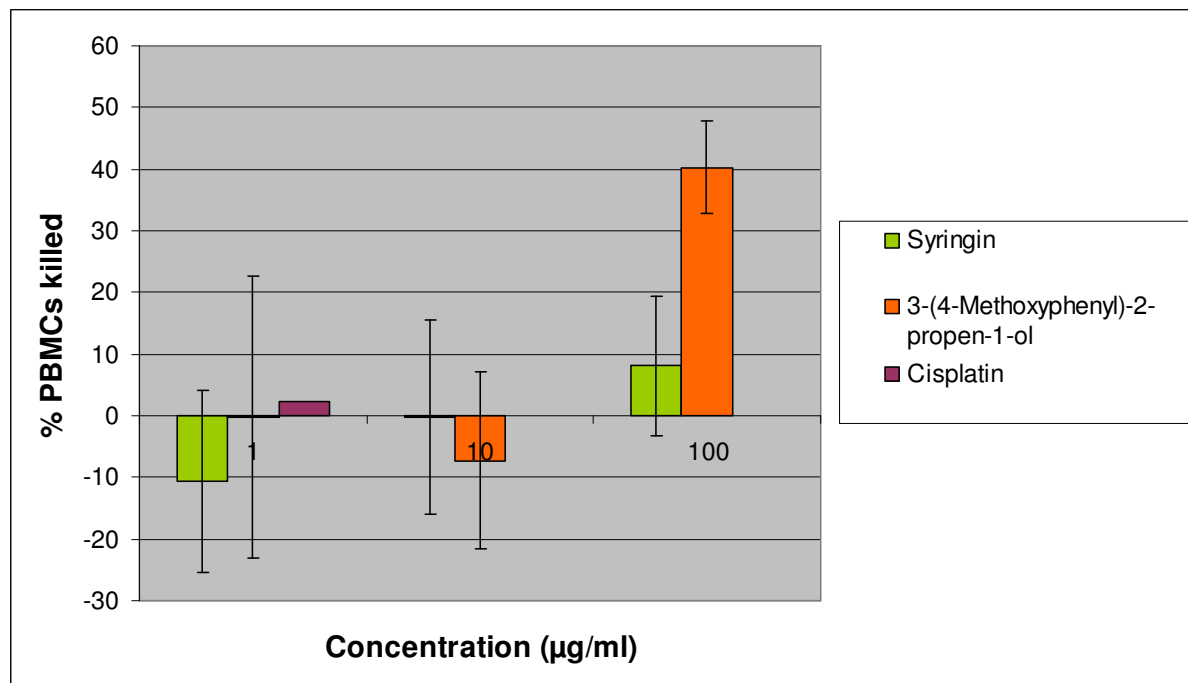
Figure 5.6 Percentage inhibition of selected compounds on peripheral blood mononuclear cells. Bars and error bars indicate mean ± SD of quadruplicates.

5.4   Discussion

5.4.1   Cytotoxicity of the first fractions from *F. vulgare* (Bioassay guided isolation)

Fractions one and two didn't have any cytotoxic activity on HeLa cells at the highest concentration tested (100 µg/ml), and are in fact stimulating the growth of HeLa cells (Table 5.1). The other fraction 3 to 12 had cytotoxic activities ranging between 56.04 ± 13.130 and 20.98 ± 4.480 (Table 5.1). Fraction 11 had the second least cytotoxic activity of 56.04 ± 13.130. According to the guidelines from the American National Cancer institute the limit for crude extracts and fractions for anticancer potential are less than 30 µg/ml, after a 72 hour of exposure. Only fractions seven and eight had cytotoxic activity less than 30 µg/ml. Fraction eight had the best cytotoxic activity and was therefore selected for further investigation.

5.4.2   Cytotoxicity of the compounds isolated from *F. vulgare*

The American National Cancer Institute's guidelines set the limit of activity for pure compounds less than 10 µg/ml, after an exposure time of 72 hours. 4-Methoxycinnamyl alcohol had high cytotoxic activity on HeLa cells at 7.82 ± 0.28 µg/ml (Table 5.2). On the other two cell lines the results were less cytotoxic at 14.21 ± 0.16 on MCF-7 and 22.10 ± 0.14 on DU145 cells respectively.

According to a previous report no cytotoxic activity were found for syringin, against HeLa, A549, and HCT116 cancer cell lines at the highest concentration tested (30 µg/ml). There are however, patents existing on the anticancer activity of this compound on MCF-7 and SNO cells. In this study syringin had high cytotoxic activity on HeLa cells at 10.26 ± 0.18 µg/ml, and less cytotoxic activity on MCF-7 cells at 21.88 ± 0.13 µg/ml (Table 5.2). It is however not toxic towards the DU145 cells with and IC_{50} of larger than 100 µg/ml the highest concentration tested. Syringin was also previously tested *in vitro* for its antioxidant activity against DPPH and since it do not have any free phenolic groups showed an $IC_{50} >$ 500 µM (Pauletti *et al.*, 2006). .

5.4.3   Cytotoxicity of the compounds Isolated *F. vulgare* on U937 cells

Cisplatin, the positive control, had high toxicity on the U937 cells at 5.71 ± 0.13 µg/ml.   4-Methoxycinnamyl alcohol had higher cytotoxic activity than cisplatin, with an IC_{50} value of 3.550 ± 0.18 µg/ml. At the concentrations of 10 and 100 µg/ml 4-methoxycinnamyl alcohol was comparable to that of cisplatin. Syringin however, had much lower cytotoxicity on the U937 cells than 4-methoxycinnamyl alcohol, at 91.14 ± 0.63 µg/ml.

5.4.4   Cytotoxicity of the compounds Isolated *F. vulgare* on PBMCs

Syringin also had the lowest cytotoxicity on the PBMCs, at all the concentration tested. Both syringin and 4-methoxycinnamyl alcohol were not cytotoxic at concentrations of 1 and

10 µg/ml on the PBMCs when compared to cisplatin. At 100 µg/ml, 4-methoxycinnamyl alcohol had the highest toxicity towards the PBMCs.

Based on all the results 3-(4-methoxyphenyl)-2-propen-1-ol was selected for further investigation to examine the mechanism involved.

5.5   References

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CHAPTER 6

Mechanistic studies of potent anticancer compounds

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There are different methods to detect whether the compounds induce cell death (specifically apoptosis) and by which mechanism these compounds induce cell death:

6.1 Different methods to detect different types of cell death with the focus on apoptosis

6.1.1 Cell cycle analysis

A crucial part of a cells life cycle is death, together with growth and differentiation (Schwartzman & Cidlowski, 1993). Novel antitumour agents from natural sources were initially discovered by testing them for cytotoxic activity (an assays), *in vivo* (models) or *in vitro* (cell lines growing) until in the 1990s when it was found that these antitumour agents (vinblastine, vincristine, colchicine, combretastatins and maytansine) use also other mechanisms together with exerting their cytotoxic action via interaction with tubulin, which encourage tubili depolymerisation (Cragg & Newman., 2005). With the taxanes and the plant-derived chemotype such as the jatrophane esters, it was revealed that these agents exhibit a different method; microtubules are "bundled" due to stabilization against depolymerization. This further led to the finding of another mechanism exerted by the plant-derived compounds (camptothecin derivatives, topotecan and irinotecan, which exert their cytotoxic action via inhibition of topoisomerse I (Cragg & Newman, 2004).

Mechanism based receptor screens were developed through progress made in the molecular cancer biology. Now anticancer drug discovery basically take place by means of high throughput screening and enhanced molecular target (linked with particular cancers) development, which allows screening of many compounds against a wide variety of targets. These screens nowadays permit probing of interactions connecting large molecules and locating new small natural product molecules as potentially improved chemotherapy drug candidates (Mukherjee *et al.*, 2001). These techniques are greatly used for plant-based medicines which are widely studied. Another extensively studied area is the mechanism of action involved in many plant-based phytochemicals and cancer cells which play important roles in the treatment of cancer and the discovery of new and improved anticancer drugs (Mukherjee *et al.*, 2001). An essential role is played in the regulation of the cell cycle progression by the cyclin-dependant kinases together with their cyclin partners, since they are intimately involved in the majority of cell cycle stages (Cragg & Newman, 2005). The cell cycle progression is delayed or arrested at specific stage/s when their activity is inhibited and a series of interactions occur within the cell cycle which then induced the cell to undergo apoptosis (Newman *et al.*, 2002; Cragg & Newman, 2005).

The human cell life cycle consist of the following stages: (1) proliferation – is a period of normal growth, (2) differentiation – when the tissue matured more of the cells will differentiate into various specialized subsets that are required for the tissue to function and starts producing the chemicals that are required for maintenance or function of the organism; and (3) cell death.

In eukaryotes, cell cycle control is a vital process (Newman *et al.*, 2002). The cell reproductive life cycle has four phases, as taken from Mukherjee *et al.* (2001): (1) G0 phase – is a stage of quiescence, during which the cell carries out its ordinary role for the organism; when there is any proliferation, then purines and pyrimidines, the building blocks for DNA synthesis, must be produced then the cell enters the; (2) G1 phase - in which nucleotides and enzymes are synthesized; (3) the S phase – it is when DNA synthesis occurs; one enzyme responsible for replication of DNA for the new cell that seems to be particularly vulnerable to exogenous plant chemicals is topoisomerase; the next stage is the: (4) G2 phase – when the cell prepares other structures needed for mitosis, (5) the M

phase is mitosis itself, where two daughter cells are produced (Figure 6.1). During the S phase the cell first checks if the DNA was entirely replicated and without damage before it will enter to the M phase (mitosis). Arrest takes place at the checkpoint (mitotic spindle checkpoint) if the spindle wasn't correctly formed or when the chromosomes weren't properly attached. Aneuploidy and/or mutations will probable be introduced if these controls are not employed and the cell divide without it, but before a cell proceed into mitosis, the damaged DNA is repaired during a permitted pausing time at a checkpoint or replication is completed (Newman *et al.*, 2002). Apoptosis occur in mammalian cells which are incapable of repairing the damage, and these controls on the cell cycle might vanish during cancer (Newman *et al.*, 2002).
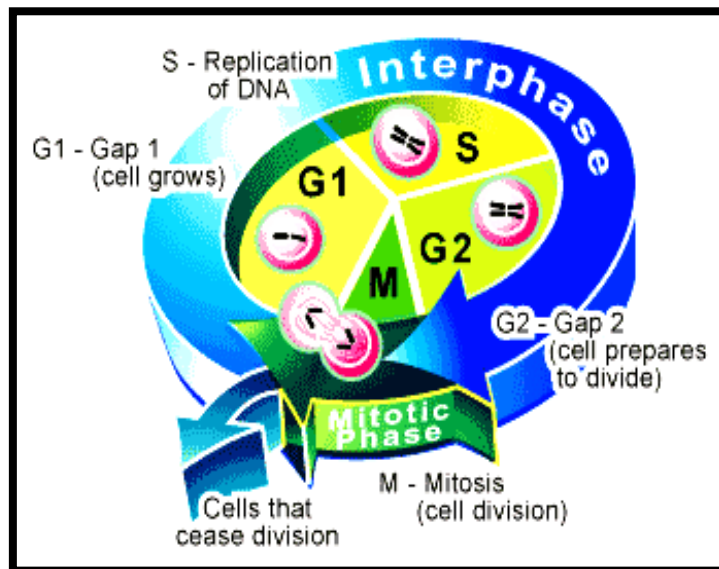
Figure 6.1 The different stages of the cell cycle G1 (cell grows), S (replication of DNA), G2-(cell prepare to divide) and M (cell division).

A cell cycle analysis assay using propidium iodide (PI) was applied for this project to give more detailed evidence for the features of apoptosis induced by the naphthoquinone derivatives. The fluorescent dye most widely-used for staining DNA in whole cells or isolated nuclei is propidium iodide (PI), since the intact plasma membrane of viable cells cannot be cross by it, although it can readily enter through damaged plasma membranes of cells in apoptosis (late stages) or dead cells (Fang, 2006). PI will intercalate into the DNA helix during the cell cycle analysis of fixed permeabilized cells.
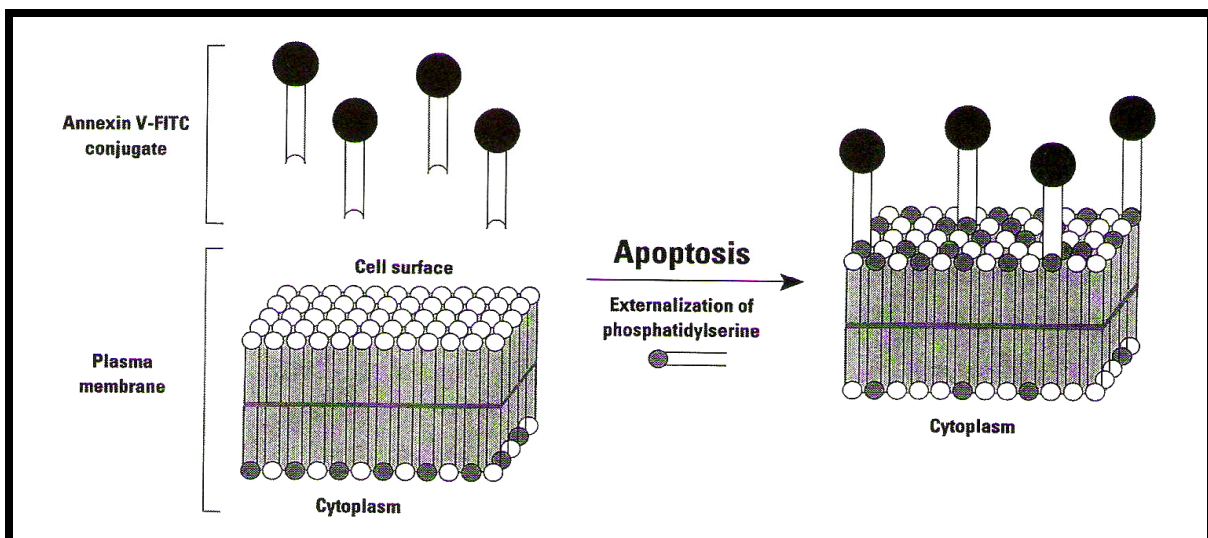
6.1.2 Annexin V-FITC/PI

All the defining characteristic aspects of apoptosis, are a complete change in cellular morphology (Kerr, 1991). One of the earliest features of apoptosis is changes in the plasma membrane. Phosphatidylserine is normally located on the inner membrane surface of viable cells. Convincing proof exist that during apoptosis of cells (in many cells if not all) the phosphatidylserine (PS), a plasma membrane phospholipid, is translocated from the inner to the outer leaflet and externalized via an active process based on kinetics and active transport of phosphatidylcholine to the extra cellular environment (Zhu & Chun, 1998; Van Engeland *et al.*, 1998).

Bohn and colleagues, was first to report on the protein Annexin V which was isolated from human placenta. They and termed it plavental protein 4 (PP4) (Van Engeland *et al.*, 1998). Annexin V was at that time also isolated by Reutelingsperger *et* al., from the umbilical cord by its good quality of anticoagulant activity and identified it as vascular-anticoagulant-α (Van Engeland *et al.*, 1998). It was later called Annexin because of the proteins homology with the family of annexin proteins. This family of structurally related proteins are called annexins. These annexins are also able to bind specifically to cellular membranes (Crutz, 1992). Annexin V (a phospholipids binding protein) has anticoagulant activity, it inhibit phospholipids-dependant reactions via high-affinity binding to phospholipids thus inhibiting their catalytic activity and is a monomer with a molecular weight of 35.8 kDa (Maurer-Fogy, 1989; Zhu & Chun, 1998) which can detect cell-surface PS. It has a very high affinity for membranes containing the negatively charged phospholipids such as PS (Darzynkiewicz, 1997a, Koopman *et al.*, 1994), specific binding is rapid and contain high calcium-dependent affinities for aminophospholipids (Zhu & Chun, 1998). In the presence of PS liposomes inhibit the binding of annexin V to cell-surface PS. The binding of a annexin V was however found to be unaffected by liposomes having other phospholipids (like phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, and sphingomyelin) (Zhu & Chun, 1998).

During apoptosis, Annexin V is used as a probe to monitor change in the distribution of PS in the plasmamembrane (Figure 6.2) and serves as a marker for apoptotic cells. It may be

conjugated to florochromes such as fluorescein isothiocyanate (FITC) and phycoerythrin (PE). The translocation of PS to the exposed membrane surface or extra cellular environment is an early stage in apoptosis, where it serves as a signal for the attack of phagocytic cells. An earlier stage of apoptosis based on nuclear changes such as DNA fragmentation can be detected by FITC coupled to annexin V. Early apoptotic cells will stain annexin-V FITC positive and PI negative where dead cells (necrotic cells) or later stages of apoptosis will stain annexin-V FITC positive and PI positive. It was also found that all cells that show chromatin condensation and DNA fragmentation after initiation of apoptosis strongly stain with annexin V (annexin V-positive cells), while annexin V-negative cells are normal (Koopman *et al.*, 1994).

The Annexin-V FITC detection kit was designed to detect specific biochemical changes in the cell surface membrane which are signature events of early apoptosis and analysis can be done by flow cytometry or by fluorescence microscope. Propidium iodide (counterstain) was also given in the kit and it was used to distinguish apoptotic cells with intact membranes from lysed, necrotic cells. Therefore, staining cells with a combination of Annexin-V FITC and PI allow flow cytometry to detect nonapoptostic live cells (annexin V negative/PI negative), early apoptotic cells (annexin V positive/PI negative), late apoptotic cells (annexin V positive/PI positive) and necrotic cells (annexin V negative/PI positive) (Darzynkiewicz, 1997). The assay can however not differentiate between cells that have died as a result of the necrotic pathway from those that have undergone apoptotic death, since the combination of annexin V and PI will stain both dead cell types positive.

Figure 6.2 Biological basis of annexin V-FITC binding assay (Zhu & Chun, 1998).

Cell populations that were detected were as follows: the viable cells were; the cells that were in the metabolically active stages of apoptosis will stain with Annexin V-FITC and not with the PI. Cells that were with compromised membranes were stained with both Annexin V-FITC and PI. Apoptotic bodies in the late-stages may enter secondary necrosis if they were not detected through phagocytosis, especially in suspension cultures and therefore stain with PI positive and not with annexin V-FITC.

For detecting apoptosis in this project the plasma membrane alteration assay with annexin-V FITC, and the cell viability assay with PI staining have been adopted. The optical properties of the fluorescent probes are given in Table 6.1.

Table 6.1 Optical properties for the fluorescent probes

Agent	Excitation	Emission
FITC	488nm	525nm
PI	536nm	620nm

6.1.3 Caspase 3 and 7 activity

Apoptosis or programmed cell death proceeds through an ordered series of steps (Slee *et al.*, 1999). A lot of work has been done on identifying endonucleases capable of cleaving DNA at internucleosomal sites (Martin & Green, 1995). Given that, in most forms of apoptosis (but not all) DNA degradation is a fixed trait (Martin & Green, 1995). It consists of the following different phases (Slee *et al.*, 1999; Huppertz *et al.*, 1999):

1. Initiation phase - the time during which the cell obtains the initial cell death activation
   signal and include the induction of the cascade which leads to the first proteolytic
   events.

2. Commitment phase - in which the cell becomes committed to apoptosis and these
   death signals become irreversible since they degrade several protein.

3. Amplification phase - which involves the multiple caspase activation, it involves the
   activation of execution caspases, recruited to assist
   in the destruction of the cell.

4. Demolition phase - consists of direct activation of caspase-mediated destruction or via activation of other enzymes (CAD/DFF45) which leads to dismantling of the cellular structures and in the end the collapse of the nucleus and the cell itself.

Protease activation is able to initiate activation and processing of other similar molecules or other proteases (such as calpain) which result in an amplified and escalating protease cascade that is vital for the processes of clotting and complement activation (Martin & Green, 1995). Besides it is a familiar topic in protease function. Apoptosis can be activated by means of a range of interactions and stimuli, but the pathway ultimately lead to the activation of the caspases. The caspase-family can be divided into three subfamilies based on their phylogenetic comparisons of structural homology and sequence similarities (Kidd 1998):

1. ICE-like caspases (includes caspases 1, 4, and 5)
2. CPP32-like caspases (includes caspases 3, 6, 7, 8, 9, and 10)
3. ICH-1 subfamily (caspase2).

The CPP32-like caspases are further classified as execution or initiator caspases due to the role that they play in the apoptosis cascade (Huppertz *et al.*, 1999).

During the apoptotic cell death pathway a family of enzymes, the caspases (Cysteinyl Aspartate-Specific Proteases) play a crucial role and mediate important key proteolytic events (Saleh *et al.*, 2004). As signified by caspase, a conserved cysteine residue functions in catalysis and substrate cleavage occurs on the carboxyl-side of an aspartate residue (Biomol AK-118, 2002). All the caspases are synthesized as single polypeptide zymogens which are triggered through proteolytic activation which arise during autocatalysis or by cleavage via an additional or active caspase or by cleavage with several serine proteases such as granzyme B and cathepsin –B. The caspases are now active caspases that were formed from tetramers. These tetramers consist of two large subunits, usually 20kDa, that were formed from the reminder of the zymogen, frequently minus the N-terminal prodomain and two small 10kDa subunits derived from the C-terminal part of the zymogen polypeptide, by cleavage at one site, or two closely spaced sites, C-terminal to the active-site cystein (Biomol AK-118, 2002). The substrate binding site is formed through residues contributed by both large and small subunits. Two binding sites

per tetramer are formed, except caspase 9 since the active mature enzyme, *in vivo,* hold on to the N-terminal prodomain as part of the large subunit which is 35 or 37 kDa.

Maintenance of the functional and structural integrity of the organs and tissues arise from the apoptosis cascade which are a tightly-regulated and controlled homeostatic mechanisms essentially required to manage the balance between proliferative activity and apoptotic activity (Erickson, 1997). Two distinct subfamilies of human caspases exist and they are functionally divided into: those involved in cytokine maturation (Caspase-1,-4 and -5) and those involved in cellular apoptosis (caspase-2, -3, -6, -7, -8, -9, and -10) (Saleh *et al.*, 2004). The highly regulated process of apoptosis can be induced, stimulated and inhibited at different stages as taken from Huppertz *et al.* (1999) follows (Figure 6.3):

(1) Apoptosis can be induced by specific ligand-receptor interactions and these include TNFα and its p55-receptor (TNF-R1), Fas-ligand (FasL) and its Fas-receptor;

(2) Activation of these receptors leads to the formation of a signalling complex that subsequently activates a family of so-called initiator caspases, e.g. caspase 8;

(3) Initiator caspases (Figure 6.4) are responsible for the first proteolytic events e.g. cleavage of cytoskeletal and related proteins including vimentin, and fodrin (a membrane-associated cytoskeletal protein, and these early apoptotic events are thought to be responsible for the characteristic blebbing of the cell surface;

(4) Cleavage of translocase (flippase) and/or activation of scramblase (floppase) leads to a subsequent flip of phosphatidylserine from the inner to the outer leaflet of the plasma membrane, which is known to be a very early event during the initiation stages of apoptosis, externalization of phosphatidylserine is used by the cells as a signal for events such as the attraction of macrophages, induction of the coagulation cascade or induction of syncytial fusion;

(5) Initiator caspases (Figure 6.4) are positioned at the top of the caspase hierarchy and subsequently they cleave and thus activate a second subpopulation of caspases known as the execution caspases and irreversible progression of the apoptosis cascade commences with activation of the latter;

(6) Execution caspase (Figure 6.4) activation is tightly regulated by the bcl-2 family of mitochondrial proteins, the members of this family promote (bad, bak, bax, bcl-x_s, bik, hrk), or inhibit (A1, bcl-2, bcl-w, bcl-x_L, bfl-1, brag-1, mcl-1, NR13) cleavage of execution caspases;

(7) Irreversible progression of apoptosis by activated execution caspases (e.g. caspase 3) requires that these enzymes remain active for critical minimum period of time which may vary from minutes to hours depending on size, type and functional state of the cell;

(8) Once activated, the execution caspases, either directly or by means of other proteases, cleave a broad array of proteins critical for cell survival which include intermediate filament proteins (such as cytokeratin 18), nuclear envelope proteins (such as lamins A and B), proteins involved in DNA maintenance and repair (such as poly-(ADP-ribose), polymerase (PARP), enzymes involved in relaxation of the DNA-helix and separation of chromosomes during mitosis (topoisomerase IIα), the catalytic subunit of DNA-dependant protein kinase (DNA-PK$_{CS}$);

(9) Execution caspases activate DFF (DNA fragmentation factor) and other endonucleases resulting in specific fragmentation of DNA;

(10) Finally, execution caspases cause characteristic intracellular shifts and activation of proteins such as of: TIAR (T-cell-restricted intracellular antigen-related protein, possibly involved in cytoplasmic DNA cleavage), which is shifted from a nuclear to cytoplasmic localization, transglutaminase II, which protects the endangered plasmalemma, by cross-linking proteins and forming large sub-plasmalemmal protein scaffolds that is shifted from a more diffuse cytoplasmic localization to directly underneath the plasma membrane.

Many plants and their bioactive compounds possess chemopreventative properties that are able to be used therapeutically and their antiproliferative activity is thought to be related via modulation of cell cycle progression and apoptosis induction which have effects on the cellular signaling processes (Kuo *et al.*, 2005).

Within whole living cells the substrate-based CR(DEVD)$_2$ Biomol CV-Caspase 3 and 7 Detection Kit can detect caspase activity. This kit was used since it is a user friendly kit which allows rapid visualization of the interacellular caspase activity in the particular cell line used. For detection of the caspase 3 and 7 activity the kit make use of the flourophore, cresyl violet coupled to the C-terminus of the optimal tetrapeptide recognition sequence DEVD (CR(DEVD)$_2$) (Biomol AK-118, 2002). Caspase 3 and 7 cleave the DEVD groups which are then detached which then lead to excitement of the mono- and un-substituted cresyl violet fluorophores fluoresce at 550-590nm.
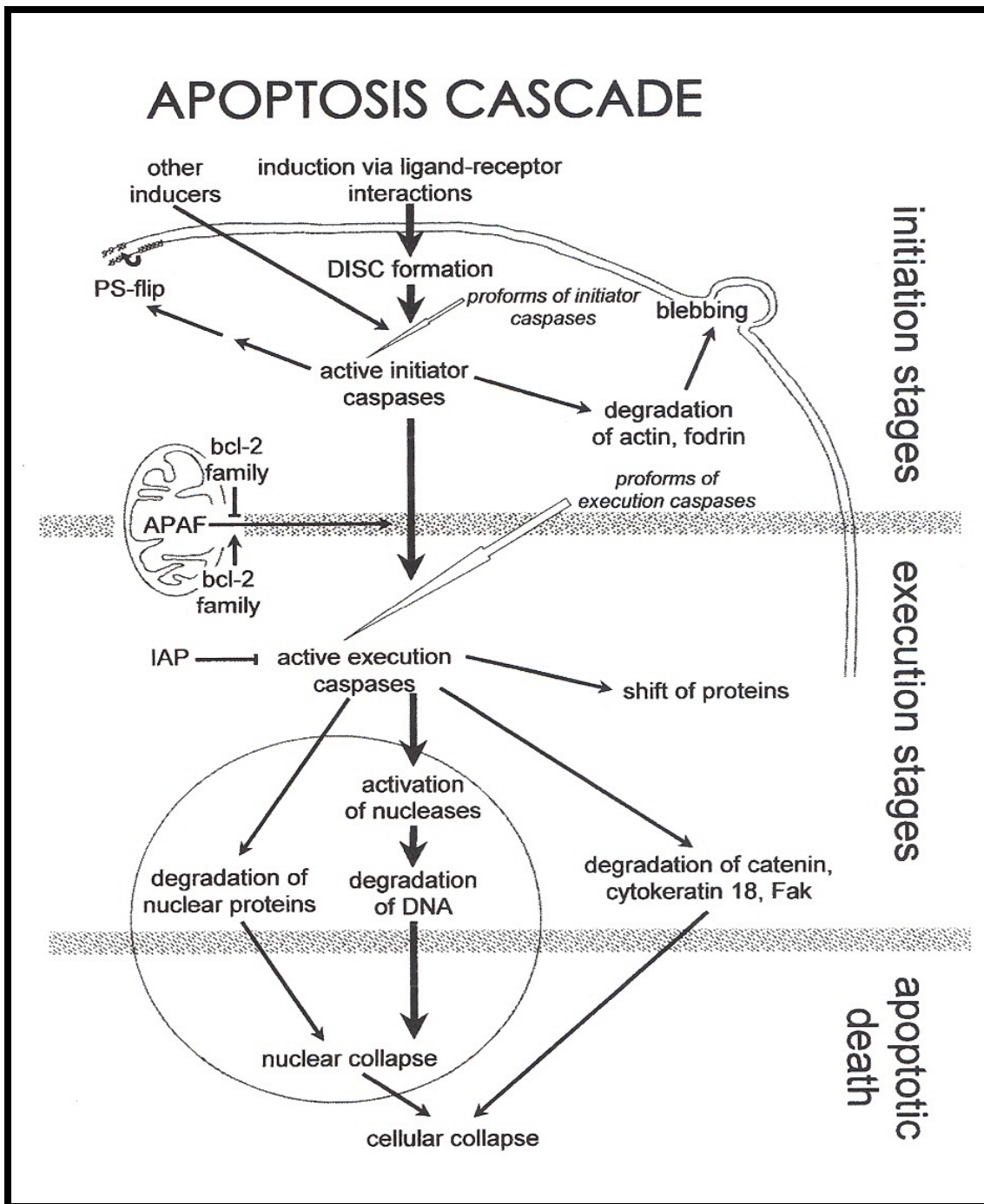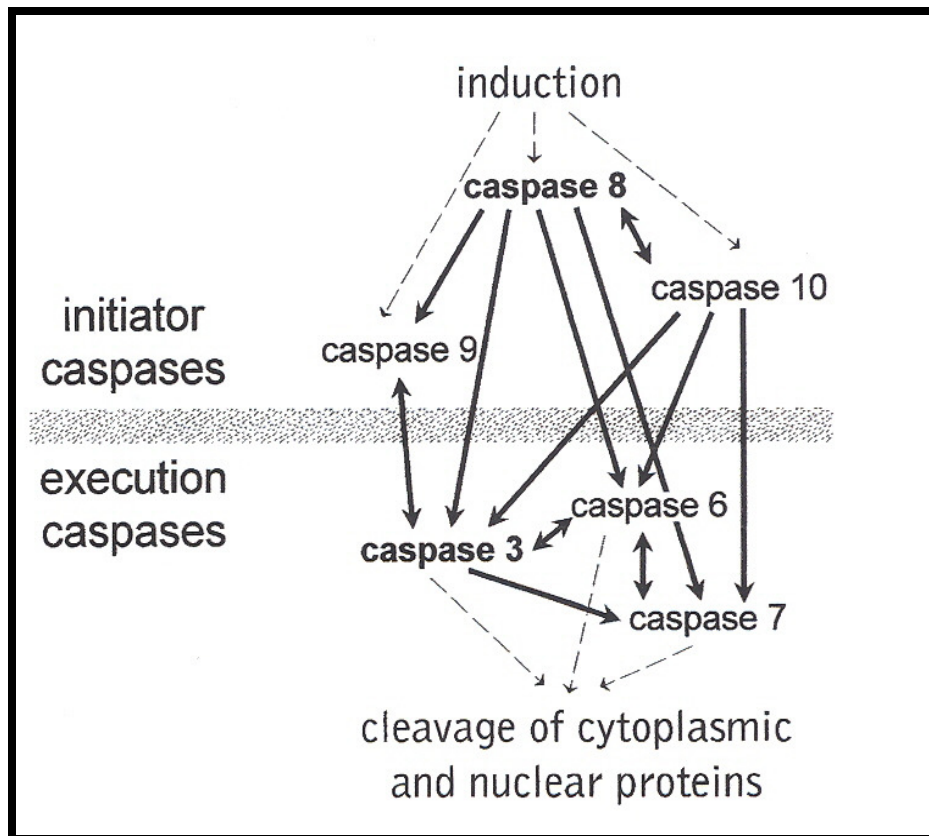
Figure 6.3 The three sequential stages of the apoptosis cascade (Huppertz *et al.*, 1999).

Figure 6.4 Induction of the initiator caspases and activation of the execution caspases which finally leads to apoptotic death as a result of the very complex cascade of events (Huppertz *et al.*, 1999).

In the non-fluorescent state this fluorogenic substrate easily enters the cell through effortless infiltration of the cell membrane and the membranes of the internal cellular organelles. The four amino acids (DEVD) caspase are the targeted sequences which are cleaved off in the presence of caspase 3 and 7 enzymes and consequently yield a red fluorescent product (Biomol AK-118, 2002). According to Huppertz *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 cytoplasm and the inactive form is localized in the mitochondria and cytoplasm. Caspase 7 was found downstream of the initiator caspases and act as an execution caspase. DEVDase-mediated production of the red fluorophore signals apoptotic activity within that particular cell (Biomol AK-118, 2002). The red fluorescent signal, which have an optimal excitation and emission wavelength pairing of 592 nm and 628 nm, could be detected by use of fluorescence microscopy or 96-well microtiter plate fluorometry.

For this experiment the 96-well microtiter plate fluorometry method were used. The induction of apoptosis could be quantitated as the amount of red fluorescence generated in the induced versus non-induced cell populations (Biomol AK-118, 2002). A higher RFU intensity was generated in cell populations which were in the more advanced stages of apoptosis. It was also important to note that a variable base-level of DEVDase activity was present in a varying quantity in all cell lines, which were seen in the non-apoptotic cell populations and may possibly have been the effect of constitutively synthesized serine proteases.

6.1.4 Acridine orange and ethidium bromide nuclear staining

Cell death is not constantly accompanied by the typical characteristics of either apoptosis or necrosis (Darzynkiewicz *et al.*, 1997a). Previous cell death experiments have explained patterns of morphological and/or biochemical changes which differed from the classical features of neither apoptosis nor necrosis (Darzynkiewicz *et al.*, 1997a). Often cell death revealed features of both apoptosis and necrosis. Hematopoietic lineage cell types have typical features of apoptosis at cell death and are most usually "primed" to apoptosis whereas epithelial type cells death are more complex and occasionally even tricky to classify (Darzynkiewicz *et al.*, 1997a). The pattern of cell death may be especially confusing since several drugs cause apoptosis as a result of the drugs-induced secondary effects on the cell. All of this makes the identification of the characteristic features of apoptosis and necrosis extremely difficult.

The frequency of apoptotic induction of THP-1 and U937 cells following exposure to the selected naphthoquinone derivatives was determined by fluorescence microscopy of nuclear stains, acridine orange and ethidium bromide (Coligan, 1995). The cells were identified as viable, apoptotic, and necrotic as described previously by Stasiauskaite *et al.* (2006).

6.1.5 DNA fragmentation

DNA fragmentation plays a major part in the cell death mechanism since it take place very early (primarily appearing several hours before cell viability starts to decrease) in the

apoptotic process. According to Bortner *et al.* (1995) three types of DNA fragmentation can be distinguished which occur throughout apoptosis: internucleosomal DNA cleavage, fragmentation into large (500-300kbp) lengths and single-strand cleavage events. It is also thought that there is a near-universal occurrence of internucleosomal cleavage in apoptostic cells and therefore it is proposed by Schwartzman and Cidlowski, (1993) that in different cell types there possibly exist a frequent mechanism by which apoptosis occur. In several cell types the presence of zinc (Zn^{2+}) inhibits apoptosis and DNA degradation. DNA degradation in isolated nuclei is also blocked because of Zn^{2+}, which suggests that it inhibits the endogenous endonuclease further highlightening the significance of DNA degradation in apoptotic cell death (Schwartzman & Cidlowski, 1993). Proteases are also capable of contributing to DNA fragmentation through the activation of endonucleases (Martin & Green, 1995). Typical DNA ladders were induced in isolated nuclei in the presence of an additional partially characterized 24 kDa protease (Martin & Green, 1995). On the other hand, no nuclease activity is displayed by protease itself. DNA fragmentation is used as a diagnostic for the occurrence of apoptosis since it can detect internucleosomal chromatin degradation in apoptotic cells which is an identifiable biochemical parameter for the start of apoptosis. Caspase activated DNase (CAD) and DNA fragmentation factor (DFF 40) are the main enzymes responsible for fragmentation during apoptosis and are selectively activated upon cleavage by caspase 3.

Agarose gel ethidium bromide staining of extracted DNA reveals the appearance of nucleosomal DNA which is believed to be the hallmark of apoptosis. Wyllie was first to observed the quantitative measure of apoptosis, internucleosomal DNA fragmentation in apoptotic cells, in 1980 (Martin *et* al., 1994). But it is clear that apoptosis is far more than the degradation of nuclear DNA during apoptosis and is a more complex nuclease-mediated process that proceeds through a well-organized multi-step fashion and not all need to be finished for cell death to occur (Warrington *et al.*, 2003). 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 fragments and more fragmentation takes place and in the end giving rise to 10 to 40 kb fragments which in the end give rise to the oligosomal DNA fragments (Warrington *et al.*, 2003; Kerr *et al.*, 1994). These oligosomal DNA fragments (Warrington *et al.*, 2003) and double stranded DNA cleavage observed at linker regions between nuclosomes (180-200 base pair fragments) (Gerschenson & Rotello,

1992) are detectable on conventional agarose gels (gel electrophoresis) as distinctive "DNA ladders", when extracted from apoptotic cells (Figure 6.4). Random cleavage of DNA and degradation of histone occur generally in necrosis. Alternatively to apoptotic cells, DNA extracted from necrotic cell develops as a diffuse smear on DNA electrophoresis (Figure 6.4). Substantial variation exist in the aspect of apoptotic DNA degradation and these factors include things such as the cell type used, the toxic agent involved and even the function of the dose of a given agent in the same cells. According to Warrington and co-workers (2003) variation in the extent and characteristic features of apoptosis may also arise from the fact that cancer cell lines are studied, in which diverse perturbations of apoptotic pathway responses may be a prominent factor not only in the events leading to the generation of such cells, but also their subsequent apoptotic behaviour. Engulfment is also a factor that must be taken in account for because it happens *in vivo* and is excluded when experiments are done *in vitro* and therefore, a different picture can possibly be revealed during *in vitro* studies than what ensues in situ.
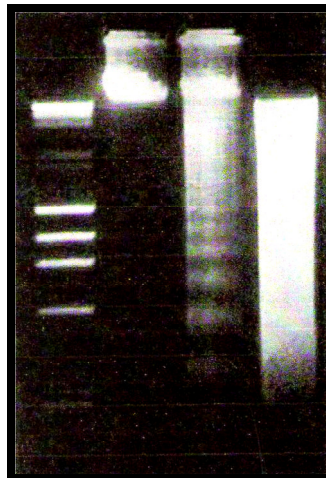
Figure 6.5 Agarose gel electrophoresis of DNA extracted from cultures of P-815 cells. Ethidium bromide stain photographed in ultraviolet light. Lane 1: DRIgest III molecular weight markers; lane 2: control culture; lane 3: culture showing extensive apoptosis induced by heating; lane 4: culture showing massive necrosis 72 hours after repeated freezing and thawing. (Kerr *et al.*, 1994).

The purpose of DNA fragmentation during apoptosis according to Martin *et al.* (1994):

1. may be to facilitate breakdown of the DNA upon uptake of apoptotic cells by phagocytes;
2. alternatively it may serve to destroy the information content of the cell and thus act as an

irreversible step in the process;

3. or it may simply be a by-product of ion redistributions that occur during apoptosis

6.2 Materials and Methods

6.2.1 Cell cycle analysis U937 cells

Compounds having obtained good anticancer activity were selected to further investigate the mechanism of action. U937 cells were grown in 25 cm^2 culture flasks (Nunc) at a density of $1x10^6$ cells/ml in 5 ml RPMI1640 complete medium. All the seeded cells were drawn with serological pipettes from the flasks and transferred to sterile 15 ml tubes. By centrifugation of the tubes at 500 x g for 5 minutes the cells were pelleted. The supernatant was removed with serological pipettes and the pellet loosened by gently tapping the tube. Thereafter, the cells were resuspended in one millilitre complete RPMI1640 medium after which the cell viability and number were determined when they were counted with trypan blue and a hemacytometer. The cells were seeded in complete RPMI1640 medium at a density of $2x10^6$ cells per millilitre in flasks. After a recovery period of 24h, the selected derivatives of 7-methyljuglone, compounds 1-5 and 19 together with 4-methoxycinnamyl alcohol were dissolved in DMSO and compounds 1-4, and 19 were added to the cells to a final concentration of 2 µg/ml and compound 5 and 4-methoxycinnamyl alcohol to a final concentrations of 0.5 µg/ml and 10 µg/ml, respectively. Cisplatin was added as a positive control to give a final concentration of 10µM, and DMSO was used as a negative control. The flasks were further incubated for 24 hours.

The Coulter® DNA Prep™ Reagents Kit (Beckman Coulter) was brought to room temperature before use. Two millilitres were drawn from the treated flasks and centrifuged for 5 minutes at 500 x g at 4°C. The supernatant was discarded and the pellet loosened in 500 µl sheath fluid (Beckman Coulter) and 500 µl lysis buffer added to all the tubes. The tubes were incubated at room temperature for 5 minutes after which 1 ml of PI was added, covered in foil and incubated for 5 minutes at 37°C. The cells were analysed on a Beckman-Coulter FC500 Flow Cytometer. A minimum of 10 000 events were acquired for each sample.

6.2.2 Cell cycle analysis MCF-7 cells

Basically the same method was followed as for the U937 cell cycle analysis, except that only the following derivatives of 7-methyljuglone 2, 3 and 5 were selected based on their activity (IC_{50}) and their quantity for testing on the MCF-7 cells. Cells were plated at 60-70 % confluency in T-25 flasks (Triple Red). After 24 hours one flask were treated with 500 nM of each compound (positive control 2-methoxyoestradiol-3,17-O,O-bis-sulphamate (STX140), Taxol and 2-MeOE2) for 48 hours. Control cells were untreated and another was treated with THF vehicle only. To harvest cells for flow cytometric DNA analysis, cells were washed with phosphate buffer saline (PBS) before being trypsinised (0.25 % trypsin 0.05 % EDTA). Medium containing non-adherent cells was also collected and pooled with the trypsinised cells. The cells and PBS washings were pelleted by centrifugation at 500 x g for 5 minutes. The same Coulter$^{®}$ DNA Prep^{TM} Reagents Kit (Beckman Coulter) (prepared as per kit instructions) was used as for the U937 cells and the cells were analysed using a flow cytometer (FACScan, Becton Dickinson).

6.2.3 Annexin V-FITC/PI U937 cells

The same cell suspensions prepared for the cell cycle analysis were used for the annexin V-FITC/PI staining. Reagents of the Annexin V-FITC Kit (Beckman Coulter) were prepared as per kit instructions and kept on ice until further use. Two millilitres were drawn from the treated flasks and centrifuged for 5 minutes at 500 x g at 4°C. The supernatant was discarded and the pellet loosened and resuspended in 1 x binding buffer after which all the tubes were kept on ice. To 100 µl of the cell suspension, 1 µl of annexin V-FITC solution and 5 µl of the dissolved PI were added and vortexed lightly. All the tubes were incubated on ice for 15 minutes in the dark. Thereafter 400 µl of the 1x buffer were added. The samples were analysed on a Beckman Coulter FC500 flow cytometer with a minimum of 10 000 events that were acquired per sample.

6.2.4 Annexin V-FITC/PI MCF-7 cells

The cells were prepared with the same method as for the MCF-7 cell cycle analysis, but using Annexin V-FITC/PI Kit (Beckman Coulter). The flasks were then treated with 500 nM of the positive control 2-methoxyoestradiol-3,17-O,O-bis-sulphamate (STX140) and the selected concentrations for each compound were the same as for the U937 cells. For this experiment only derivatives 2, 3 and 5 of 7-methyljuglone were selected based on their activity (IC_{50}) on the MCF-7 cells. A flask with control cells were also left untreated (only medium) and another were treated with the THF vehicle only. The cells were harvested with the same method as for the MCF-7 cell cycle analysis and were analysed using a flow cytometer (FACScan, Becton Dickinson).

6.2.5 Caspase 3/7 activity U937 cells

The Caspase 3/7 detection kit (Biomol) was used and the reagents were prepared as described in the directions of the kit.

The U937 cells and compounds were prepared the same as for the cell cycle analysis and annexin V-FITC. The compounds were added to the cells and controls prepared and incubated for 24 hours. The flasks were mixed gently by hand so that the cells mix well because they tend to settle while standing. Two millilitres of RPMI 1640 medium containing cells and compounds, positive control, and negative control were removed from each flask and transferred into separate 2 ml Eppendorf tubes. The eppendorf tubes were centrifuged and 290 µl of RPMI 1640 medium were added and the cell suspension were transferred to a black microtiter plate. At least 2×10^5 cells per 100 µl aliquot was added per well.

Ten microlitres of the 30x CR(DEVD)$_2$ working solution were added to the 290 µl treated cells and mixed gently by aspiring and expelling the cells with a pipette. The black microtiter plate was wrapped in foil and incubated for 120 minutes at 37°C under 5% CO_2. The cells tend to settle and were gently resuspended every 20 minutes to ensure the CR(DEVD)$_2$ is evenly dispersed among all the cells. The plates were read with the ThermoLabsystem Fluoroskan AscentFL fluorescence microplate reader at excitation and emission wavelengths of 544:620 nm and 544:590 nm.

6.2.6 Acridine orange and ethidium bromide nuclear staining U937 and THP-1 cells

The frequency of apoptotic induction of THP-1 and U937 cells following exposure to the selected naphthoquinone derivatives was determined with nuclear stains, acridine orange and ethidium bromide, by fluorescent microscopy (Gu & Belury, 2005). The U937 cells and THP-1 cells were maintained the same as in section 6.2.1. Morphological changes of the U937 and THP-1 cells photographed, fluorescent microscope (Olympus BX40), following exposure to compounds1 to 4, 6 and 19 at final concentration of 2 µg/ml and compound 5 at a final concentration of 0.5 µg/ml, for 24 hours. Equal quantities of cell suspensions (25 µl) were mixed with a mixture of acridine orange and ethidium bromide (100 µg/ml). On microscope slides 15 µl of the mixtures were viewed under a fluorescent microscope. Each microscope slide was viewed immediately after preparation. Two hundred cells per replicate were counted and each compound was done in triplicate. During this study the cells were scored as viable, apoptotic or necrotic as judged by nuclear morphology and membrane integrity (Gu & Belury, 2005). Viable normal cells were easily distinguished with their bright green nuclei and intact structure where the viable apoptotic cells were bright green with their highly condensed or fragmented nuclei. Non-viable normal cells were identified with their bright orange chromatin with organized structure where as the non-viable cells with apoptotic nuclei were with bright orange chromatin which is highly condensed or fragmented (Gu & Belury, 2005).

6.2.7 DNA fragmentation U937 cells

To determine whether the selected naphthoquinone compounds induced U937 cells death was caused by apoptosis, we examined the DNA fragmentation. U937 cells were treated with 4-methoxycinnamyl alcohol at a concentration of 10 µg/ml for 12, 24, 48, 72 and 96 hours after which the modulations of DNA fragmentation by these compounds were detected by using a FexiGene DNA kit (QIAGEN). Untreated cells was incubated at the same experimental conditions and times to serve as a control. At the end of each time of treatment the DNA was extracted following the manufacturer's instructions. The DNA was resolved on a 1 and 2% TAE (tris/acetate/EDTA) electrophoresis agarose gel (White Sci).

6.3 Results

6.3.1 Cell cycle analysis

6.3.1.1   Cell cycle analysis U937 cells

To establish whether the compounds inhibited cell growth by blocking cells in a certain phase of the cell cycle and/or inducing apoptosis, cellular DNA was stained with PI and cells analyzed using flow cytometry after 24 hours (Figure 6.6 and Table 6.2) and 48 hours (Figure 6.6 and Table 6.2). The sub-G1 phase of the cell cycle was used to calculate the percentage of apoptotic cells (Chang *et al.*, 1998).

Table 6.2 shows that, at 24 hours, cisplatin, when compared with the control at 24 hours, did not increase the number of apoptotic cells (Sub G1). There was, however, an increase in percentage at G0/G1, which was expected. At 48 hours cisplatin did give an increase in the percentage of apoptotic cells from 1.53 % to 13.59 %, when compared to the control as well as in the G0/G1 phase.

Compounds 1 and 4 did show a small increase in the percentage of apoptotic cells at 24 hours, and a higher percentage than the control at 48 hours. There were increases in the percentage apoptotic cells for both 1 and 4 from 1.53 % to 11.63 % and 1.53 % to 10.94 %, respectively.   Compounds 2 and 3 did not show any increase in the percentage of apoptotic cells at 24 hours when they were compared with the control, but showed higher percentages than the control at 48 hours. At 48 hours compounds 2 and 3 both showed increase in the percentage of SubG1, from 1.53 % to 17.78 % and 1.53 % to 15.76 %, respectively.
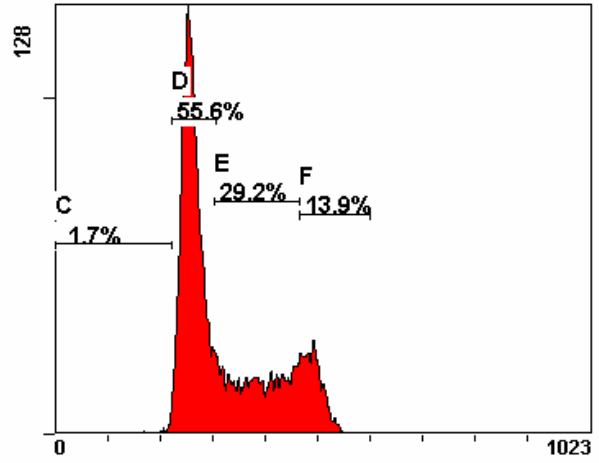
Compound 5 showed, at 24 hours, a good increase in the percentage of apoptotic cells, from 15.03 % to 34.45 %, and at 48 hours there was an increase from 1.53% to 22.12%. With compound 19, a much lower number of apoptotic cells were observed, from 15.03 %
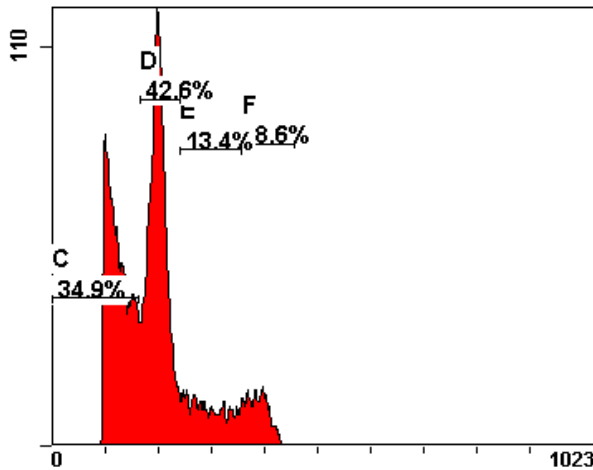
to 3.15 %, at 24 hours, and at 48 hours almost the same number as for the control were found.
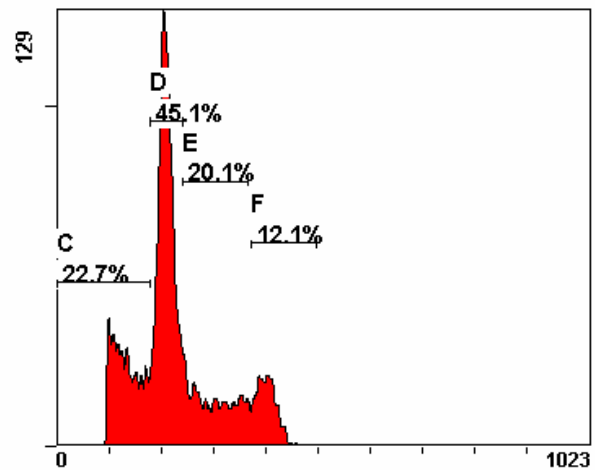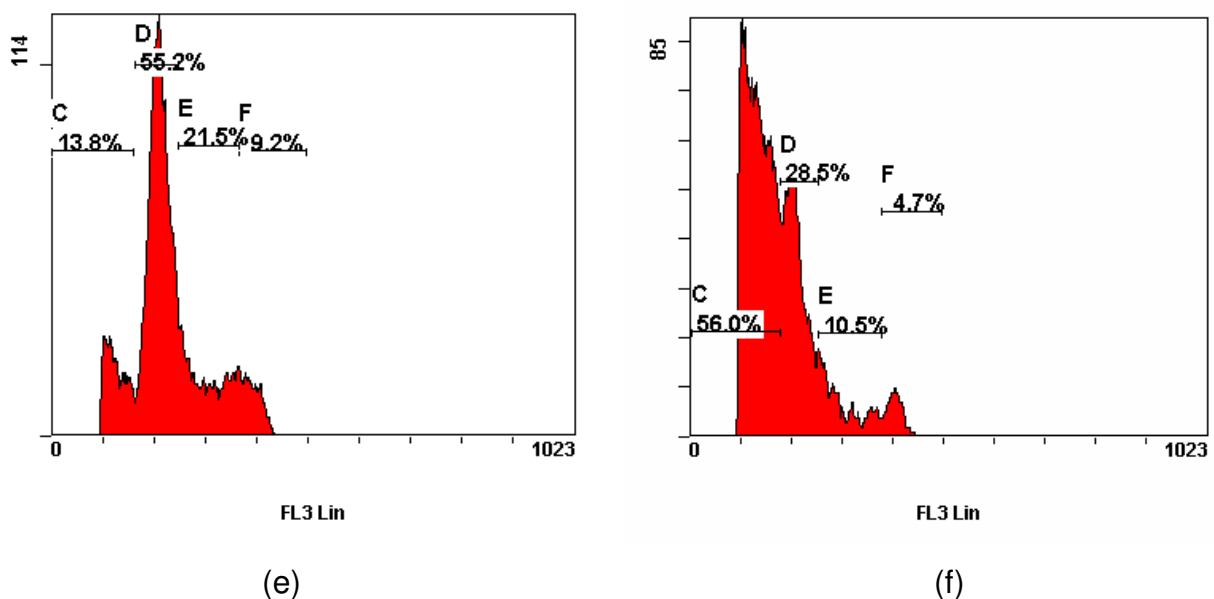

(a)

(b)

(c)

(d)

(e)   (f)
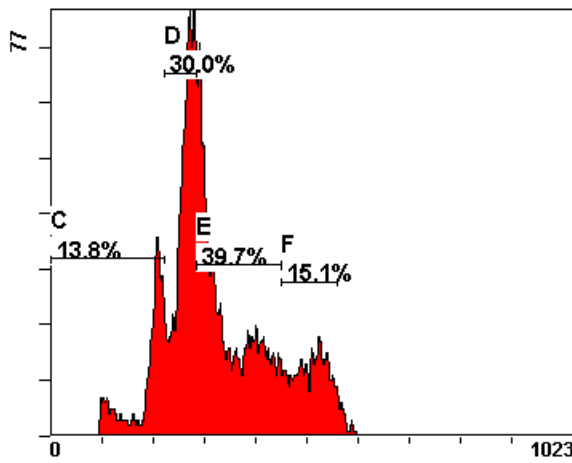
Figure 6.6 DNA content histograms of U937 cell cycle analysis (a) Control after 24 hours (b) Control after 48 hours (c) Compound 5 after 24 hours (d) Compound 5 after 48 hours (e) Cisplatin after 24 hours (f) Cisplatin after 48 hours (Keyes: C=sub-G1 peak; D=G_0/G_1 peak; E=S peak; F=G_2/M peak).

Table 6.2 Results of cell cycle analysis using nuclear PI staining. U937 cells were exposed to the compounds at the indicated concentrations for 24 hours and 48 hours before cell cycle analysis was performed.

Cell Cycle analysis 24 hours				
Treatment	**Sub G1 (%)**	**G0/G1 (%)**	**S (%)**	**G2/M (%)**
Control	15.03	38.20	33.84	12.93
Cisplatin (10µM)	13.51	56.02	21.14	9.33
Compound 1 (2µg/ml)	17.15	61.03	16.27	5.55
Compound 2 (2µg/ml)	14.44	63.98	16.68	4.90
Compound 3 (2µg/ml)	8.57	69.40	17.33	4.70
Compound 4 (2µg/ml)	18.26	65.79	11.45	4.50
Compound 5 (0.5µg/ml)	34.45	42.53	13.93	9.09
Compound 19 (2µg/ml)	3.15	53.17	27.59	16.09

Cell cycle analysis 48 hours				
Treatment	Sub G1 (%)	G0/G1 (%)	S (%)	G2/M (%)
Control	1.53	53.24	29.46	15.77
Cisplatin (10µM)	13.59	56.06	21.13	9.22
Compound 1 (2µg/ml)	11.63	65.34	15.76	7.27
Compound 2 (2µg/ml)	17.78	62.45	13.94	5.83
Compound 3 (2µg/ml)	15.76	63.47	14.21	6.56
Compound 4 (2µg/ml)	10.94	65.35	15.78	7.93
Compound 5 (0.5µg/ml)	22.12	45.83	19.99	12.06
Compound 19 (2µg/ml)	1.22	55.56	25.60	17.62

Table 6.3 shows that most cells were in the Sub G1, or apoptotic, peak after cisplatin treatment for 48 hours, compared to only 5.80% of the control cells. 4-Methoxycinnamyl alcohol gave similar results to the control.

Table 6.3 Results of cell cycle analysis using nuclear PI staining. U937 cells were exposed to 4-methoxycinnamyl alcohol at the indicated concentration (10 µg/ml) for 48 hours before cell cycle analysis was performed.

Cell Cycle analysis 48 hours				
Treatment	Sub G1 (%)	G0/G1 (%)	S (%)	G2/M (%)
Control	5.80	43.51	34.63	16.06
Cisplatin (10 µM)	71.82	18.53	7.52	2.13
4-methoxycinnamyl alcohol (10 µg/ml)	5.10	47.12	32.73	15.05

6.3.1.2 Cell cycle analysis MCF-7 cells

For the MCF-7 cell cycle analysis, at 24 hours and 48 hours (Table 6.4), the positive control, 2-methoxyoestradiol-3,17-O,O-bis-sulphamate (STX140), showed an increase in the number of apoptotic cells from 2.34 % to 13.57 % and 2.48 % to 30.22 %, respectively,

and in the G2/M phase from 22.50 % to 72.81 % at 24 hours and from 12.69 % to 44.26 % at 48 hours.

Again compounds 2 and 3 did not show any increase in the percentage of apoptotic cells at 24 hours. At 48 hours, both compounds showed increases in the percentage of SubG1, from 2.48 % to 4.30 % and 2.48 % to 4.54 %, respectively.

Even from 24 hours compound 5 appeared to be the best, it showed a good increase in the percentage of apoptotic cells, from 2.34 % to 35.54 %.

Table 6.4 Results of cell cycle analysis using nuclear PI staining. MCF-7 Cells were exposed to the compounds at the indicated concentrations for 24 and 48 hours before cell cycle analysis was performed.

| Cell Cycle analysis 24 hours				
Treatment	Sub G1 (%)	G0/G1 (%)	S (%)	G2/M (%)
Control	2.34	47.45	27.71	22.50
Vehicle	1.22	53.54	22.63	22.61
STX140	13.57	7.44	6.18	72.81
Compound 2 (2µg/ml)	2.32	54.47	21.35	21.86
Compound 3 (2µg/ml)	2.25	52.62	22.51	22.62
Compound 5 (0.5µg/ml)	35.54	36.42	16.74	11.30
Cell Cycle analysis 48 hours				
Treatment	Sub G1 (%)	G0/G1 (%)	S (%)	G2/M (%)
Control	2.48	72.32	12.51	12.69
Vehicle	1.43	69.38	13.54	15.65
STX140	30.22	11.40	14.12	44.26
Compound 2 (2µg/ml)	4.30	62.55	19.82	13.33
Compound 3 (2µg/ml)	4.54	51.76	22.10	21.60
Compound 5 (0.5µg/ml)	3.59	63.65	19.48	13.28

6.3.2 Annexin V-FITC/PI

### 6.3.2.1   Annexin V-FITC/PI staining U937 cells

To confirm apoptosis, Annexin V-FITC/PI staining was done on U937 cells.   From the results (Table 6.5) it is evident that there were a number of apoptotic cells observed by all the compounds.   However, a large number of cells did undergo necrosis and compound 5 showed the least number of necrotic cells of all the derivatives at 24 and 48 hours (Figure 6.7).   At 48 hours the results were more or less the same as at 24 hours.   Cisplatin was the only treatment that had a higher percentage apoptotic cells compared to necrotic cells (Figure 6.7).
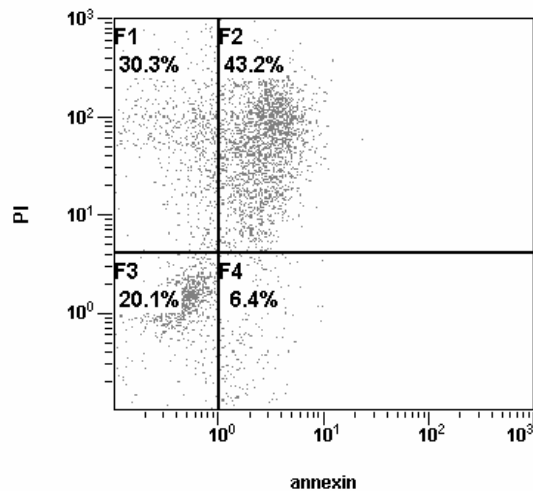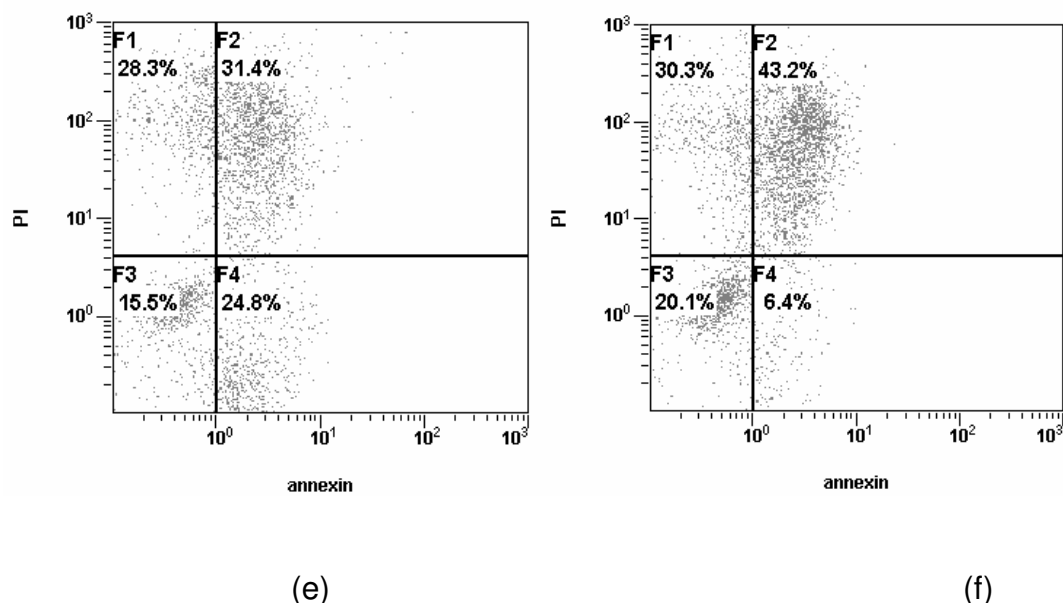

(a)

(b)

(c)

(d)

(e)   (f)
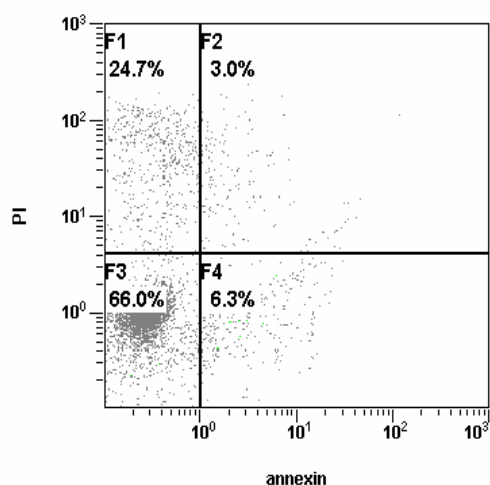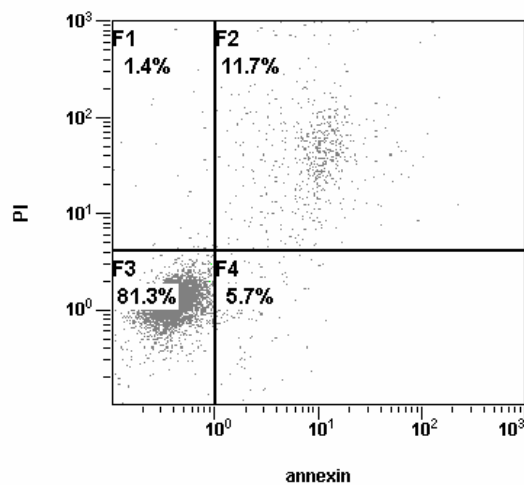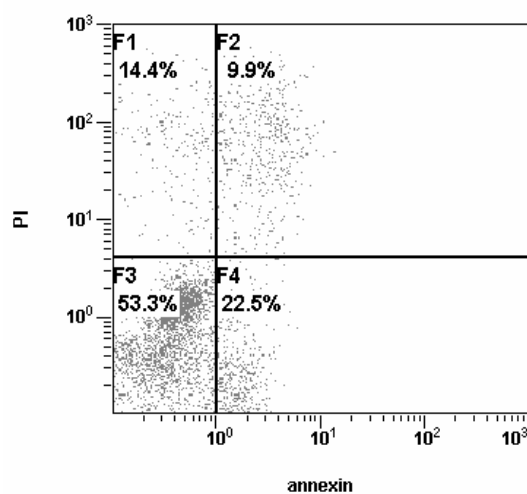
Figure 6.7 U937 annexin V-FITC/PI stained (a) after 24 hours on exposure to control (b) after 48 hours on exposure to control (c) after 24 hours on exposure to cisplatin (d) after 48 hours on exposure to cisplatin (e) after 24 hours on exposure to compound 5 (f) after 24 hours on exposure to compound 5.

Table 6.5 Summary of U937 annexin V-FITC/PI staining after 24 and 48 hours.

Annexin V-FITC/PI 24 hours				
Treatment	Viable (%)	Apoptotic (%)	Late Apoptotic (%)	Necrotic (%)
Control	66.05	6.37	3.08	24.50
Cisplatin (10µM)	40.56	35.00	16.05	8.39
Compound 2 (2µg/ml)	13.52	12.69	12.14	61.65
Compound 3 (2µg/ml)	4.46	9.94	34.80	51.25
Compound 4 (2µg/ml)	6.12	16.49	26.36	51.03
Compound 5 (0.5µg/ml)	14.96	26.75	37.37	20.92

Annexin V-FITC/PI 48 hours

Treatment	Viable (%)	Apoptotic (%)	Late Apoptotic (%)	Necrotic (%)
Control	81.33	5.70	11.73	1.24
Cisplatin (10µM)	20.72	11.59	32.38	35.31
Compound 2 (2µg/ml)	4.19	3.03	29.91	62.87
Compound 3 (2µg/ml)	2.14	1.53	29.18	67.15
Compound 4 (2µg/ml)	2.19	12.04	33.13	52.64
Compound 5 (0.5µg/ml)	22.00	4.42	33.81	39.77

6.3.2.2 Annexin V-FITC/PI staining MCF-7 cells

Apoptotic cells were defined as the cells positive for Annexin V and negative for propidium iodide (M2 on histogram). With the MCF-7 cells (Table 6.6), compound 5 (Figure 6.8) and 2 gave the highest percentages of apoptotic cells, higher than the positive control 2-methoxyoestradiol-3,17-O,O-bis-sulphamate (STX140), and compound 3 a lower percentage of apoptotic cells. At 48 these percentages were however decreasing and compound 3 gave the same result as the positive control.

(a)   (b)
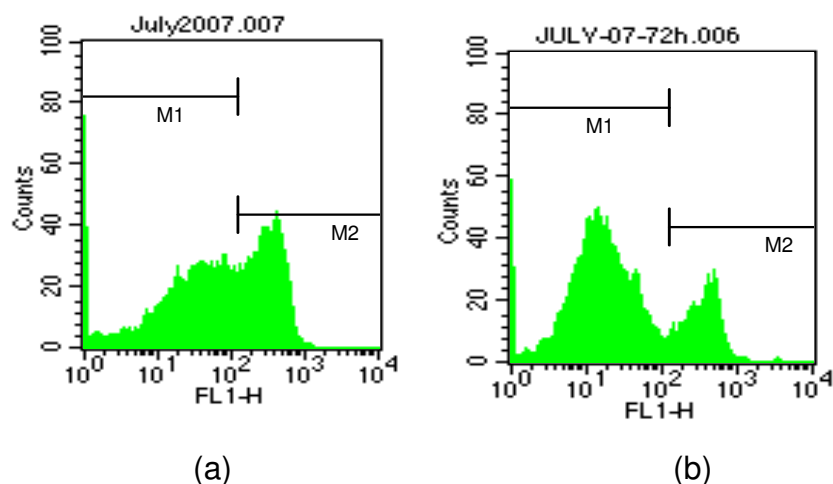
Figure 6.8 MCF-7 annexin V-FITC/PI staining of cells treated with compound 5 (a) after 24 hours (b) after 48 hours

Table 6.6 Annexin V-FITC/PI results for MCF-7 cells at 24 and 48 hours.

Annexin V-FITC/PI 24 hours		
Treatment	**Apoptotic (%)**	**Viable, Late Apoptotic and Necrotic (%)**
Control	13.34	86.66
Vehicle	16.45	83.55
STX 140	30.02	69.98
Compound 2	44.95	55.05
Compound 3	20.35	80.65
Compound 5	44.57	55.43
Annexin V-FITC/PI 48 hours		
Treatment	**Apoptotic (%)**	**Viable, Late Apoptotic and Necrotic (%)**
Control	18.94	81.06
Vehicle	17.38	82.62
STX 140	32.84	67.57
Compound 2	22.32	77.68
Compound 3	32.43	67.16
Compound 5	27.42	72.58

6.3.3 Caspase 3 and 7

6.3.3.1 Caspase 3 and 7 activity after 24 hours

From table 6.7 it is clear that after incubation cisplatin did activate caspase 3/7 activity, by 6.61 % (Figure 6.9). After incubation, compound 3 and 5 inhibited caspase 3/7 activity (-4.13 % and -3.24 % respectively), while compound 19 did activate caspase 3/7 (7.40 %).
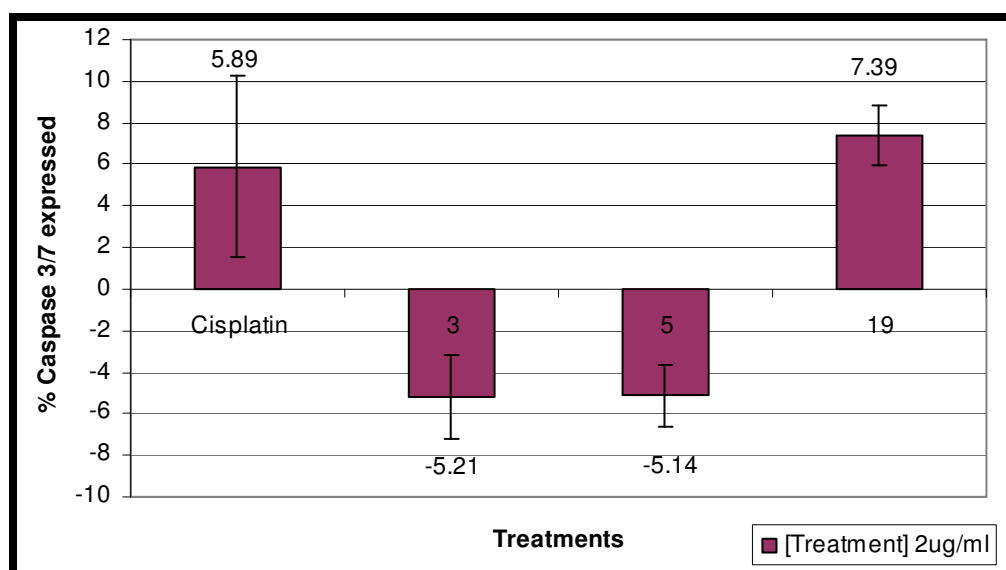
Figure 6.9 Percentage caspase 3/7 expression after a 24 hour treatment in U937 cells (544:620).

Table 6.7 Summary of caspase 3 and 7 results.

Treatment	Caspase 3/7 activity (% above control)
Cisplatin	6.61
Compound 3	-4.13
Compound 5	-3.24
Compound 19	7.40

6.3.4 Acridine orange and ethidium bromide nuclear staining U937 and THP-1 cells

Apoptosis was detected by using DNA-binding fluorescent dyes (Gu & Belury, 2005). The U937 and THP-1 cells exhibited marked morphological changes such as: membrane blebbing, dense chromatin and nuclei were fragmented into apoptotic bodies, as compared with the untreated control which did not exhibit these apoptotic characteristics (Figure 6.10 and Table 6.8).

(a)

(b)

(c)

(d)

(e)

(f)

(g)

(h)

(i)
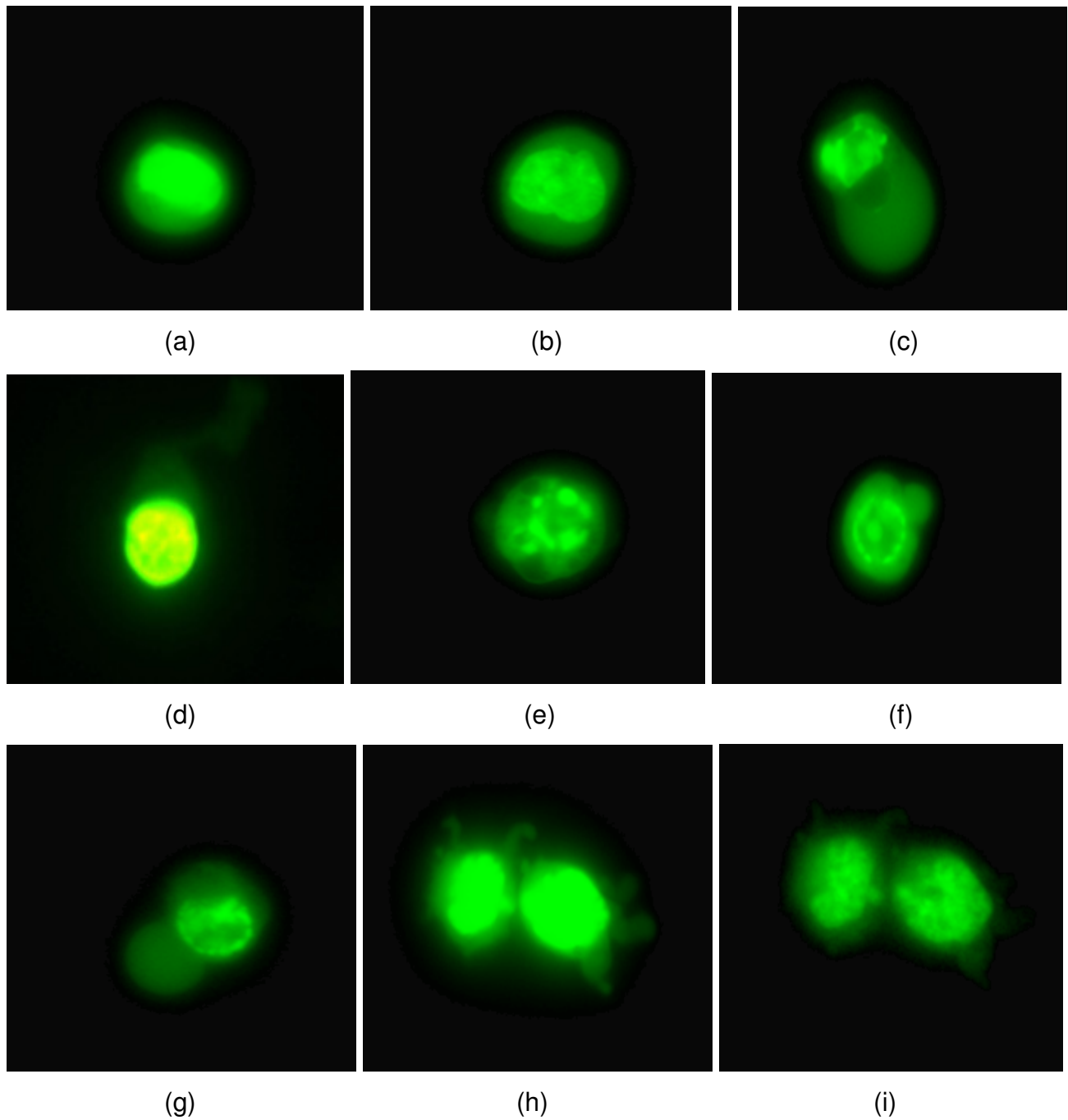
Figure 6.10 (a) Control THP-1 cells viable (b) Control cells with DMSO (c) nuclear fragmentation (d) orange nuclei (e) nuclear fragmentation (f) blebbing (g) dumbbell (h) blebbing (i) nuclear fragmentation.

Table 6.8 Summary of the morphological changes in U937 and THP-1 cells due to exposure to the naphthoquinone derivatives as determined by fluorescence microscopy of nuclear stains, acridine orange and ethidium bromide.

U937 cells			
Treatments	**% Viable cells**	**% Apoptotic cells**	**% Necrotic cells**
Control cells with only medium	98.50	0.23	1.27
Control cells with DMSO	72.77	23.91	3.32
Compound 1	23.79	65.12	11.09
Compound 2	16.50	78.53	4.97
Compound 3	14.59	77.74	7.67
Compound 4	7.30	87.38	5.32
Compound 5	0.67	79.94	19.39
Compound 6	22.41	62.09	15.50
Compound 19	7.53	88.26	4.21
THP-1 cells			
Treatments	**% Viable cells**	**% Apoptotic cells**	**% Necrotic cells**
Control cells with only medium	96.32	0.08	3.60
Control cells with DMSO	84.94	8.44	6.62
Compound 1	4.32	65.03	30.65
Compound 2	10.05	55.26	34.69
Compound 3	5.69	64.33	29.98
Compound 4	6.97	80.59	12.44
Compound 5	3.54	59.25	37.21
Compound 6	2.85	53.18	43.97
Compound 19	8.95	76.07	14.98

6.3.5 DNA fragmentation

A DNA fragmentation assay was used confirm that 4-methoxycinnamyl alcohol did not lead to the gradual accumulation of fragments after exposure U937 cells for 72 hours (Figure 6.11).
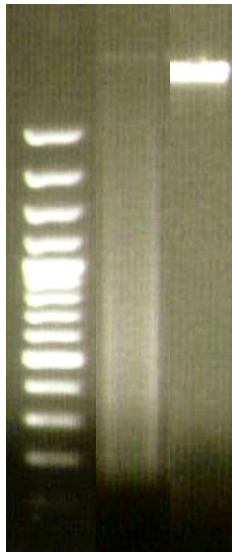
Figure 6.11 First lane loading dye, second lane 4-methoxycinnamyl alcohol with characteristic necrotic smear and third lane control cells without treatment.

6.5 Discussion

6.5.1   Cell cycle analysis

Apoptosis assessment by using the state and content of nuclear DNA is one of its main characteristics that are readily assessed with flow cytometric quantitation of red fluorescence from fixed propidium iodide-stained, RNase-treated cells (Warrington *et al.,* 2003). Sub-G1 events on the DNA histograms were where apoptotic activity was heralded (Nicoletti *et al.*, 1991).

To establish whether or not the naphthoquinones inhibited cell growth by blocking cells in a certain phase of the cell cycle and/or induce apoptosis, cellular DNA was analysed (Peng *et al.*, 2006) and stained with PI and the cells analyzed using flow cytometry (Koduru *et al.,* 2007).

6.5.1.1   Cell cycle analysis U937 cells

There was no increase in the number of apoptotic cells with cisplatin treatment after 24 hours, but there was an increase in the G0/G1 phase from 38.20 % to 56.02 %, and therefore a delay in the cell cycle at this phase. Previous reports showed a delay in the S phase (Qin and Ng, 2002) but it can be that cisplatin delays the cell cycle late in the G0/G1 phase or early in the S phase and with the results there is an overlap between the two phases and therefore a delay was found in the G0/G1 phase. At 48 hours cisplatin showed an increase in the number of apoptotic cells. The lower percentages found at all the other phases of the cell cycle were expected because of the increase at G0/G1. This means that, due to the delay in the G0/G1 phase (or early S phase), apoptosis was induced and the cells did not continue further with the cell cycle.

Compounds 1 and 4 did show a small increase in the percentage of apoptotic cells at 24 hours, suggesting an early induction of apoptosis. This was confirmed at 48 hours. At both 24 and 48 hours there was an increase in the percentage cells in the G0/G1 phase and a decrease in all the other cell cycle phases, which was expected. With the delay at the G0/G1 phase, the cells cannot follow through with the other phases of the cell cycle and therefore became apoptotic.

Compounds 2 and 3 did not show any increase in the percentage of apoptotic cells at 24 hours when they were compared with the control. Despite this there were percentage increases for both at the G0/G1 phase, indicating cell cycle arrest which could potentially lead to apoptosis. This was confirmed at 48 hours, when compounds 2 and 3 both showed increases in the percentage of SubG1, from 1.53 % to 17.78 % and 1.53 % to 15.76 %, respectively. Again there were delays for both compounds at the G0/G1 phase and as a consequence, a decrease in all the other phases because these cells could not continue through the cell cycle and therefore became apoptotic.

Of all the compounds tested, compound 5 appeared to be the best. Even from 24 hours a good increase was observed in the percentage of apoptotic cells, the same was found at 48 hours. There was no increase at any of the other cell cycle phases which might show that this compound does not delay the cell cycle at all and may use another mechanism to induce apoptosis. Therefore, this compound could be considered for further investigation.

The least active compound was 19. At 24 hours a much lower number of apoptotic cells was observed, and at 48 hours the same number as for the control were found. This could mean that this compound induces necrosis rather than apoptosis to kill the cells, even at an early stage, and therefore, this could lead to inflammation. If this is the case, this compound is not a good choice for further investigation; however, the results would have to be confirmed using another method of apoptosis detection.

All the compounds except compound 19 showed an increase in apoptosis at 48 hours and all for them caused a delay at the G0/G1 phase, except compound 5. This was expected because all these compounds have the same chemical structure except for the functional groups, confirming the results.

The cell cycle analysis of 4-methoxycinnamyl alcohol indicates that it did not arrest the cells at any of the cell cycle stages and no increase in apoptotic cell death was observed. This suggests that 4-methoxycinnamyl alcohol possibly induced necrotic cell death to kill the cells but further investigation is needed to verify this.

6.5.1.2   Cell cycle analysis MCF-7 cells

The positive control, 2-methoxyoestradiol-3,17-O,O-bis-sulphamate STX140, showed an increase in the G2/M phase, and therefore, a delay in the cell cycle at this phase. Lower percentages found at the other phases of the cell cycle were expected because of the increase at G2/M. This means that, due to the delay in the G2/M phase, apoptosis was induced and the cells did not continue further with the cell cycle.

Despite no increase in the percentage of apoptotic cells for compounds 2 and 3 at 24 hours, there were percentage increases for both at the G0/G1 phase, indicating cell cycle arrest which could potentially lead to apoptosis. This was confirmed at 48 hours, where both compounds showed increase in the percentage of SubG1, from 2.48 % to 4.30 % and 2.48 % to 4.54 %, respectively. But this time there were delay for both compounds at the G2/M phase and as a consequence, a decrease in the other phases because these cells could not continue through the cell cycle and therefore, became apoptotic.

Of all the compounds tested, compound 5 appeared to be the best. Even from 24 hours it showed a good increase in the percentage of apoptotic cells, from 2 34 % to 35.54 %, showing that this compound induces apoptosis even faster than the positive control, STX140. There was a small increase from 2.48 % to 3.59 % at 48 hours. There were no increases for any of the other cell cycle phases which might show that compound 5 does not delay the cell cycle at all and may use another mechanism to induce apoptosis. Therefore, this compound is the best of all the compounds tested and should be considered for further investigation.

None of the compounds significantly affected the cell cycle, but 2 and 5 caused a rapid induction of apoptosis (24 hours) with compound 3 less potent and slightly delayed (48 hours). All three compounds appear to only induce apoptosis transiently.. Based on the cell cycle data and the lack of any obvious changes in morphology, at least at this concentration, these compounds do not interact with microtubules. The relative lack of toxicity of these compounds at 2 µg/ml in this study was slightly surprising, since, based on the IC_{50} values, greater cell kill might be expected. It could however, be because the cells were trypsinized. It would be interesting to carry out similar tests over a longer time period, since 72 to 96 h is the 'standard' assay time for most cell proliferation experiments and, the shorter proliferation time assay used in this case, may be overestimating the efficacy of the compounds.

6.5.2 Annexin V-FITC

6.5.2.1 Annexin V-FITC/PI staining U937 cells

The sub-G1 peak from cell cycle analysis is a convenient tool to get some indication of apoptosis. However, results need to be confirmed with other methods. The annexin V-FITC dual labelling experiment on U937 cells was done to confirm and also to distinguish between apoptosis and necrosis. It can be seen that the percentage viable cells was already quite low after 24 hours, with relatively high percentage apoptosis. The increase in percentage viability of the control cells from 66.05 % after 24 hours to 81.33 % after 48 hours can be ascribed to handling during subculturing. At 24 hours, 24.50 % of the control cells were necrotic, indicating physical damage. This percentage decreased to 1.24 % at 48 hours, showing that the necrotic cells present at 24 hours have died and are not detected by the flow cytometer due to discriminator settings to ignore particles that are too small.

Cisplatin gave the highest percentage apoptosis after 24 and 48 hours, with compound 5 being the second best, supporting the results obtained from cell cycle analysis where compound 5 had the highest percentage cells in the sub-G1 phase. It should be noted that compound 5 was tested at a concentration four times lower than the other compounds, due to its high toxicity on U937 cells and this makes the results even more significant. Compounds 2, 3 and 4 did not induce apoptosis to any significant degree, although the relatively high percentages seen in the late apoptotic quadrant after 24 hours may suggest that apoptosis induction occurred very soon after exposure and shorter incubation times may have been better in this case. This is supported by the very low percentage of viable cells seen after only 24 hours. The high percentages of necrotic cells observed for compounds 2, 3 and 4 raises some concern as this indicates that not all the cells were killed through induction of apoptosis. This could lead to serious complications such as inflammation in patients.

6.5.2.2 Annexin V-FITC/PI staining MCF-7 cells

The positive control (STX140) gave the highest percentage apoptosis only after 48 hours. Compounds 2 and 5 caused rapid induction of apoptosis at 24 h with an increase from 13.34 % to 44.95 % and 13.34 % to 44.57 % for both compounds, whereas, compound 3 was less potent and slightly delayed at 48 hours. All the compounds except STX140 and compound 3 showed a reduction in apoptosis showing that these compounds have a much quicker induction time than STX140.

6.5.3 Caspase 3/7 activity after 24 hours

The activation of caspase 3 is one of the most common hallmarks of apoptosis although not all apoptotic pathways necessarily activate this enzyme. The caspase 3/7 assay results show that cisplatin caused activation of these enzymes above the levels detected in untreated control cells, as expected (Table 6.7). Compounds 3 and 5 did not lead to the activation of caspase 3/7, despite the fact that apoptosis was detected during cell cycle analysis. This suggests that compounds 3 and 5 induce apoptosis through a different apoptotic pathway. Surprisingly, compound 18 did activate caspase 3/7, although no apoptosis was seen during cell cycle analysis. It is possible that the induction and execution of apoptosis by compound 18 is slower than with the other compounds, and although caspase 3/7 activity was increased at 24 hours, no increase was observed in the number of apoptotic cells at 24 or 48 hours. The increase in G0/G1 observed at 24 hours from 38.20 % to 53.17 % might be the first indication of cell cycle arrest (U937 cells) that could lead to apoptosis induction.

6.5.4 Acridine orange and ethidium bromide nuclear staining U937 and THP1 cells

Under the fluorescence microscope live cells were fluorescing green because of the acridine orange and the dead cells fluoresced orange because of the ethidium bromide. The U937 and THP-1 cells exhibited condensed chromatin, fragmented nuclei and the appearance of apoptotic bodies, resulting from exposure to the selected naphthoquinone

derivatives. From the results on the U937 cells (Table 6.8), the control cells had high percentage viable cells (98.50 %). The few necrotic cells, in the control, might have been caused by damage to the cells when they were transferred to the plates. Compounds 1 and 6 had the lowest percentages of apoptotic cells, 65.12 % and 62.09 %, respectively. These two compounds (1 and 6) had high percentages of viable cells (23.79 % and 22.41 %) compared to the 7.53 % of compound 19. Compounds 1 and 6 also had high percentages of necrotic cells (11.09 % and 15.50 %) when compared to several of the other derivatives. A much lower percentage of necrotic cells were found for compound 19, 4.21 %. Compounds 4 and 19 had the highest percentages of apoptotic cells, 87.38 % and 88.26 % respectively. The lowest percentage of viable cells 0.67 % and the highest percentage of necrotic cells (19.39 %), of all the derivatives, were from compound 5 with 79.94 % apoptotic cells. Of all the derivatives, compound 19 had the least percentage of necrotic cells at 2.21 %. Both compounds 2 and 4 had few necrotic cells and moderate apoptotic cells when compared to the other derivatives.

From the THP-1 results (Table 6.8) a high percentage viable cells were found with the control cells that were treated only with medium, 96.32 %, and also few necrotic cells as with the U937 control cells. The DMSO containing cells also had a high percentage of viable cells (84.94 %) with few apoptotic and necrotic cells, 8.44 % and 6.62 % respectively. Compound 4 had showed to have the highest percentage of apoptotic cells 80.59 % together with 12.44 % necrotic cells. Where the other remaining compounds 1, 2, 3, 5, 6 and 19 had fewer apoptotic cells than the aforementioned compounds and had more necrotic cells, 65.03 %, 55.26 %, 64.33 %, 59.25 %, and 53.18 % apoptotic cells and 30.65 %, 34.69 %, 29.98 %, 37.21 % and 43.97 %, in that order.

6.5.5 DNA fragmentation

The purpose of DNA fragmentation during apoptosis is still uncertain, but it is said to be probably one of the most useful tests for apoptosis. To determine whether the selected isolated compound, 4-methoxycinnamyl alcohol, induced U937 cells death via apoptosis. When the U937 cells were cultured with the 4-methoxycinnamyl alcohol (5 µg/ml) for 12, 24, 48, 72 and 96 hours and it revealed a typical necrotic smear on the agarose gel, confirming our cell cycle results.

6.6 References

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CHAPTER 7

Discussion and conclusion

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7.1 Discussion and conclusion

Cancer is a group of diseases (more than 100 different diseases), characterized by uncontrolled growth and spread of abnormal cells. Each year many more people are newly diagnosed with cancer. Currently cancer is treated by surgery, radiation, hormones and chemotherapy. Both radiation and chemotherapy induce apoptosis that suppress tumour growth. Failure to activate apoptosis is one of the major obstacles to the successful treatment of cancer with drugs. The loss of specificity of current therapies as well as high toxicity towards the non-cancerous cells led to the seeking of a new anticancer drugs from plants which launches the mechanism of apoptosis in the amelioration of this group of diseases.

The ethanol extract of six plant species namely: *Artemisia afra* (leaves)*, Centella asiatica* (leaves)*, Euphorbia ingens* (stem)*, Euclea natalensis* (roots)*, Foeniculum vulgare* (seeds)*, Hypoxis hemerocallidea* (corms)*,* and *Tulbaghia violacea* (leaves) were tested for its cytotoxicity against four human cancer cell lines at concentrations ranging from 0.78 to 100 µg/ml. From all the ethanol extracts, *Foeniculum vulgare* was selected based on its cytotoxic activity towards HeLa cells, the highest cytotoxicity at 20.73 ± 0.065 µg/ml and DU145 cells at 56.41 ± 0.281 µg/ml, was selected for isolation of the bioactive compound/s.

The ethanol extract of *Foeniculum vulgare* was fractionated using column chromatography with hexane and ethyl acetate at different ratios as eluent. The cytotoxicity of these

fractions was determined. Fraction eight exhibited the highest cytotoxic activity at 20.98 ± 4.48 µg/ml. Therefore, this fraction was subjected to further separation by column chromatography. 4-Methoxycinnamyl alcohol and syringin was isolated and its structure was determined by NMR spectroscopic methods. On MCF-7 and DU145 cells, 4-methoxycinnamyl alcohol, were less cytotoxic at 14.21 ± 0.16 and 22.10 ± 0.14 µg/ml, respectively. Syringin is less cytotoxic on MCF-7 cells (21.88 ± 0.13 µg/ml) and was not toxic towards the DU145 cells with an IC_{50} of larger than 100 µg/ml the highest concentration tested. Both 4-methoxycinnamyl alcohol and syringin was then tested for its cytotoxicity against U937. 4-Methoxycinnamyl alcohol had its highest cytotoxic activity on HeLa cells at 7.82 ± 0.28 µg/ml whereas syringin had its highest cytotoxicity on HeLa cells at 10.26 ± 0.18 µg/ml and therefore these two isolated compounds were selected for cytotoxicity on PBMCs.

Previously 7-methyljuglone was isolated from *E. natalensis* and had toxicity towards cancer cells. Therefore, 7-methyljuglone and its 5-hydroxy, 5-acetoxy-, 5-alkoxy- and 1,2,4,5-tetra-*O*-acetate derivatives were synthesized to establish their structure-activity relationships as well as to see if there is an induction of apoptosis of a few selected derivatives, and to provide insight into possible mode(s) of action of some of the selected derivatives. From the fifty percent inhibitory concentration values it was clear that the 5-hydroxy (8-Halogen) (1-5) group derivatives had the greatest inhibitory effect of all the derivatives. The 1,2,4,5-tetra-*O*-acetate derivatives (16,17,18) had the least anticancer activity of all the derivatives. The only promising result given by these derivatives (1,2,4,5-tetra-*O*-acetate derivatives) is compound 17 (HeLa 8.389 ± 4.51 µg/ml and SNO 9.389 ± 3.52 µg/ml). The 5-ethoxy derivatives (8, 10 and 12), had 50% reduction in cell viability at 11.10 ± 2.00 µg/ml and less on all four cancer cell lines, with 10 (without the Cl at the 8 position) having the best cytotoxic activity of the 5-ethoxy derivatives with an IC50 value as low as 3.25 ± 1.24 µg/ml on DU145 cells. Compound 7 and the other two 5-methoxy derivatives, 9 and 11, also had activities of between 10.63 ± 1.56 µg/ml and 3.10 ± 0.76 µg/ml on the four cell lines. This time compound 9 had the best activity of the 5-methoxy derivatives on MCF-7 cells with an IC_{50} = 3.10 ± 0.76 µg/ml. Of all the 5-hydroxy (8-Halogen) group derivatives (1-5), compounds 4 gave the best cytotoxic effect on all the cell lines with IC_{50} values between 2.24 ± 0.90 µg/ml and the lowest value 2.07 ± 2.106 µg/ml. The other 5-hydroxy (8-Halogen) derivatives also had good activity on all the cell lines with

IC_{50}= 2.11 ± 0.67 and higher (highest IC_{50}= 12.37 ± 1.25 µg/ml) on all four the tested cell lines. Compound 18 was most effective (IC_{50} = 1.09 ± 0.22 µg/ml) while the parent compound 6 was less active than several of these derivatives.

All the selected 7-methyljuglone derivatives: 8-Chloro-5-hydroxy-7-methyl-1, 4-naphthoquinone (1); 8-Bromo-5-hydroxy-7-methyl-1, 4-naphthoquinone (2); 8-Fluoro-5-hydroxy-7-methyl-1, 4-naphthoquinone (3); 8-Chloro-5-hydroxy-6-methyl-1,4-naphthoquinone (4); 5-Hydroxy-6-methyl-1,4-naphthoquinone (5); 5-Hydroxy-7-methyl-1,4-naphthoquinone (6); and 2,5-dihydroxy-7-methyl-1,4-naphthoquinone (19) as well as both isolated compounds 4-methoxycinnamyl alcohol and syringin were tested for their cytotoxicity against U937 cells and also peripheral blood mononuclear cells.

On the peripheral blood mononuclear cells, cisplatin, the positive control, had high toxicity on the U937 cells at 5.71 ± 0.13 µg/ml. The first isolated compound 4-methoxycinnamyl alcohol had higher cytotoxic activity than cisplatin, with an IC_{50} value of 3.550 ± 0.18 µg/ml. At the concentrations of 10 and 100 µg/ml 4-methoxycinnamyl alcohol was comparable to that of cisplatin. Syringin however, had much lower cytotoxicity on the U937 cells than 4-methoxycinnamyl alcohol, at 91.14 ± 0.63 µg/ml. Syringin also had the lowest cytotoxicity on the PBMCs, at all the concentration tested. Both syringin and 4-methoxycinnamyl alcohol were not cytotoxic at concentrations of 1 and 10 µg/ml on the PBMCs when compared to cisplatin. At 100 µg/ml, 4-methoxycinnamyl alcohol had the highest toxicity towards the PBMCs. Based on the results on and PBMCs cells, 4-methoxycinnamyl alcohol was selected for further investigation to examine the mechanism which this compound uses to inhibit the cancer cells.

On the U937 cells all the selected derivatives were toxic at 5 and 10 µg/ml. Cisplatin, the positive control, had the highest toxicity/percentage of inhibition even at 1 µg/ml. 5-Hydroxy-6-methyl-1,4-naphthoquinone (Compound 5) was the most toxic of all the selected compounds, and the other compounds had IC_{50} values between 1 and 5 µg/ml and were therefore less toxic. 8-Chloro-5-hydroxy-6-methyl-1,4-naphthoquinone (Compound 4) were the least toxic (U937 cells) of the selected compounds. On the PBMCs compound 3 and 6 and 19 were the least cytotoxic, whilst the other compounds showed much greater cytotoxicity. Compound 6 had the lowest IC_{50} at 18.40 µg/ml and was followed by

compound 19 IC_{50} at 53.97 µg/ml, therefore more toxic towards PBMCs), than compound 3 which had an IC_{50} of 188.70 µg/ml. The IC_{50} values of the other compounds (compounds 1, 2, 4 and 5) were less than 1,00 µg/ml, the lowest concentration tested, and therefore very cytotoxic towards PBMCs.

At 24 hours, cisplatin cell cycle analysis, when compared with the control at 24 hours, did not increase the number of apoptotic cells (Sub G1). There was, however, an increase in percentage at G0/G1, which was expected. At 48 hours cisplatin did give an increase in the percentage of apoptotic cells from 1.5% to 13.5%, when compared to the control as well as in the G0/G1 phase. Compounds 1 and 4 did show a small increase in the percentage of apoptotic cells at 24 hours, and a higher percentage as the control at 48 hours. There were increases in the percentage apoptotic cells for both 1 and 4 from 1.5% to 11.6% and 1.5% to 10.9%, respectively. Compounds 2 and 3 did not show any increase in the percentage of apoptotic cells at 24 hours when they were compared with the control, but showed higher percentages than the control at 48 hours. At 48 hours compounds 2 and 3 both showed increases in the percentage of SubG1, from 1.5% to 17.7% and 1.5% to 15.7%, respectively. Compound 5 showed, at 24 hours, a good increase in the percentage of apoptotic cells, from 15.0% to 34.4%, and at 48 hours there was an increase from 1.5% to 22.1%. With compound 18, a much lower number of apoptotic cells were observed, from 15.0% to 3.1%, at 24 hours, and at 48 hours almost the same number as for the control were found. 4-Methoxycinnamyl alcohol cell cycle analysis, indicate that it did not arrest the cells at any of the cell cycle stages and no increase in apoptotic cell death was observed, possibly suggesting necrotic cell death.

For the MCF-7 cell cycle analysis, at 24 hours and 48 hours, the positive control, 2-methoxyoestradiol-3,17-O,O-bis-sulphamate (STX140), showed an increase in the number of apoptotic cells from 2 % to 13 % and 2% to 30 %, respectively, and in the G2/M phase from 24% to 74% at 24 h and from 14% to 45 % at 48 h. Again compounds 2 and 3 did not show any increase in the percentage of apoptotic cells at 24 hours. At 48 hours, both compounds showed increases in the percentage of SubG1, from 2% to 4% and 1% to 4%, respectively. Even from 24 hours compound 5 appeared to be the best, it showed a good increase in the percentage of apoptotic cells, from 2% to 35%.

To confirm apoptosis, Annexin V-FITC/PI staining was done on U937 cells. From the results it was evident that there were a number of apoptotic cells observed by all the compounds. However, a large number of cells did undergo necrosis and compound 5 showed the least number of necrotic cells of all the derivatives at 24 and 48 hours. At 48 hours the results were more or less the same as at 24 hours. Cisplatin was the only treatment that had a higher percentage apoptotic cell compared to necrotic cells.

With the MCF-7 cells, compound 5 and 2 gave high percentages of apoptotic cells, higher than the positive control 2-Methoxyoestradiol-3,17-O,O-bis-sulphamate (STX140), and compound 3 a lower percentage of apoptotic cells. At 48 and 72 hours these percentages were however decreasing.

The activation of caspase 3 is one of the most common hallmarks of apoptosis although not all apoptotic pathways necessarily activate this enzyme. It was clear that after incubation cisplatin did activate caspase 3/7 activity, 6.6%. After incubation, compound 3 and 5 inhibited caspase 3/7 activity (-4.1 and -3.2 respectively), while compound 18 did activate caspase 3/7, 7.4%.

Nuclear staining showed that the derivatives exhibit various characteristic features of apoptotic cell death such as blebbing, dense chromatin and nuclear fragmentation, whereas 4-methoxycinnamyl alcohol revealed a typical necrotic smear on the agarose gel.

Considering the findings from cell cycle analysis, caspase 3/7 activation, annexin V-FITC dual labelling and toxicity towards cancer cells and PBMCs together, 5-hydroxy-6-methyl-1,4-naphthoquinone (5) is the most toxic and exerts its effects through the induction of apoptosis. The apoptotic pathway that is activated appears not to involve caspase 3 activation. Unfortunately, 5-hydroxy-6-methyl-1,4-naphthoquinone (5) was also most toxic to the non-cancerous peripheral blood mononuclear cells and therefore has the potential to lead to serious toxicity in patients. Other compounds, such as 8-fluoro-5-hydroxy-7-methyl-1, 4-naphthoquinone (3) and 2,5-dihydroxy-7-methyl-1,4-naphthoquinone (18), were much less toxic to PBMCs but were also less potent in killing the cancer cell lines. There should be a balance between efficiency and general toxicity and therefore 8-fluoro-5-hydroxy-7-methyl-1, 4-naphthoquinone (3) and 2,5-dihydroxy-7-methyl-1,4-naphthoquinone (18) may

be better candidates than the more potent, but toxic, 5-hydroxy-6-methyl-1,4-naphthoquinone (5). 4-Methoxycinnamyl alcohol, alternatively had necrotic cell death activity.

7.2 Recommendations for future work

Further studies are required to understand the effect of different functional group substitutions of the isolated compounds and what effect it might have on the activity as well as the mode of inhibition. For the naphthoquinone derivatives and the isolated compounds the following could also be performed: the activation of the other caspases, inhibition of topoisomerase, microtubule inhibition effect, tyrosine kinase inhibition, the synergistic effect of the naphthoquinone derivatives with doxorubicin or other combined therapies, stability of the formulation as well as absorption of naphthoquinones in the human body.

Even though a large number of molecules exhibit anticancer activity *in vitro*, only few are able to induce anticancer activity without killing normal cells in clinical trials. There is however, a large gap between *in vitro* and *in vivo* studies. Therefore, new strategies are needed for discovering new anticancer agents that validate their efficacy and safety.

CHAPTER 8

Acknowledgments

Chapter 8

Acknowledgments

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CHAPTER 9

Appendices

Chapter 9

Appendix A

9.1 ^{1}H-NMR and ^{13}C-NMR of isolated compounds from *Foeniculum vulgare*

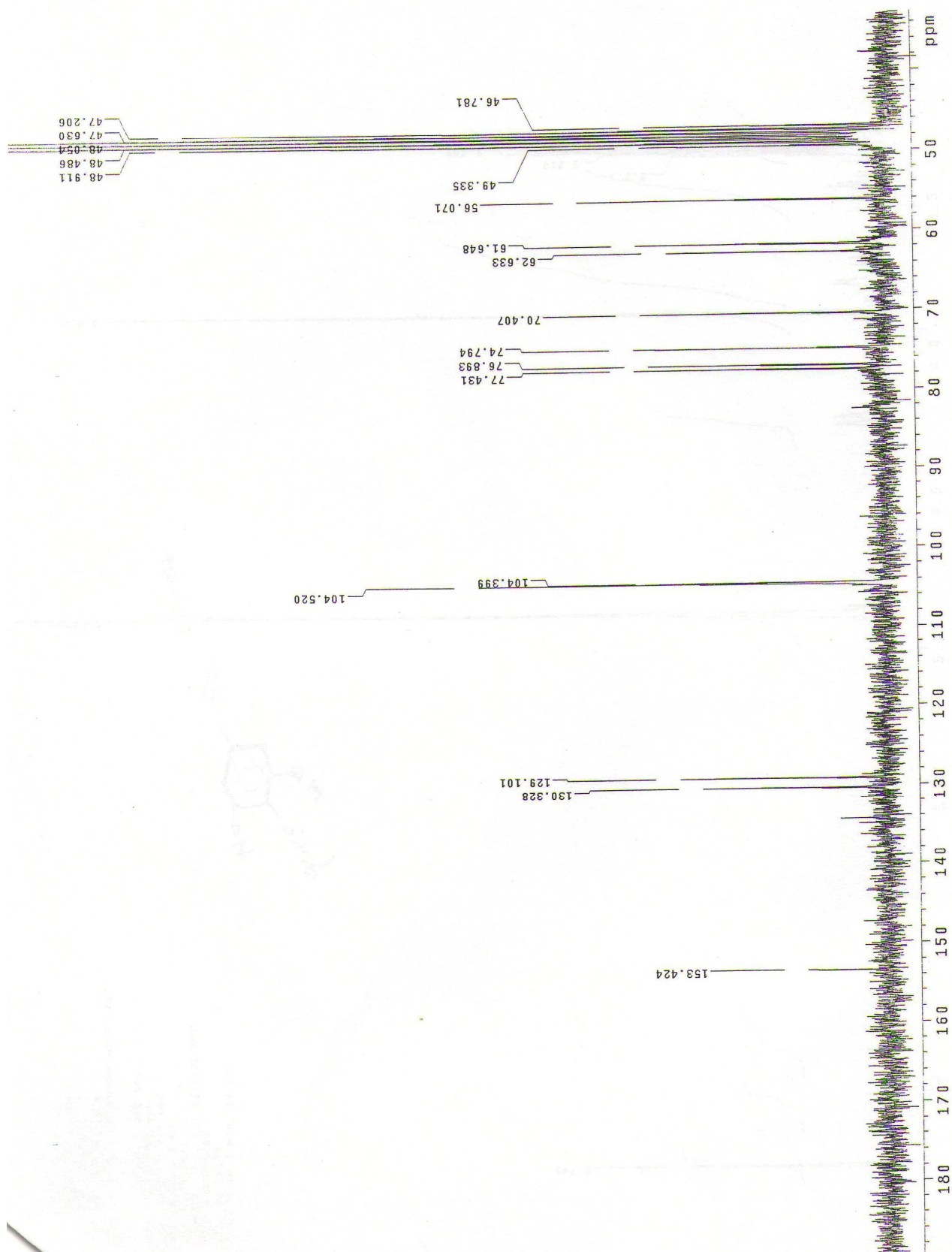**Figure 9.1** ^{13}C-NMR spectrum of syringin isolated from *Foeniculum vulgare.*

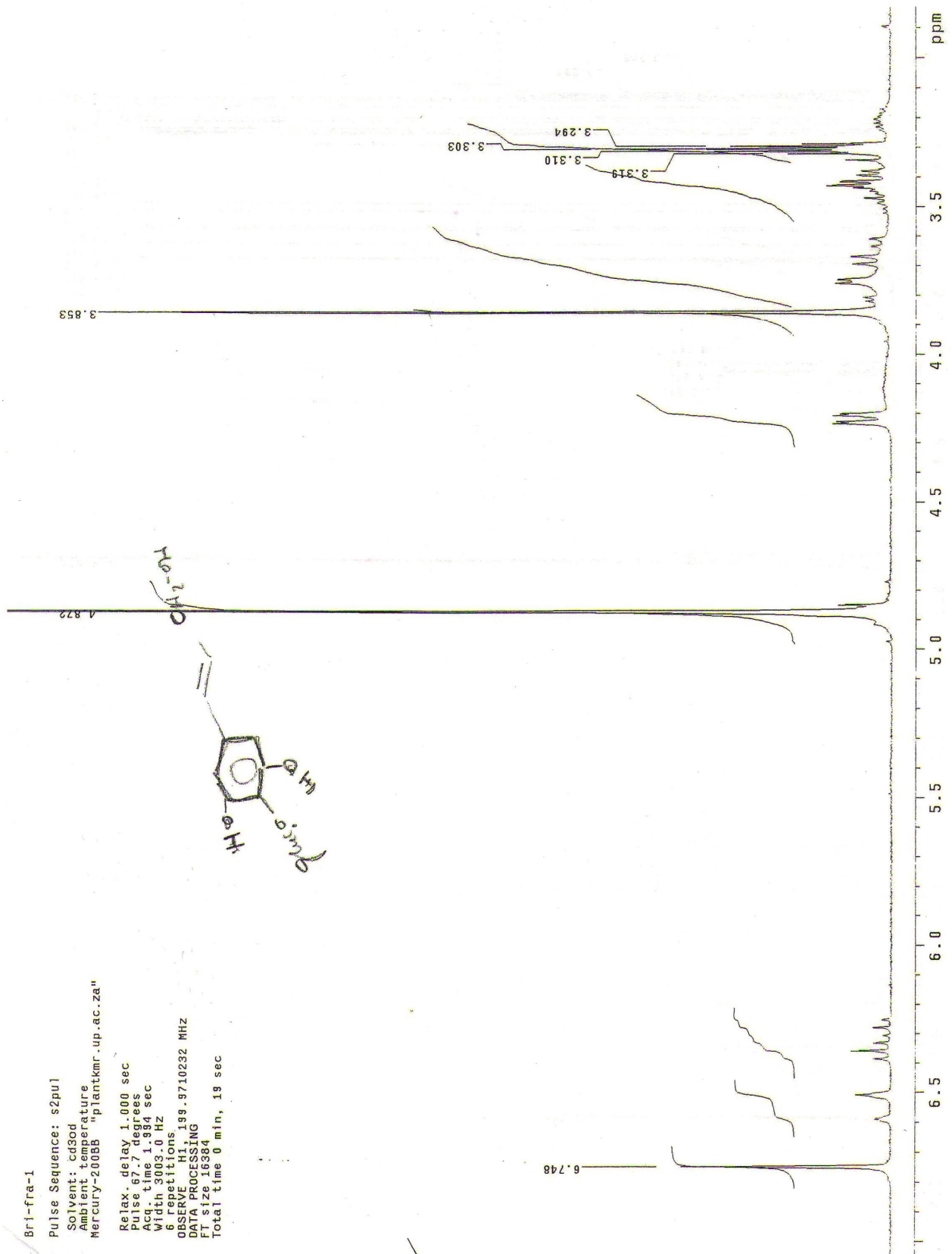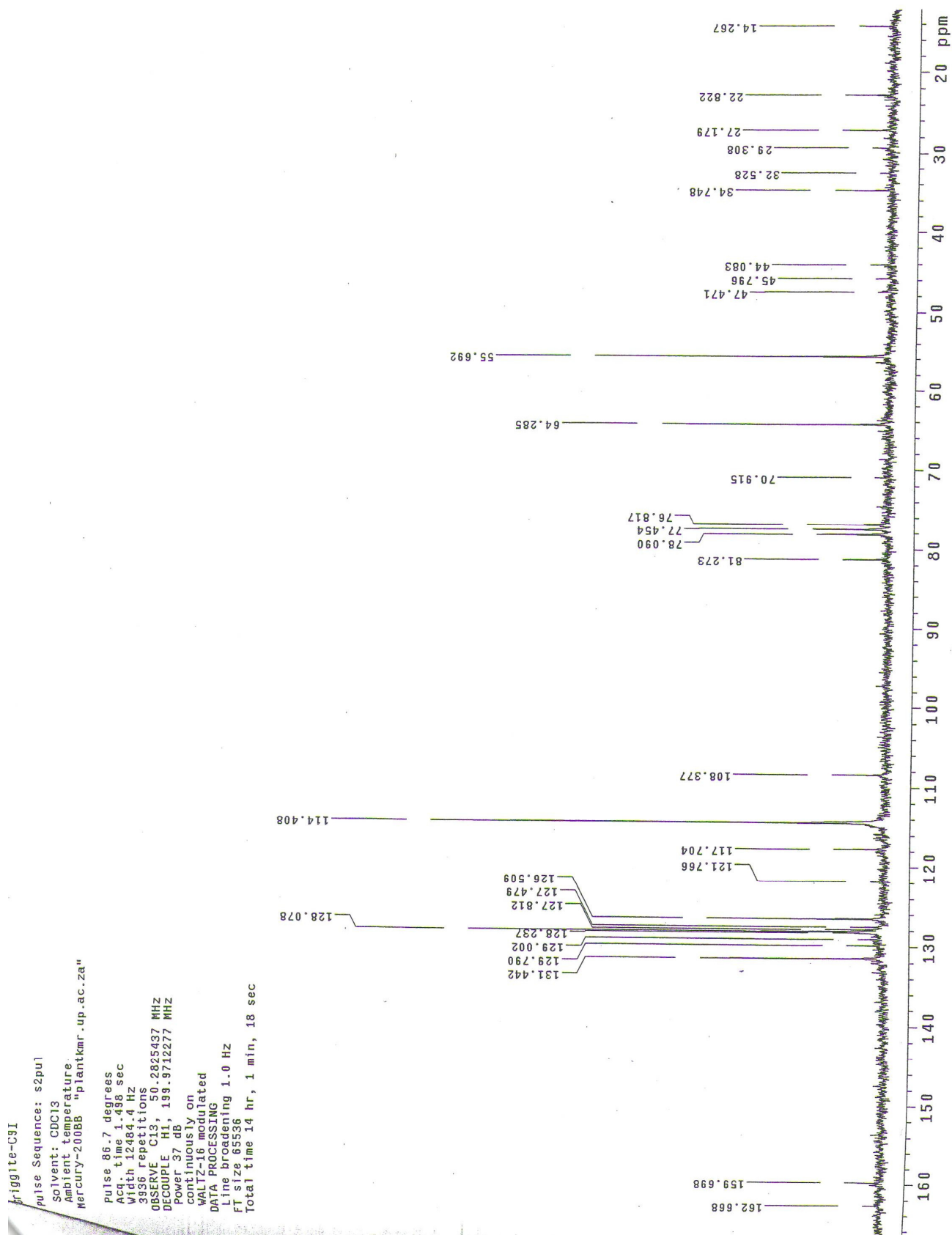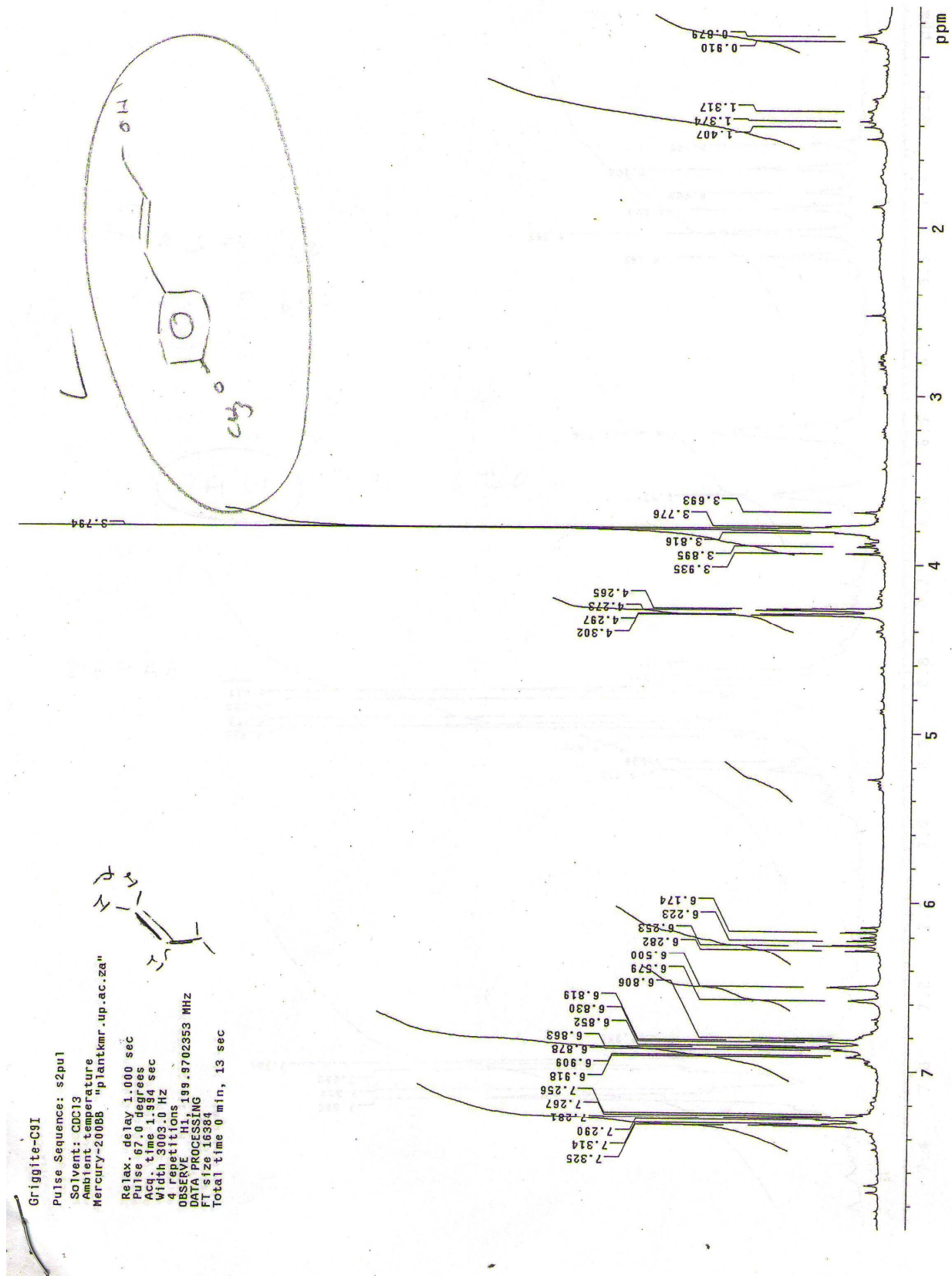**Figure 9.2** ^{1}H-NMR spectrum of syringin isolated from *Foeniculum vulgare.*

Figure 9.3 ^{13}C-NMR spectrum of 4-methoxycinnamyl alcohol isolated from *Foeniculum vulgare*.

Figure 9.3 ^{1}H-NMR spectrum of 4-methoxycinnamyl alcohol isolated from *Foeniculum vulgare*.

Appendix B

9.2 Publications and conference presentations resulting from this thesis

9.2.1 Publications

Manuscripts:

Binneman, B., Lall, N., Mahapatra, A., van de Venter, M., du Plessis-Stoman, D., Boukes, G, Houghton, P., Meyer, J.J.M. (2008) Selective induction of apoptosis by 7-methyljuglone and its synthetic naphthoquinone derivatives on human cancer cell lines. Cancer Letters. Submitted

Binneman, B., Lall, N., Mahapatra, A., van de Venter, M., du Plessis-Stoman, D., Boukes, G (2008) Cytotoxic active compounds from *Foeniculum vulgare*. In preparation.

Books:

Chapter 1 and 2 are being prepared for a chapter in a book.

9.2.2 Conference presentations

National

SAAB conference (2007) (poster presentation), <u>Binneman, B</u>, Mahapatra, A, Meyer, J.J.M., Lall, N. Evaluation of medicinal plants and synthesized naphthoquinones on cancer cells.

SAAB conference (2008) (oral presentation), <u>Binneman, B</u>, Lall, N., Mahapatra, A., Newman, S.P., Jones, L., Van De Venter, M., Houghton, P., Meyer, J.J.M. Selective induction of apoptosis in U937 cells by 8-fluoro-5-hydroxy- AND 2,5-dihydroxy-7–methyljuglone.

CANSA Cancer Research in Action (2008) (poster presentation), <u>Binneman, B.</u>, Lall, N., Mahapatra, A., van de Venter, M., du Plessis-Stoman, D., Boukes, G, Houghton, P., Meyer, J.J.M. Selective induction of apoptosis by potent anticancer plants extracts and /or compounds.

International

World Congress on Medicinal and Aromatic Plants (WOMAP) (2008) (oral presentation), <u>Binneman, B.</u>, Lall, N., Mahapatra, A., van de Venter, M., du Plessis-Stoman, D., Boukes, G, Houghton, P., Meyer, J.J.M. Evaluation and mechanistic studies of selected medicinal plants and purified compounds on human cancer cell lines.