CHAPTER 1

Introduction

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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).

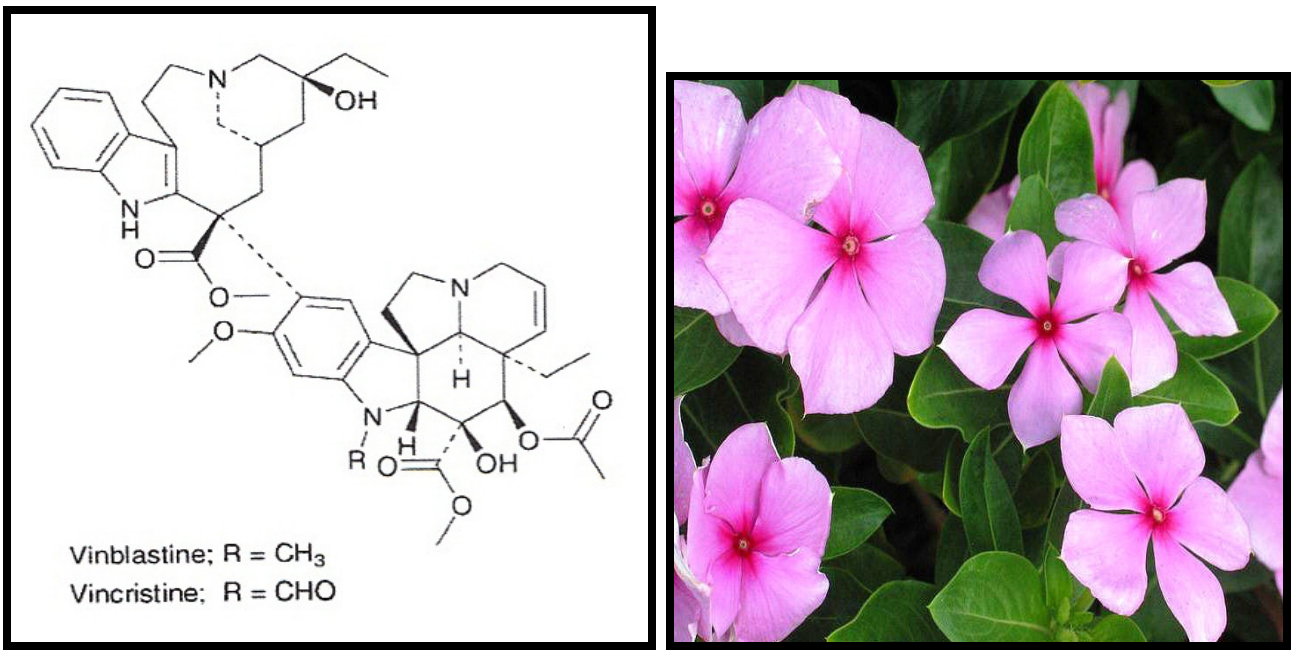
In the 1950s the search for anti-cancer agents from plants sources started to intensify. It was also in this time when the discovery and development of vinblastine and vincristine (vinca alkaloids), and the isolation of the cytotoxic podophyllotoxins took place (Cragg & Newman, 2005). This led to the 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).

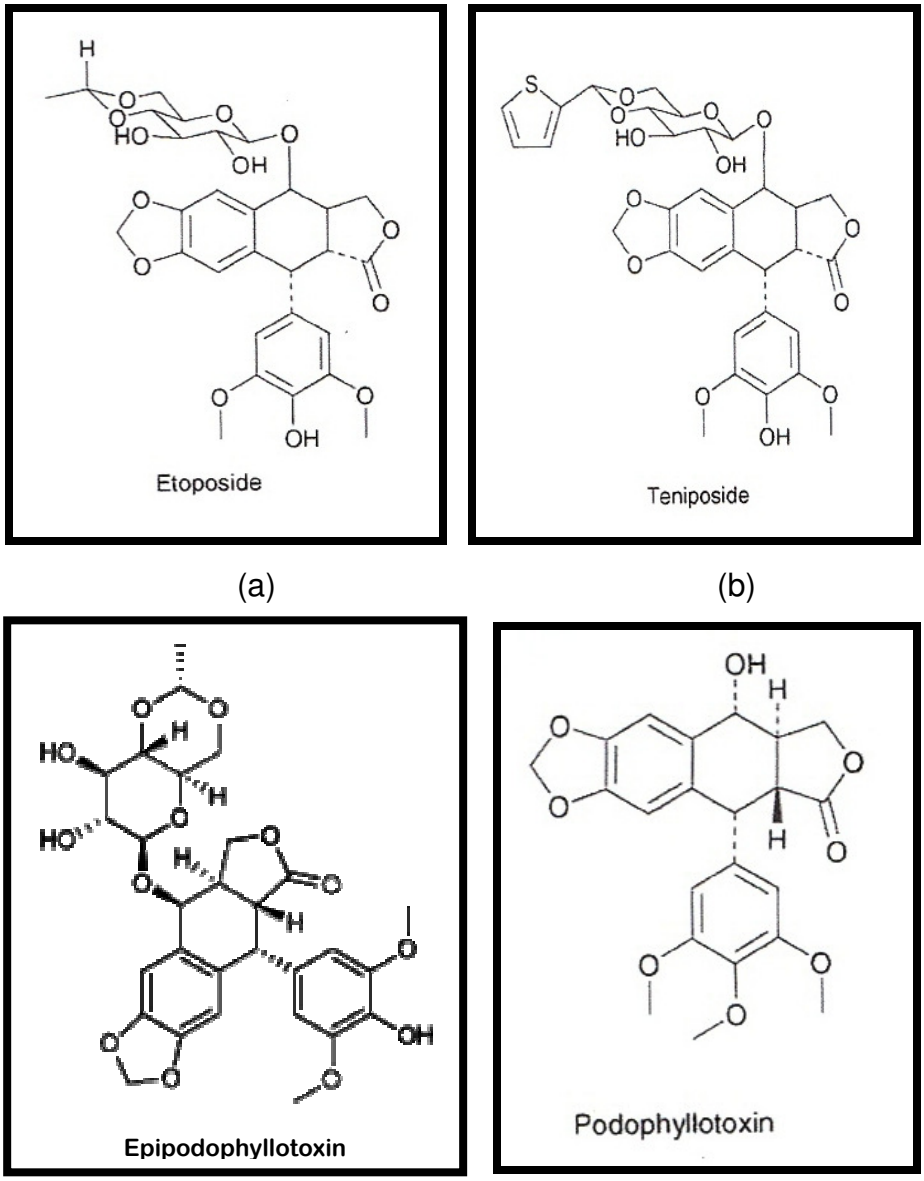
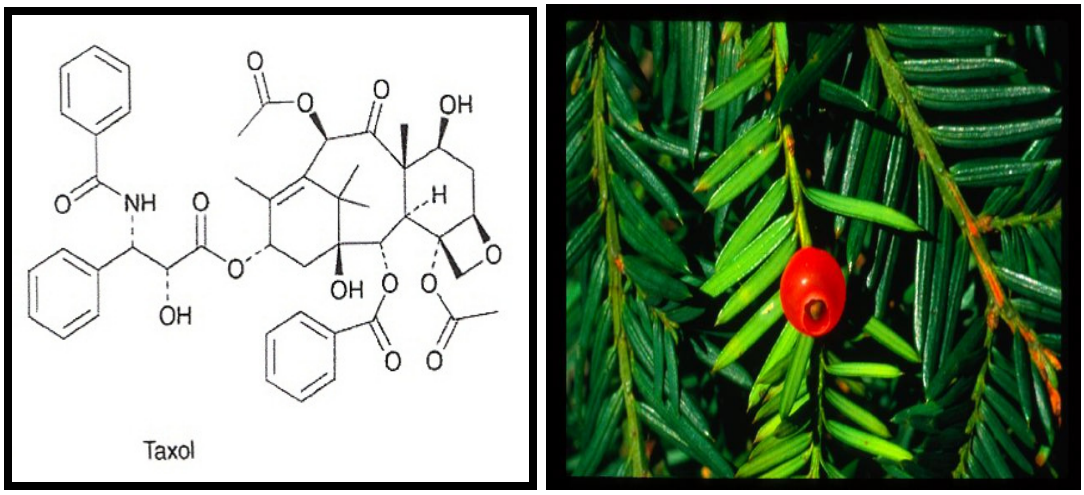


Figure 1.2 Semi-synthetic derivatives of epipodophyllotoxin, an isomer of podophyllotoxin (a) etoposide and (b) teniposide which are clinically active (c) epipodophyllotoxin (d)

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).

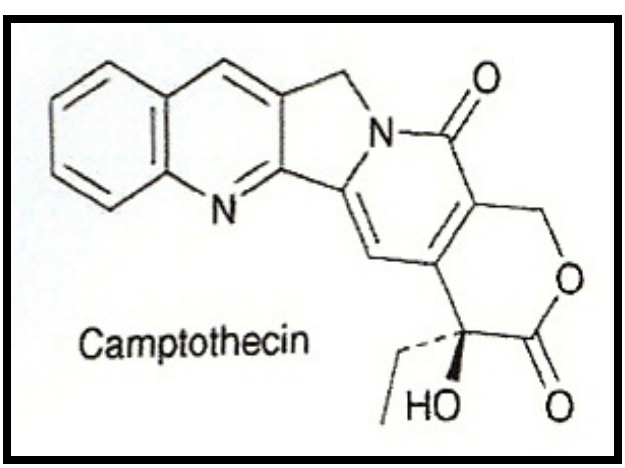


Figure 1.4 A natural alkaloid 'camptothecin' from *Camptotheca acuminate* (http://hortiplex.gardenweb.com/plants/jour/p/56/gw1007256/1452951021731136.jpeg) (Cragg *et al.*, 2005).

'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.

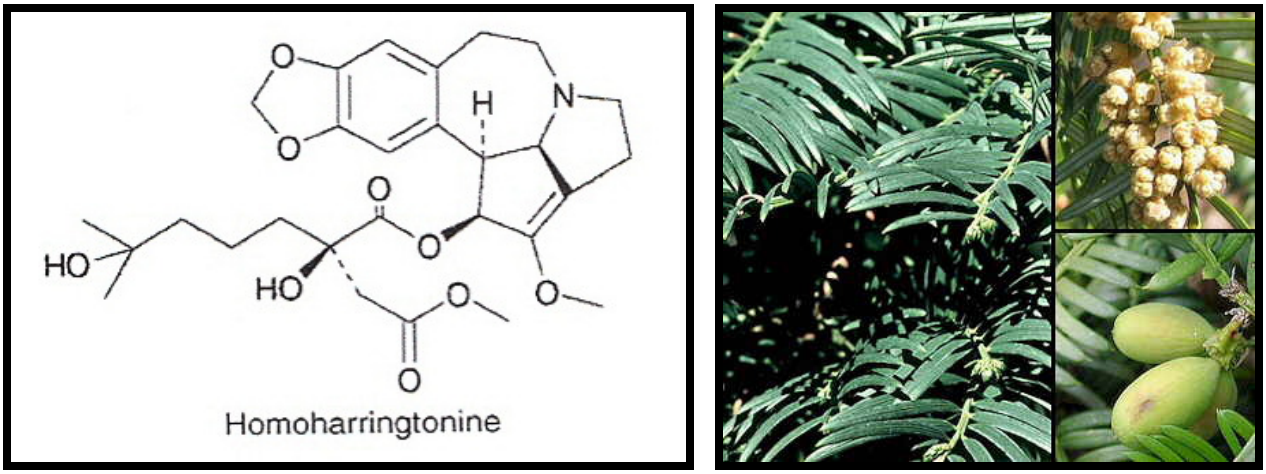


Figure 1.5 (a) 'Homoharringtonine' isolated from the Chinese tree (b) *Cephalotaxus harringtonia* var. *drupacea* (Cragg & Newman, 2005) (http://www.arbolesornamentales.com/Cephalotaxusharri.jpg).

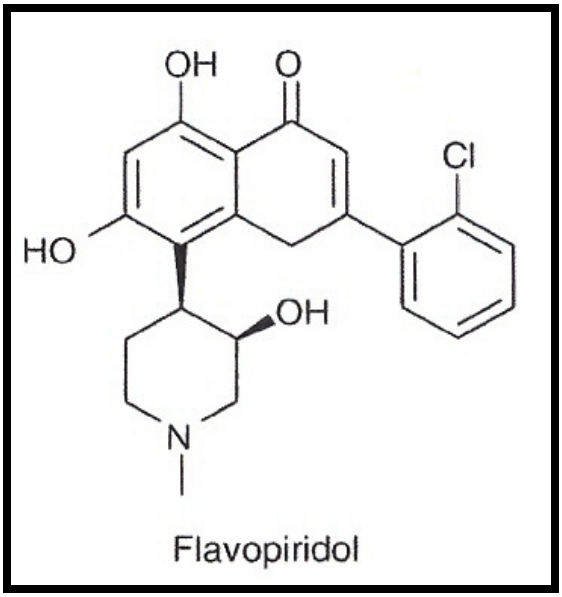
By systematically screening plant extracts, clinically active agents with antitumour properties can be isolated so that their metabolism can be explained so that more active molecules might be synthesized (Hill, 2001).

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).

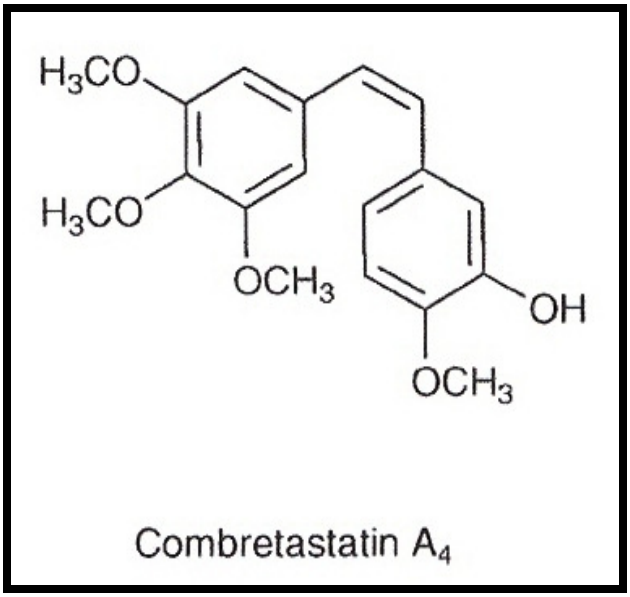
In the 1970s the NCI and United States Department of Agriculture (USDA) were working together with the South Africa Botanical Research Institute on a random collection

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).



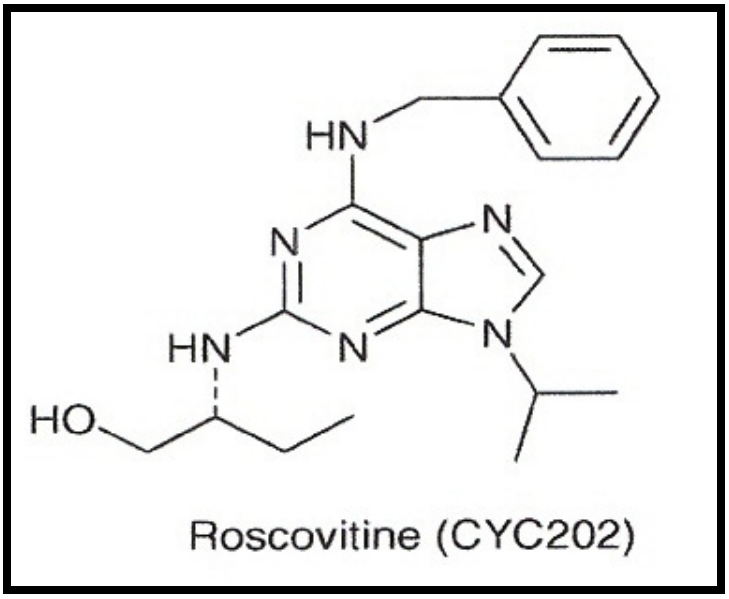
Flavopiridol

(a)



Combretastatin A_4

(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

Akerele, O. (1994) Nature's medicinal bounty: do not throw it away. World health Forum 14: 390-395.

Altmann, K.H., Gertsch, J. (2007) Anticancer drugs from nature-natural products as a unique source of new microtubule-stabilizing agents. Natural product reports. 24: 327-357.

Calixto, J.B. (2000) Efficacy, safety, quality control, marketing and regulatory guidelines for herbal medicines (phytotherapeutic agents). Brazilian Journal of Medicinal and Biological research. 33: 170-189.

Cassady, J.M., Douros, J.D. (Eds.) (1980) Anticancer agents based on natural product models. Academic Press, INC, New York.

Chang, Y.T., Gray, N.S., Rosania, G.R., Sutherlin, D.P., Kwon, S., Norman, T.C., Sarohia, R., Leost, M., Meijer, L., Schultz, P.G. (1999). Synthesis and application of functionally diverse 2,6,9-purine libraries as CDK inhibitors. Chemistry & Biology 6: 361–375.

Cotton, C.M. (1996). Ethnobotany principles and applications. John Wiley and Sons, Chichester. p: 1-18 and 216-244.

Cragg, G.M., Newman, D.J. (2005) Plants as a source of anti-cancer agents. Journal of Ethnopharmacology 100: 72-79.

Darzynkiewicz, Z., Traganos, F., Wu, J.M., Chen, S. (2000) Chinese herbal mixture PC SPES in treatment of prostate cancer. International journal of oncology. 17(4):729-736.

Ellis, C.G. (1986) Medicinal plant use-a survey. Veld and Flora. 72:72-74.

Fang, R. 2006. Investigation of some traditional Chinese medicines used to treat cancer. A thesis submitted to the University of London for the degree of Doctor of Philosophy,

Drug discovery group, Pharmaceutical Sciences Research Division, School of Biomedical & Health Sciences, King's College London, University of London.

Fennell, C.W., Lindsy, K.L., McGaw, L.J., Sparg, S.G., Stafford, G.I., Elgorashi, E.E., Grace, O.M., van Staden, J. (2004) Assessing African Medicinal plants for efficacy and safety: pharmacological screening and toxicology. Journal of ethnopharmacology. 94:205-217.

Finimh, A.C. (2001) Encyclopaedia of medicinal plants. Doring Kindersley. London. 1-53.

Guallo, V.P., McAlpine, J., Lam, K.S., Baker, D., Petersen, F. (2006) Drug discovery from natural products. J. Ind. Microbiol. Biotechnol. 33: 523-531.

Hartwell, J.L. (1982) Plants used against cancer. Quarterman, Lawrence, MA.

Hill, B.T. (2001) Vinflunine, a second generation novel vinca alkaloid with distinctive pharmacological profile, now in clinical development and prospects for future mitotic blockers. Current pharmaceutical design. 7(13):1199-1212.

Itokawa, H., Wang, X., Lee, K.-H., (2005) Homoharringtonine and related compounds. In: Cragg, G.M., Kingston, D.G.I., Newman, D.J. (Eds.), Anticancer Agents from Natural Products. Brunner-Routledge Psychology Press, Taylor & Francis Group, Boca Raton, FL, pp. 47–70 (Chapter 4).

Johnstone, R.W., Ruefli, A.A., Lowe, S.W. (2002) Apoptosis: a link between cancer genetics and chemotherapy. Cell. 108:153-164.

Kingston, D.G.I., (2005) Toxol and its analogs. In Cragg, G.M., Kingston, D.G.I., Newman, D.J. (Eds.) Anticancer agents from natural products. Brunner-Routledge Psychology Press, Taylor & Francis Group, Boca Raton, FL, pp. 89-122 (Chapter 6).

Kuo, P., Hsu, Y., Lin, C. (2005) The chemopreventive effects of natural products against human cancer cells. International journal of applied science and engineering. 3(3):203-214.

Lall, N. (2000) Isolation and identification of Naphthoquinones from *Euclea natalensis* with activity against *Mycobacterium tuberculosis*, other pathogenic bacteria and herpes simplex virus. University of Pretoria Press. Pretoria.

Lam, K.S. (2007) New aspects of natural products in drug discovery. Trends in Microbiology. 15(6): 279-289.

Lee, K. (1999) Novel antitumour agents from higher plants. Antitumour agents 197: 569-596.

Lewis, W.H., Lewis, M.P.E. (1995) Medicinal plants as sources of new therapeutics. Annals of the Missouri Botanical Garden. 82:16-24.

Li, Q., Sham, H.L. (2002) Discovery and development of antimitotic agents that inhibit tubulin polymerisation for the treatment of cancer. Expert Opinion on Therapeutic Patents 12: 1663–1701.

Lucci, A., Han, T., Liu, Y., Giuliano, A.E., Cabot, M.C. (1999) Multidrug resistance modulators and doxorubicin synergize to evaluate ceramide levels and elicit apoptosis in drug-resistant cancer cells. American cancer society. Cancer 86(2): 300-311.

Mabogo, D.E.N. (1990) The ethnobotany of the Vhavenda. M.Sc. thesis, University of Pretoria.

Mann, J. (2002) Natural products in cancer chemotherapy:past, present and future. Nature Rev Cancer 2: 143–148.

Mans, D.R.A., Da Rocha, A.B., Schwartsmann, G. (2000) Anti-cancer drug discovery and development in Brazil: targeted plant collection as a rational strategy to aquire candidate anti-cancer compounds. The Oncologist. 5:185-198.

McChesney, D.J. (1993) Biological and chemical diversity and the search for new pharmaceuticals and other bioactive natural products. In Kinghorn A.D. & Balandrin

M.F. (1993) Human medicinal agents in plants. American Chemical Society, Washington. p: 38-47.

Meijer, L., Raymond, E. (2003) Roscovitine and other purines as kinase inhibitors. From starfish oocytes to clinical trials. Accounts of Chemical Research 36: 417–425.

Mukherjee, A.K., Basu, S., Sarkar, N., Ghosh, A.C. (2001) Advances in Cancer Therapy with Plant Based Natural Products. *Current Medicinal Chemistry*. 8: 1467-1486.

Ndubani, P., Hojer, B. (1999). Traditional healers and the treatment of sexually transmitted illnesses in rural Zambia. Journal of Ethnopharmacology 67: 15-25.

Newman, D.J., Cragg, G.M., Snader, K.M. (2003) Natural products as source of new drugs over the period 1981-2002. Journal of Natural Products. 66(7): 1022-1037.

O'Niell ,M.J. (1993) The renaissance of plant research in pharmaceutical industry. In Kinghorn A.D. & Balandrin M.F. (1993) human medicinal agents in plants. American Chemical Society, Washington. p: 48-55.

Park, E.J., Pezzuto, J.M. (2002) Botanicals in cancer chemoprevention. Cancer and metastasis reviews. 21;231-255.

Phillips & Drew. (1992) Global Pharmaceutical Review.

Philp, R.B. (2004) Herbal remedies: the good, the bad, and the ugly. Journal of Complementary and Integrative Medicine. 1: 1-11.

Rates, S.M.K. (2001) Plants as source of drugs. Toxicon. 39: 603-631.

Sausville, E.A., Zaharevitz, D., Gussio, R., Meijer, L., Louarn-Leost, M., Kunick, C., Schultz, R., Lahusen, T., Headlee, D., Stinson, S., Arbuck, S.G., Senderowicz, A. (1999)

Cyclin-dependent kinases: initial approaches to exploit a novel therapeutic target. Pharmacology & Therapeutics 82: 285-292.

Steenkamp, V. (2003) Traditional herbal remedies used by South African women for gynaecological complaints. Journal of Ethnopharmacology. 86: 97-108.

Steenkamp, V., Gouws, M.C. (2006) Cytotoxicity of six South African medicinal plant extracts used in the treatment of cancer. South African journal of botany, 72: 630-633.

Taylor, J.L.S., Rabe, T., McGaw, L.J., Jäger, A.K. & Van Staden, J. (2001) Towards the science validation of traditional medicinal plants. Plant Growth Regulation. 34: p 23-37.

Tazi, J., Bakkour, N., Soret, J., Zekri, L., Hazra, B., laine, W., Baldeyrou, B., Lansiaux, A., Bailly, C. (2005) Selective inhibition of topoisomerase I and various steps of spliceosome assembly by diospyrin derivatives. Molecular pharmacology. 67(4):1186-1194.

Tshikalange, T.E., Meyer, J.J.M., Hussein, A.A. (2005) Antimicrobial activity, toxicity and the isolation of a bioactive compound from plants used to treat sexually transmitted diseases. Journal of ethnopharmacology 96:515-519.

Van Wyk, B.E, Van Oudtshoorn, B. & Gericke, N. (1997) Medicinal plants of South Africa. Briza publications, Pretoria, South Africa.

Vickers, A. (2002) Botanical medicines for the treatment of cancer: Rationale, overview of current data, and methodological considerations for phase I and II trials. Cancer investigation. 20:1069-1079.

Vickers, A., Zollman, C. (1999) ABC of complementary medicine: herbal medicine. Br. Med. J. 319 (7216):1050-1053.

Wagner, H. (1999) New approaches in phytopharmacological research. Pure Appl. Chem. 71(9): 1649-1654.

Wani, M.C., Taylor, H.L., Wall, M.E., Coggon, P., Mcphail, A.T. (1971) Plant antitumor agents. VI. The isolation and structure of taxol, a novel antileukemic and antitumor agent from *Taxus brevifolia*. *J. Am. Chem. Soc.* 93: 2325–2327.

World Health Organization (1993) WHO guidelines for the assessment of herbal medicines. Geneva, Switzerland.

http://www.biologie.uni-regensburg.de/Botanik/Schoenfelder/kanaren/images/Catharanthus_roseus.jpg

http://www.nature.com/nchembio/journal/v3/n8/images/nchembio.2007.10-COMP19.gif

http://arnica.csustan.edu/boty3050/medicinal/taxus_brevifolia.jpg

http://hortiplex.gardenweb.com/plants/jour/p/56/gw1007256/1452951021731136.jpeg)

http://www.arbolesornamentales.com/Cephalotaxusharri.jpg

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).

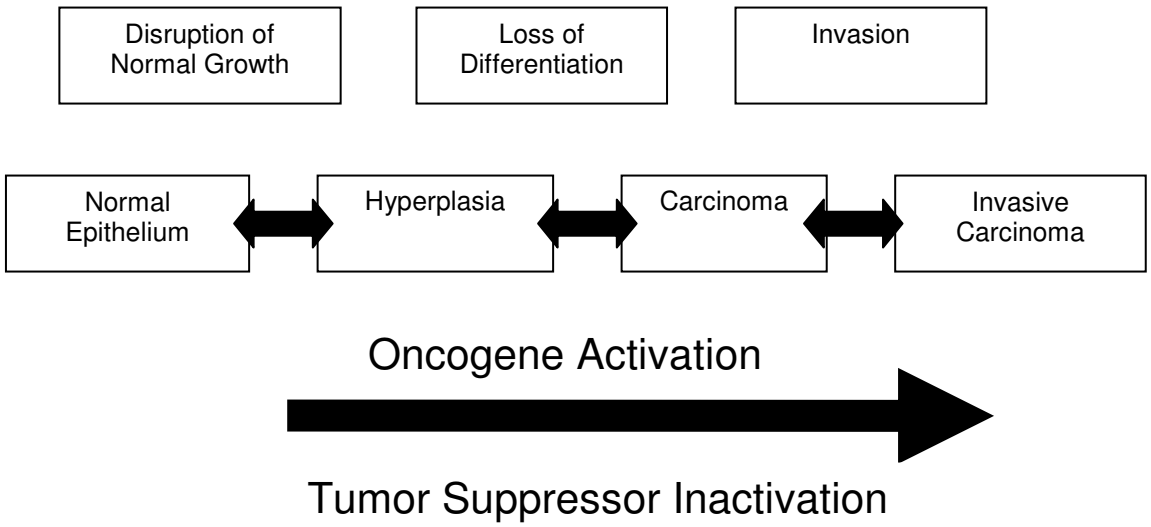


Figure 2.1 The upper row represents disturbances in growth, differentiation, and tissue integrity that lead to the phenotypes that characterize the different stages of cancer, shown in the lower row (Baudino, 2004).

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).

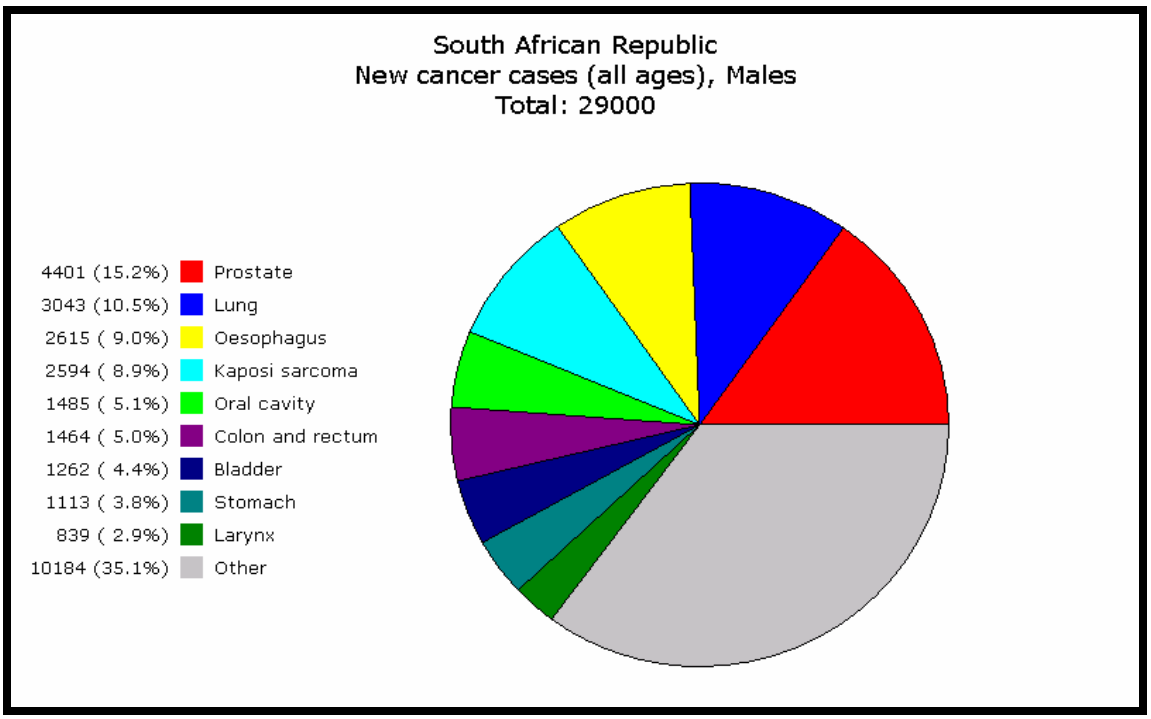


Figure 2.2 New cancer cases for males of all ages in South Africa (http://www-dep.iarc.fr/.).

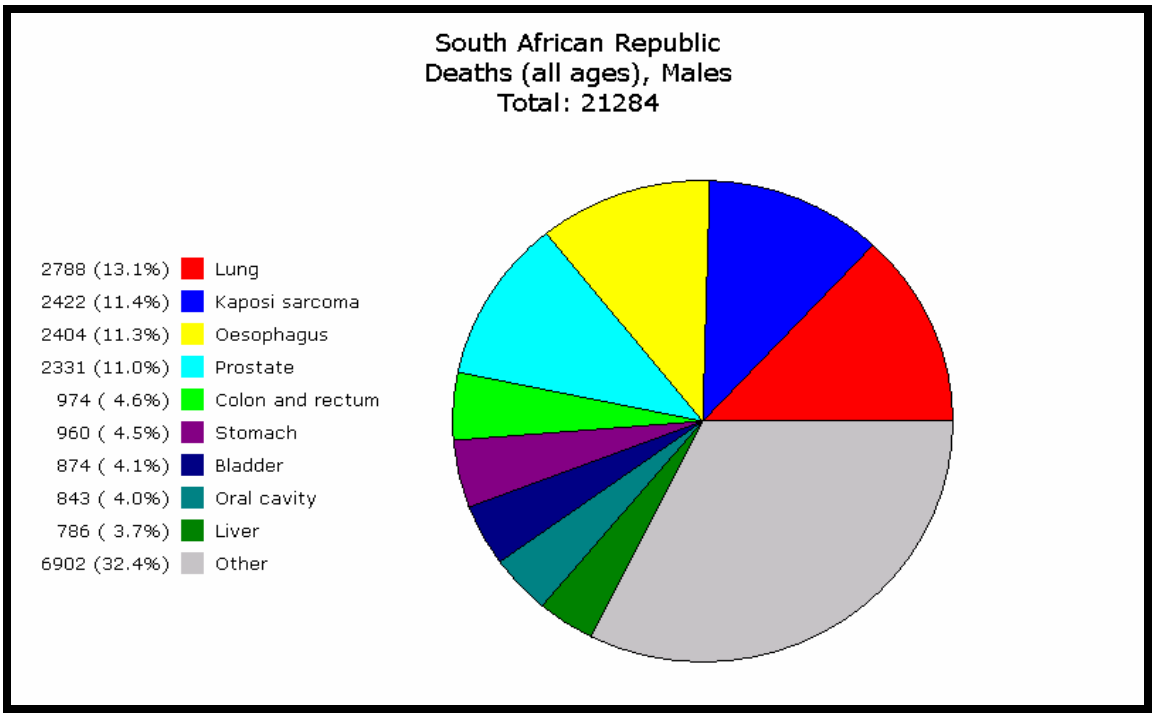


Figure 2.3 Cancer deaths for males of all ages in South Africa (http://www-dep.iarc.fr/.).

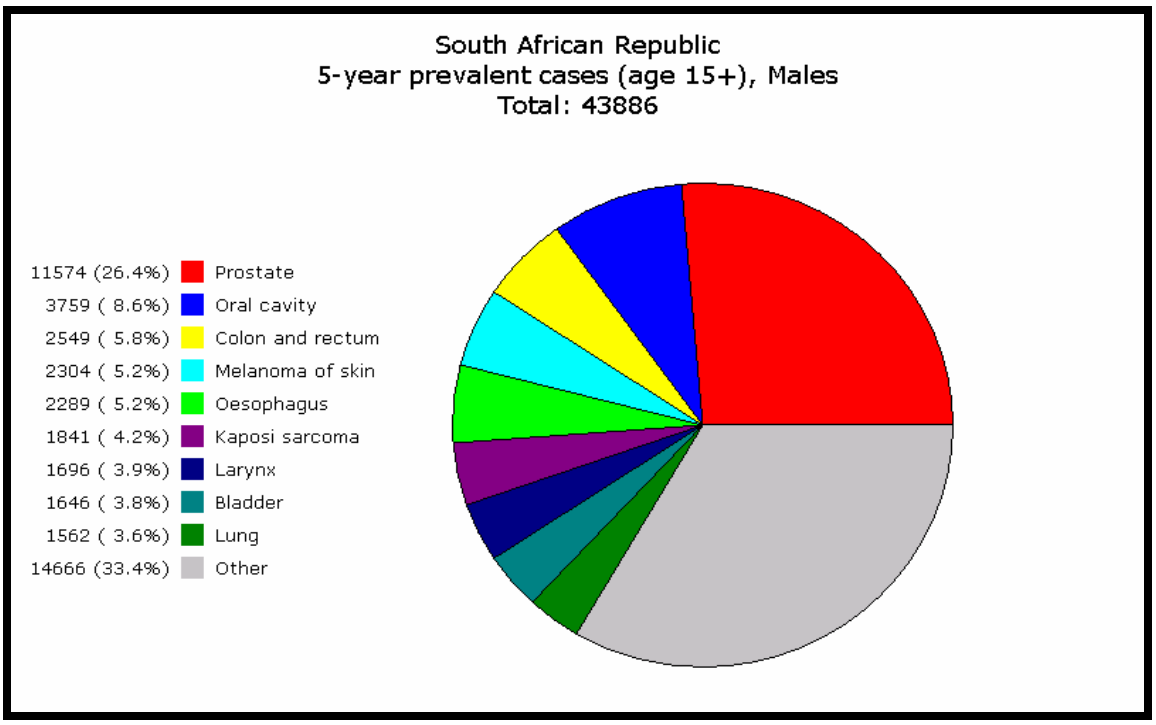


Figure 2.4 The 5-year prevalent cases for males 15 years and older in South Africa (http://www-dep.iarc.fr/.).

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).

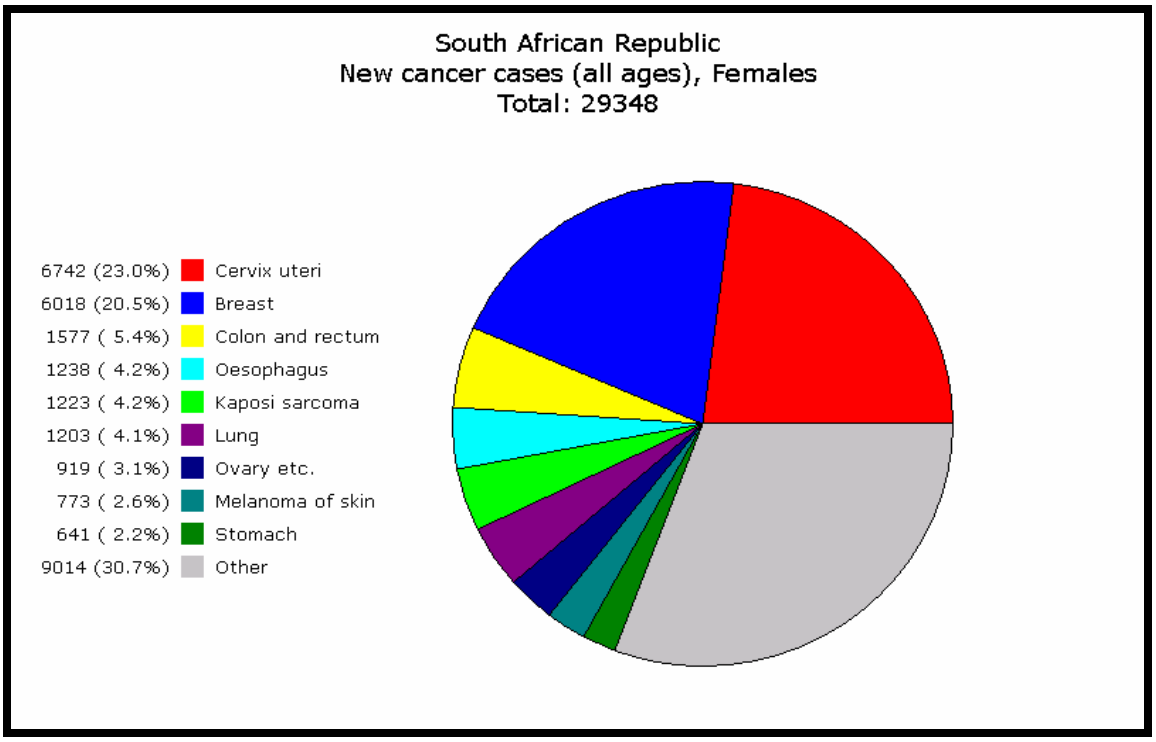


Figure 2.5 New cancer cases for females of all ages in South Africa (http://www-dep.iarc.fr/.).

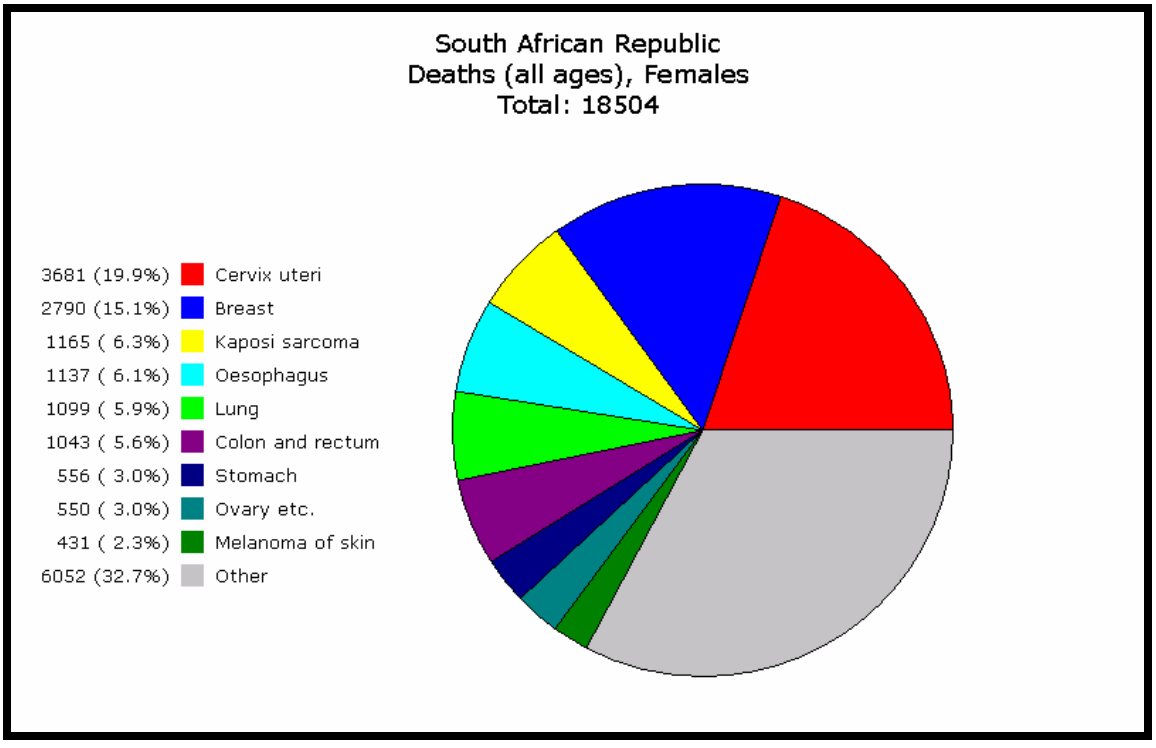


Figure 2.6 Cancer deaths for females of all ages in South Africa (http://www-dep.iarc.fr/.).

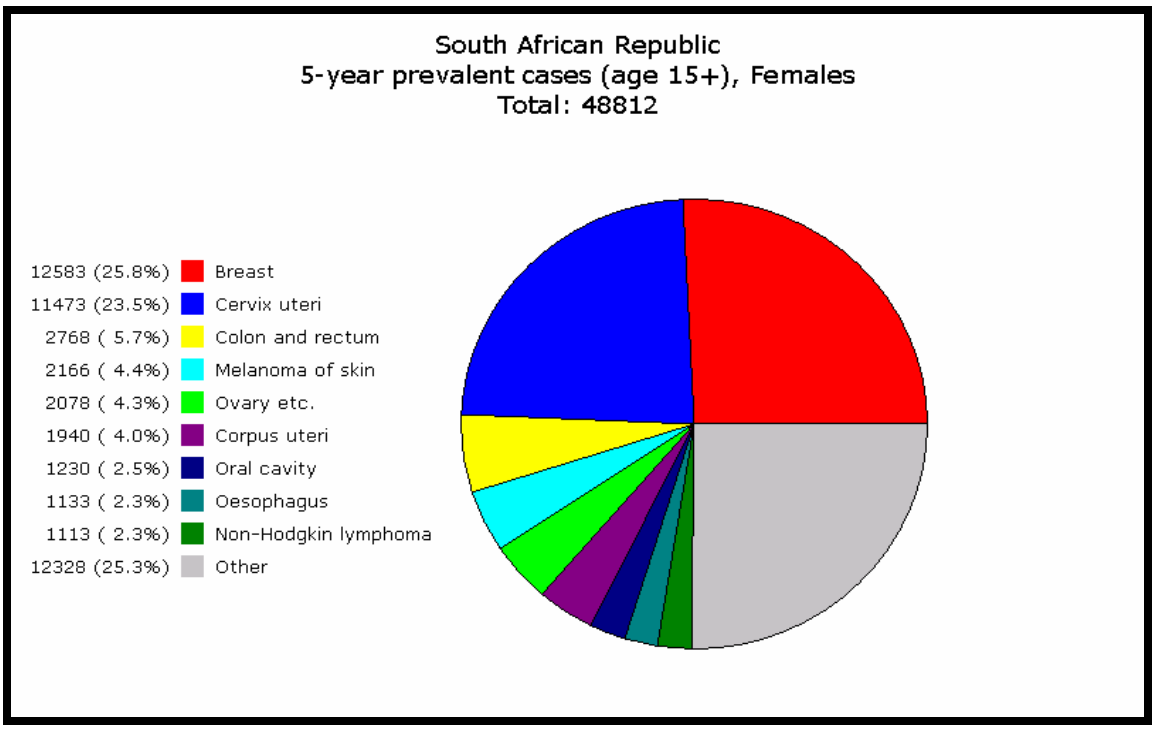


Figure 2.7 The 5-year prevalent cases for females 15 years and older in South Africa (http://www-dep.iarc.fr/.).

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).

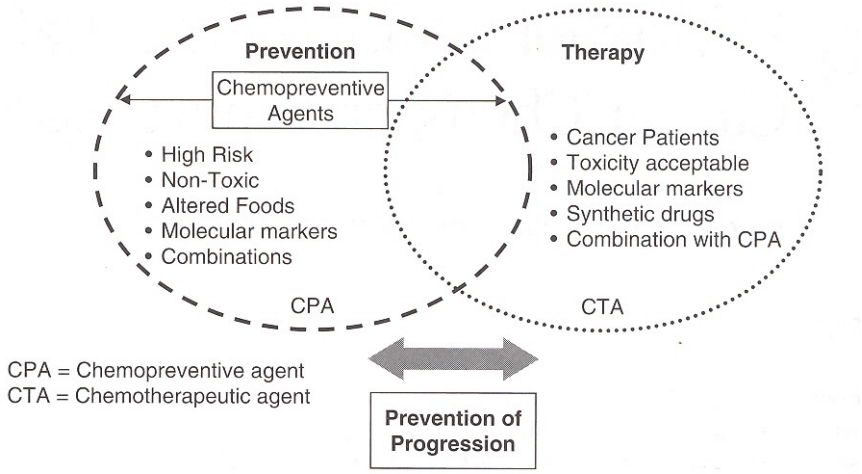


Figure 2.8 Schematic diagram showing the range of efficacy of chemopreventative agents (Bagchi and Preuss, 2005).

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.

2.3 References

Angell, M., Kassirer, P.J. (1998) Alternative medicine-the risks of untested and unregulated remedies. The New England journal of medicine 339: 839-841.

Bagchi, D., Preuss, H.G. (2005) Phytopharmaceuticals in cancer chemoprevention. CRC Press LLC.

Baudino (2004) Cancer research. Sigma.

Buja, L.M., Eigenbrodt, M.L., Eigenbrodt, E.H. (1993) Apoptosis and necrosis basic types and mechanisms of cell death. *Archives of pathology & laboratory medicine.* 117(12): 1208-1214.

Cancer facts & figures 2006

Cotter TG, Lennon SV, Glynn JG, Martin SJ (1990) Cell death via apoptosis and its relationship to growth, development and differentiation of both tumour and normal cells. Anticancer Res10:1153–1159

Cummings, J., Ward, T.J., Ranson, M., Dive, C. (2004) Apoptosis pathway-targeted drugs-from the bench to the clinic. Biochemica et Biophysica Acta. 1705: 53-66.

Darzynkiewicz, Z., Juan, G., Li, X., Gorczyca, W., Murakami, T., Traganos, F. (1997) Cytometry in cell necrobiology: analysis of apoptosis and accidental cell death (necrosis). Cytometry. 27:1-20.

Darzynkiewicz, Z. (1997) Methods in analysis of apoptosis and cell necrosis. In The Purdue Cytometry CD-ROM Volume 3, J. Parker, C. Stewart, Guest Eds., J.Paul Robinson, Publisher. Purdue University, West Lafayette, 1997.

Edinger, A.L., Thompson, C.B. (2004) Death by design: apoptosis, necrosis and autophagy. Current opinion in Cell Biology. 16: 663-339.

Ernst, E., Cassileth B.R. (1998) The prevalence of complementary/alternative medicine in cancer:a systematic review. Cancer 83: 777-782.

Fang, R. (2006). Investigation of some traditional Chinese medicines used to treat cancer. A thesis submitted to the University of London for the degree of Doctor of Philosophy, Drug discovery group, Pharmaceutical Sciences Research Division, School of Biomedical & Health Sciences, King's College London, University of London.

Hagg, M., Biven, K., Ueno, T., Rydlander, L., Bjorklund, P., Wiman, K.G., Shoshan, M., Linder, S. (2002) A novel high-through-put assay for screening of pro-apoptotic drugs. Investigational New Drugs. 20(3): 253-259.

Halestrap, A. (2005) A pore way to die. Nature. 434:578-579.

Hsu, Y., Kuo, P., Lin, L., Lin, C. (2005) Asiatic acid, a triterpene, induces apoptosis and cell cycle arrest through activation of extracellular signal-regulated kinase and p38 mitogen-activated protein kinase pathways in human breast cancer cells. The journal of pharmacology and experimental therapeutics. 313(1):333-344.

Huppertz B., Frank, H.G., Kaufmann P. (1999). The apoptosis cascade-morphological and immunohistochemical methods for its visualization. Anat Embriol 200:1-18.

Johnstone, R.W., Ruefli, A.A., Lowe, S.W. (2002) Apoptosis: a link between cancer genetics and chemotherapy. Cell. 108:153-164.

Kerr, J.F.R., Winterford, C.M., Harmon, B.V. (1993) Apoptosis its significance in cancer and cancer therapy. *Cancer.* 73(8): 2013-2026.

Kerr, J.F.R., Winterford, C.M., Harmon, B.V. (1994) Apoptosis its significance in cancer and cancer therapy. Cancer. 73(8): 2013-2026.

Kinghorn, A.D., Farnsworth, N.R., Soejarto, D.D., Cordell, G.A., Swanson, S.M., Pezzuto, J.M., Wani, M.C., Wall., M.E., Oberlies, N.H., Kroll, D.J., Kramer, R.A., Rose, W.C., Vite, G.D., Fairchild, C.R., Peterson, R.W., Wild, R. (2003) Novel strategies for the discovery of plant-derived anticancer agents. Pharmaceutical biology. 41: 53-67.

Lee, A.T.C., Azimahtol, H.L.P., Tan, A.N. (2003) Styrylpyrone derivative (SPD) induces apoptosis in a caspase-7-dependant manner in the human breast cancer cell line MCF-7. *Cancer cell international*. 3:16-24.

Martin, S.J., Green, D.R., Cotter, T.G. (1994) Dicing with death: dissecting the components of the apoptosis machinery. *TIBS*. 19: 26-30.

Mukherjee, A.K., Basu, S., Sarkar, N., Ghosh, A.C. (2001) Advances in Cancer Therapy with Plant Based Natural Products. *Current Medicinal Chemistry*. 8: 1467-1486.

Kuan, N., Passaro, E. (1998) Apoptosis: programmed cell death. Archives of surger. 133: 773-775

Newman, D.J., Cragg, G.M., Snader, K.M. (2003) Natural products as source of new drugs over the period 1981-2002. Journal of Natural Products. 66(7): 1022-1037.

Nwoga, I.A. (1994) Traditional healers and perceptions of the causes and treatment of cancer. Cancer Nursing 17: 470-478

Park, E.J., Pezzuto, J.M. (2002) Botanicals in cancer chemoprevention. Cancer and metastasis reviews. 21: 231-255.

Potten, C.S., Wilson, J. (2004) Apoptosis: the life and death of cells. Cambridge University Press.

Raff, M. (1998) Cell suicide for beginners. Nature, 396:119-122.

Schraub, S. (2000) Unproven methods in cancer: a worldwide problem. Support Care Cancer 8 :10–15.

Schwartzman, R.A., Cidlowski, J.A. (1993). Apoptosis: the biochemistry and molecular biology of programmed cell death. Endocrine reviews. 14(2): 133-151.

Studzinski, G.P. (1995) Cell growth and apoptosis a practical approach. IRL Press at the Oxford University Press.

Szende, B., Zalatnai, A., Schally, A.V. (1989) Programmed cell death (apoptosis) in pancreatic cancers of hamsters after treatment with analogs of both luteinizing hormone-releasing hormone and somstostatin. Proc. Natl. Acad. Sci. 86: 1643-1647.

Treasure, J. (2005) Herbal medicine and cancer: an introductory overview. Seminars in Oncology Nursing. 21(3): 177-183.

Trump, B.F., Berezesky, I.K., Chang, S.H., Phelps, P.C. (1997) The pathways of cell death: oncosis, apoptosis and necrosis. Toxicologic pathology. 25(1): 82-88.

Van Engeland, M., Nieland, L.J.W., Ramaekers, F.C.S., Schutte, B., Reutelingsperger, C.P.M. (1998) Annexin V-Affinity Assay: A review on an apoptosis detection system based on phosphatidylserine exposure. *Cytometry*. 31: 1-9.

Westphal, S., Kalthoff, H. (2003) Apoptosis: targets in pancreatic cancer. Molecular cancer. 2: 6-19.

WHO (2006) Cancer: Diet and physical activity's impact.

WHO, 2003

IARC, 2002

International Union Against Cancer (UICC) (2005) Prevention: Mom was right- you should eat your vegetables. eNews, December.

http://www.mrc.ac.za

http://www.cansa.co.za

http://www-dep.iarc.fr/

http://www.doh.gov.za/.docs/research/vol5-4cancer.html

CHAPTER 3

Anticancer activity of traditionally used plant extracts

Chapter 3

Anticancer activity of traditionally used plant extracts

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

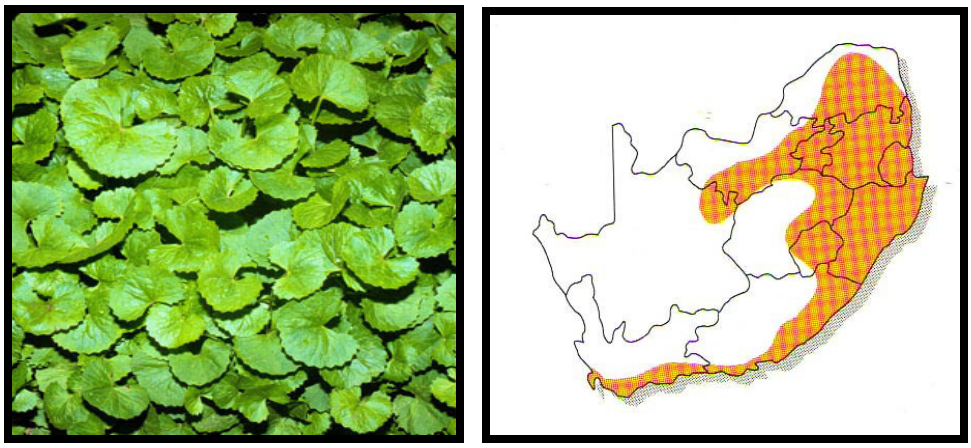
3.3.1 Ehhnobotanical use of *Centella asiatica*

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).



(a) (b)

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.

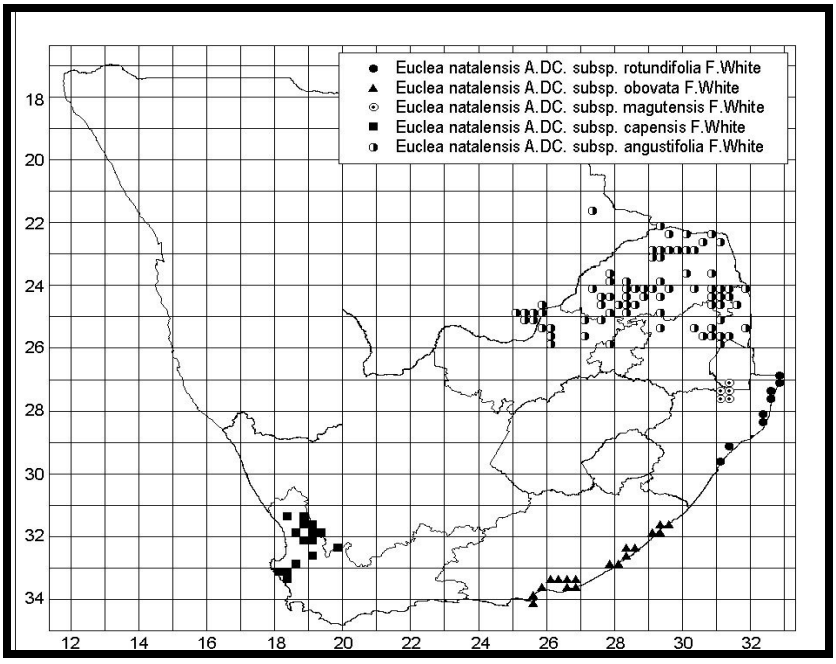


Figure 3.4 The distribution of the subspecies of *E. natalensis* in South Africa.

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.

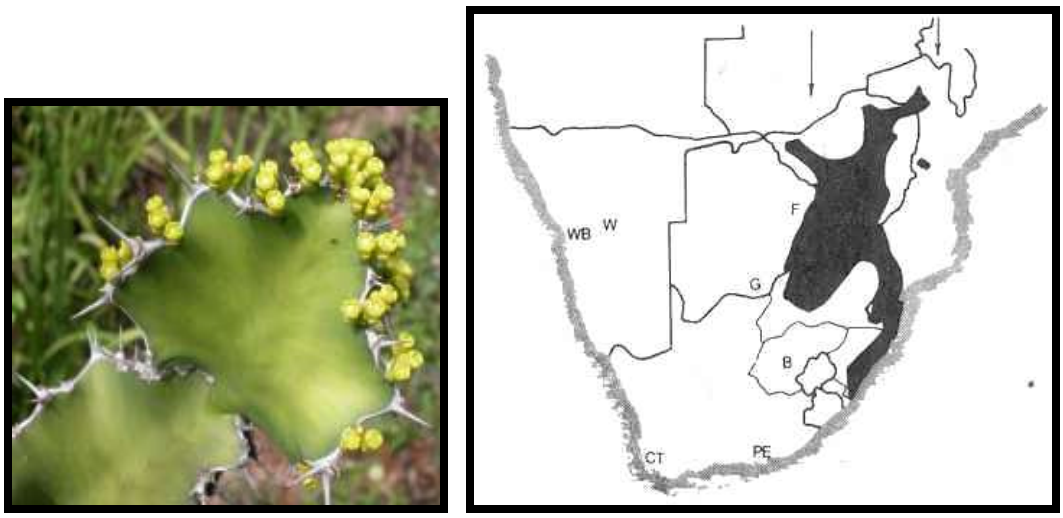


Figure 3.5 (a) The yellowish-green inflorescence of *E. ingens*. (b) The distribution of *E. ingens* in Southern Africa (www.csdl.tamu.edu/FLORA1/imaxxeup1.htm) (Palgrave, 1981).

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*

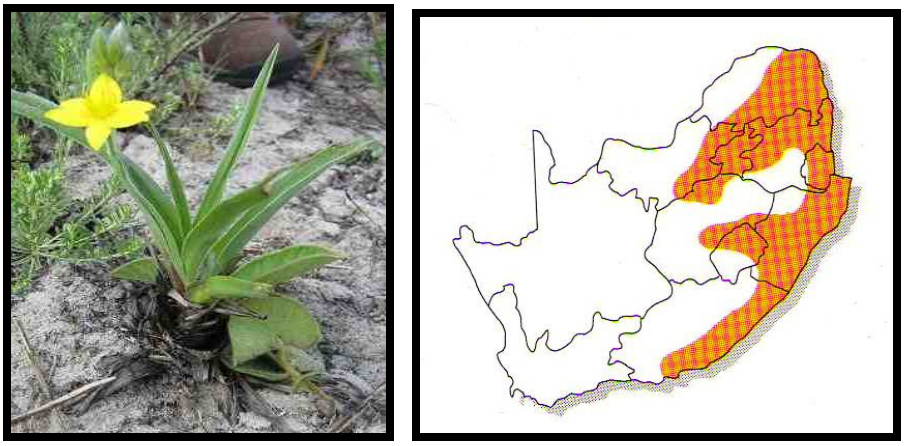
Linnaeus, in 1759, coined from the Greek words *hypo* (‘below) and *oxy* (‘sharp), the epithet *Hypoxis,* which refers to the fruit that are pointed at the base (Drewes & Khan, 2004).

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.



(a) (b)

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.

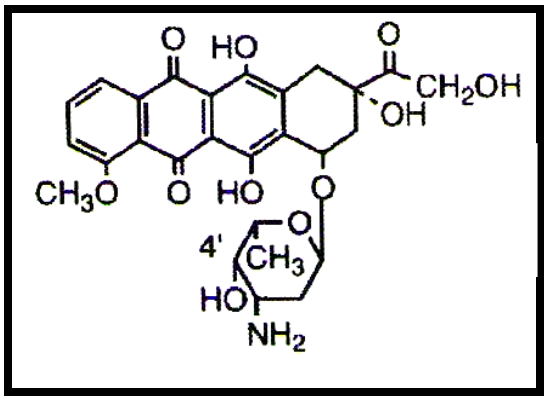


Figure 3.9 Quinonoid doxorubicin (Hutchinson & Colombo, 1999).

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).

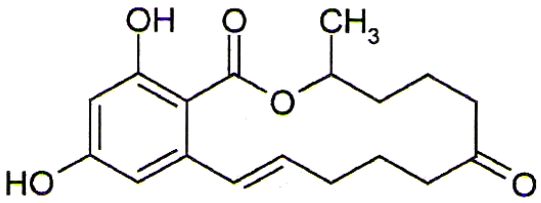


Figure 3.10 Zearalenone a non-steroidal estrogenic mycotoxin and phytoestrogen (Stopper *et al*., 2005).

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).

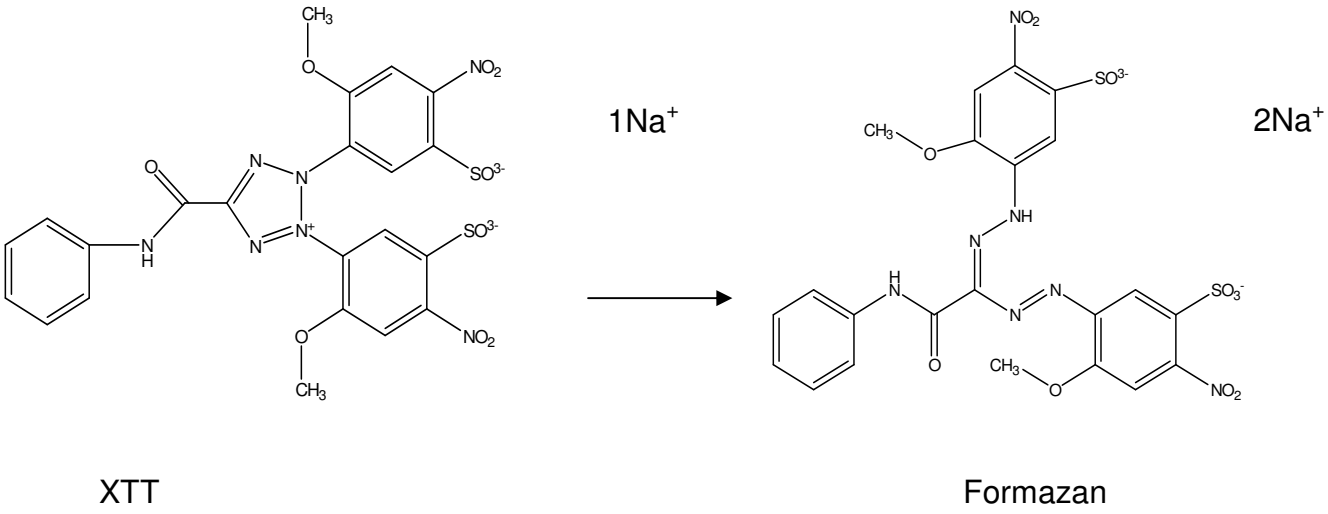


Figure 3.11 Metabolization of XTT to water soluble formazan salt by viable cells (Roche, 2004).

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°C. 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°C then stopped by the addition of complete medium. About $1x10^5$ 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 $1x10^5$ 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.

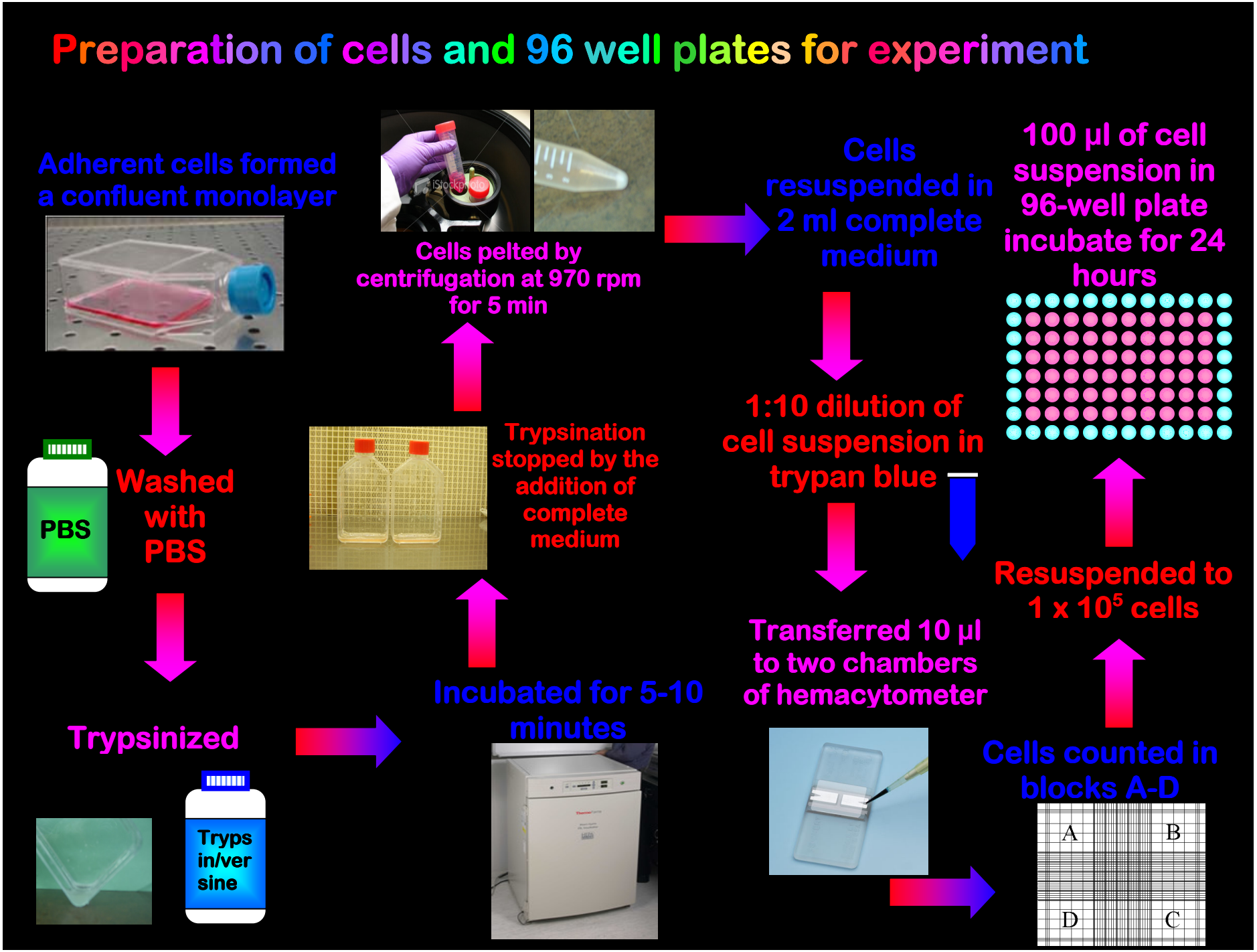


Figure 3.12 Schematic representation of the preparation of the cells and 96-well plates for the experiment.

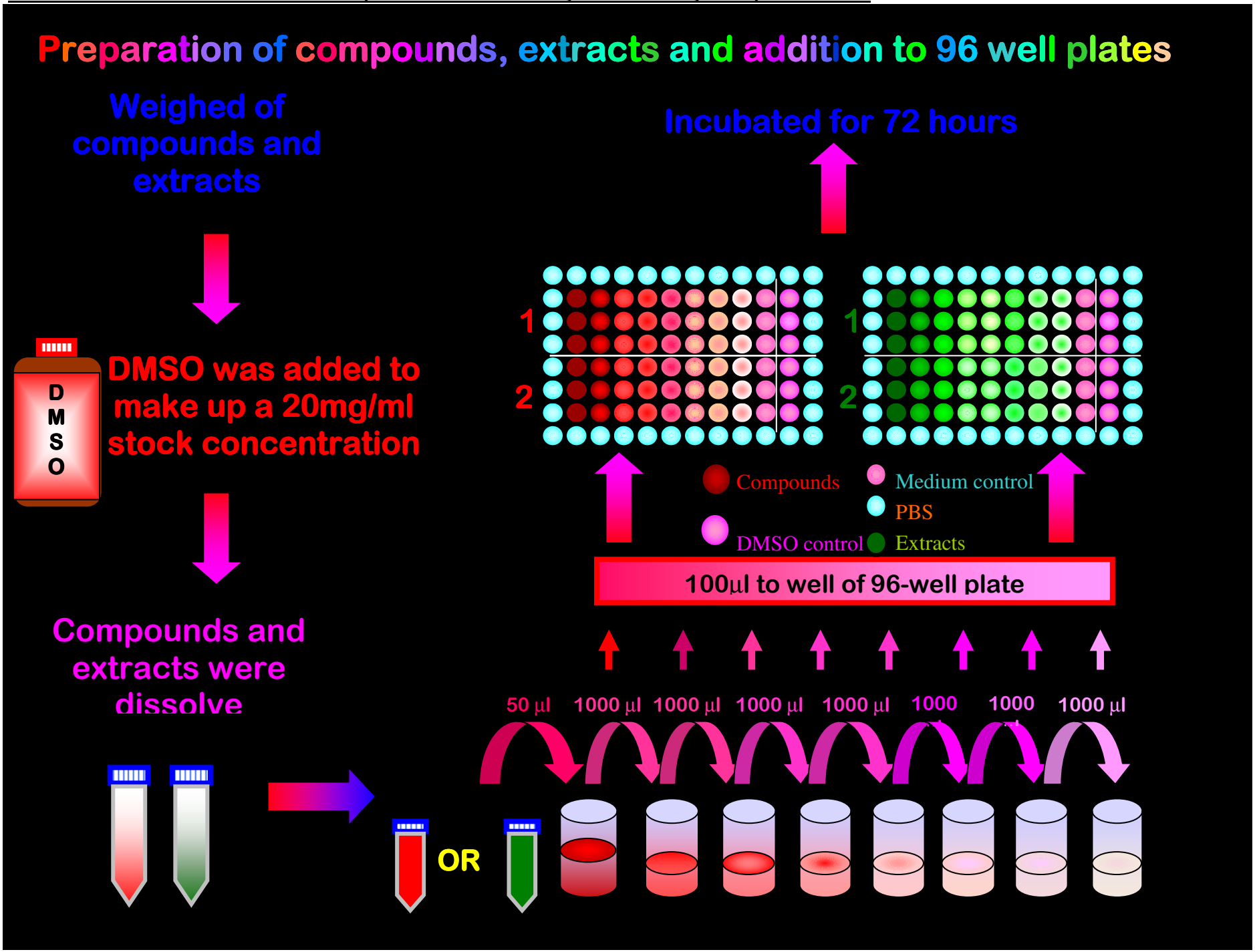


Figure 3.13 Schematic representation of the preparation of extracts/compounds for addition to the 96-well plates which contain the cells

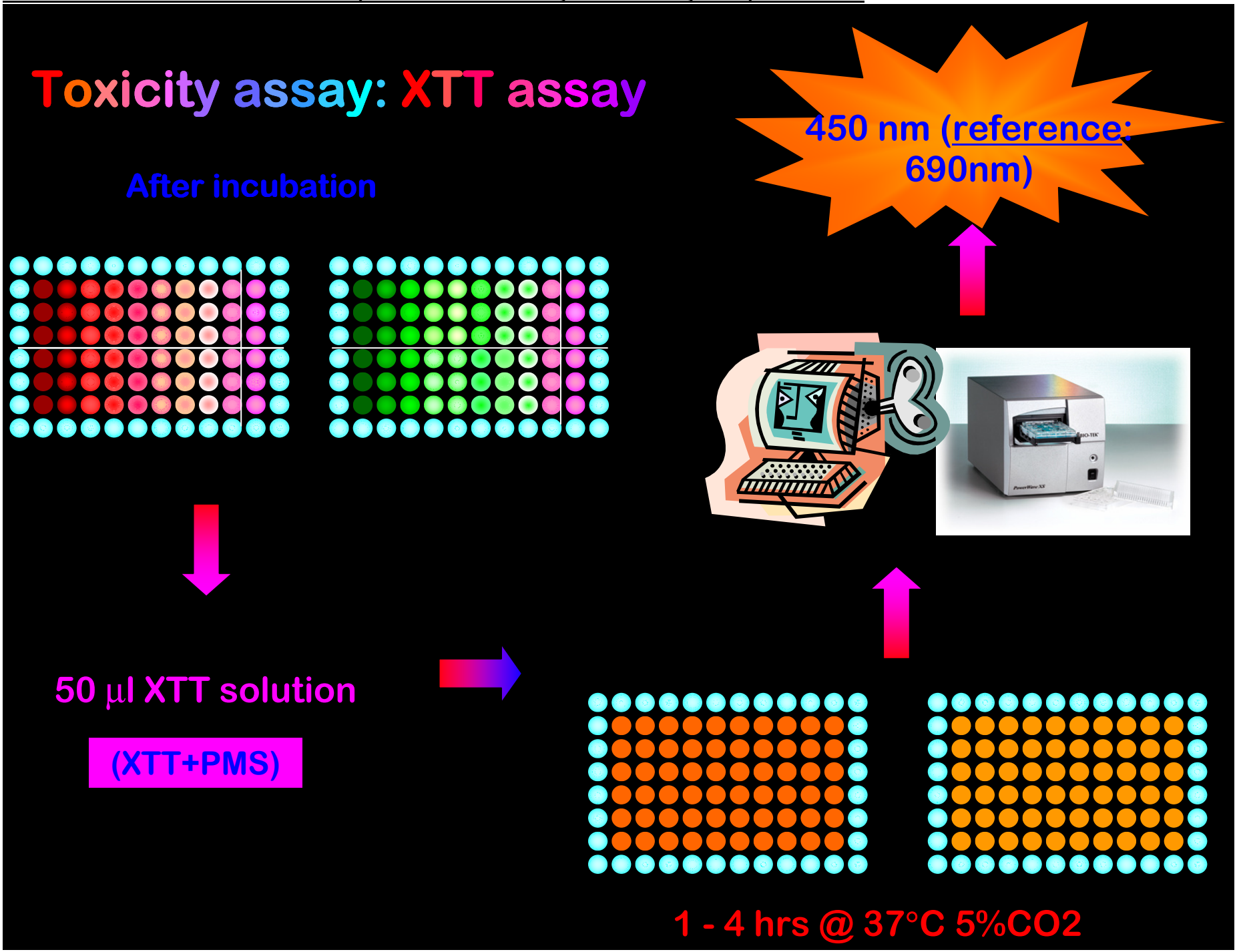


Figure 3.14 Schematic representation of the XTT assay

3.12 Results

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

	MCF-7	HeLa	SNO	DU145	Vero
Plant extracts	**IC_{50} (µg/ml) ± SD**	**IC_{50} (µg/ml) ± SD**	**IC_{50} (µg/ml) ± SD**	**IC_{50} (µg/ml) ± SD**	**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

Abid-Essefi S., Ouanes, Z., Hassen, W., Baudrimont, I., Creppy, E., Bacha, H. (2004) Cytotoxicity, inhibition of DNA and protein syntheses and oxidative damage in cultured cells exposed to zearalenone. Toxicology in Vitro. 18(4): 467-474.

Áurea Cruz Costa, M., Lopes, M.H., Margarida, I.P., Ferreira, M.A. & Alves, A.C. (1976) Naphthoquinones and triterpenoids of *Euclea divinorum*. Phytochemistry 15: 829.

Babu, B.H., Shylesh, B.S., Padikkala, J. (2002) Tumour reducing and anticarcinogenic activity of *Acanthus ilicifolius* in mice. *Journal of Ethnopharmacology*. 79: 27-33.

Babu, T.D., Kuttan, G., Padikkala, J. (1995) Cytotoxicity and anti-tumour properties of certain taxa of Umbelliferae with special reference to *Centella asiatica* (L.) Urban. *Journal of Ethnopharmacology*. 48: 53-57.

Blanco-Molina, M., Tron, G.C., Macho, A., Lucena, C., Calzado, M.A., Munoz, E., Appendino, G. (2001) Ingenol esters induce apoptosis in Jurkart cells through an AP-1 and NF-KB independent pathway. Chemistry & Biology. 8: 767-778.

Bungu, L., Frost, C.L., Brauns, S.C., Van de Venter, M. (2006) *Tulbaghia violacea* inhibits growth and induces apoptosis in cancer cells *in vitro*. African Journal of Biotechnology. 5(20): 1936-1943.

Damjanović, B., Lepojević, Ž., Živković, V., Tolić, A. (2005) Extraction of fennel (*Foeniculum vulgare* Mill.) seeds with supercritical CO_2: Comparison with hydrodistillation. Food Chemistry. 92: 143-149.

Drewes, S.E. & Khan, F. (2004) The African potato (*Hypoxis hemerocallidea*): a chemical-historical perspective. South African Journal of Science. 100: 425-430.

Dyer, R.A., Codd, L.E. & Rycroft, H.B. (1963) Flora of South Africa. 26: 80-99.

Fennell, C.W., Lindsy, K.L., McGaw, L.J., Sparg, S.G., Stafford, G.I., Elgorashi, E.E., Grace, O.M., van Staden, J. (2004) Assessing African Medicinal plants for efficacy and safety: pharmacological screening and toxicology. Journal of ethnopharmacology. 94:205-217.

Ferreira, M.A., Áurea Cruz Costa, M., Alves, A.C. & Lopes, M.H. (1973) Euclein: A new binaphthoquinones from *Euclea pseudebenus*. Phytochemistry. 12: 433-435.

Ferreira, M.A., Áurea Cruz Costa, M., Alves, A. & Lopes, M.H. (1974) Naphthoquinones from *Euclea pseudebenus*. Phytochemistry. 13: 1587-1589.

Hagg, M., Biven, K., Ueno, T., Rydlander, L., Bjorklund, P., Wiman, K.G., Shoshan, M., Linder, S. (2002) A novel high-through-put assay fo screening of pro-apoptotic drugs. Investigational New Drugs. 20(3): 253-259.

Hutchinson, C.R., Colombo, A.L. (1999) Genetic engineering of doxorubicin production in *Streptomyces peucetius*: a review. Journal of Industrial Microbiology & Biotechnology. 23: 647-652.

Jakupovic, J., Kuhnke, J., Schuster, A., Metwally, M.A., Bohlmann, F. (1988) Glaucolides and guaianolides from *Artemisia afera*. Phytochemistry. 27: 1129-1134.

Javidnia, K., Dastgheib, L., Mohammadi Samani, S., Nasiri, A. (2003) Antihirsutism activity of fennel (fruits of *Foeniculum vulgare*) extract a double-blind placebo controlled study. Phytomedicine. 10: 455-458.

Khan, M.R. (1985) Isolation of 4, 8-Dihydroxy-6-methyl-1-tetralone from the rootbark of *Euclea natalensis*. Planta Medica. 6: 356.

Kim, I.H., Son, H.Y., Cho, S.W., Ha, C.S., Kang, B.H. (2003) Zearalenone induces male germ cell apoptosis in rats. *Toxicol. Lett.* 138: 185–192.

Kitajima, J., Ishikawa, T., Tanaka, Y. (1998a) Water-soluble constituents of fennel. I. Alkyl glycosides. Chem. Pharm. Bull. 46(10): 1643-1646.

Kitajima, J., Ishikawa, T., Tanaka, Y. (1998b) Water-soluble constituents of fennel. II. Four *erythro*-Anethole glycol glycosides and two *p*-hydroxyphenylpropylene glycol glycosides. Chem. Pharm. Bull. 46(10): 1591-1594.

Kitajima, J., Ishikawa, T., Tanaka, Y., Ono, M., Ito, Y., Nohara, T. (1998c) Water-soluble constituents of fennel. V. Glycosides of aromatic compounds. Chem. Pharm. Bull. 46(10): 1587-1590.

Kitajima, J., Ishikawa, T., Tanaka, Y., Ida, Y. (1999a) Constituents of fennel. X. New Chromanone and phenylethanoid glycosides, and *theo*-epoxyanethole. Chem. Pharm. Bull. 47(10): 1448-1450.

Kitajima Kitajima, J., Ishikawa, T., Tanaka, Y., Ida, Y. (1999b) Water-soluble constituents of fennel. IX. Glucides and nucleosides. Chem. Pharm. Bull. 47(7): 988-992.

Mahomed, I.M., Ojewole, J.A. (2003) Hypoglycemic effect of hypoxis hemerocallidea corm (African potato) aqueous extract in rats. Methods find exp clin pharmacol. 25:617-623.

Mills, E., Cooper, C., Seely, D., Kanfer, I. (2005) African herbal medicines in the treatment of HIV: *Hypoxis* and Sutherlandia. An overview of evidence and pharmacology. Nutrition journal. 4:19-24.

Momtaz, S. (2007) Plant-derived multifunctional actives for skin hyperpigmentation from *Ceratonia siliqua* (l.) and *Sideroxylon inerme* (L.). MSc thesis, University of Pretoria, South Africa.

Montoya, J., Varela-Ramirez, A., Shanmugasundram, M., Martinez, L.E., Primm, T.P., aguilera, R.J. (2005) Tandem screening of toxic compounds on GFP-labeled

bacteria and cancer cells in microtiter plates. Biochemical and biophysical research communications. 335:367-372

Motoki, Y., Fujita, S., Fujihara, Y., Okada, Y., Nomura, M. (2003) Skin whitening effects of estragole derivatives. Journal of oleo science. 52(9): 495-498.

Mukinda J.T. (2005) Acute and chronic toxicity of the flavonoid- containingplant, Artemisia afra in rodents. University of the Western Cape Press. Western Cape, South Africa.

Nakagawa, Y., Suzuki, T. (2003) Cytotoxic and xenoestrogenic effects via biotransformation of *trans*-anethole on isolated rat hepatocytes and cultured MCF-7 human breast cancer cells. Biochemical Pharmacology. 66: 63-73.

O'Brien, P.J. (1991) Molecular mechanisms of quinine cytotoxicity. Chemico-biological interactions. 80(1): 1-41.

Ojewole, J.A.O (2002) Antiinflammatory properties of *Hypoxis hemerocallidea* corm (african potato) extracts in rats. Methods Find Exp Clin Pharmacol. Methods and Findings in Experimental and Clinical Pharmacology. 24(10): 685–687

Oktay, M., Gülcin, I., Küfrevioğli, Ö.I. (2003) Determination of in vitro antioxidant activity of fennel (*Foeniculum vulgare*) seed extracts. Lebensm.-Wiss. U.-Technol. 36: 263-271.

Opferkuch, H.J., Hecker, E. (1973) Ingol-a new macrocyclic diterpene alcohol from Euphorbia ingens. Tetrahedron Letters. 37: 3611-3614.

Opferkuch, H.J., Hecker, E. (1982) On the active principles of the spurge family (Euphorbiaceae). IV. Skin irritant and tumour promoting diterpene esters from *Euphorbia ingens* E.Mey. J Cancer Res Clin Oncol. 103(3): 255-268.

Palgrave, K.C. (1981) Trees of Southern Africa. Struik Publishers. Cape Town, South Africa.

Palgrave, K.C. (1991) Trees of Southern Africa. Struik Publishers. Cape Town, South Africa.

Parejo, I., Viladomat, F., Bastida, J., Schmeda-Hirschmann, G., Burillo, J., Codina, C. (2004) Bioguided isolation and identification of the nonvolatile antioxidant compounds from fennel (*Foeniculum vulgare* Mill.) waste. Journal of Agricultural and Food Chemistry. 52: 1890-1897.

Powis, G. (1987) Metabolism and reactions of quinonoid anticancer agents. Pharmacology & therapeutics. 35: 57-162.

Pujol, J. (1990) The herbalist handbook. Natur Africa Jean Pujol Natural healers Foundation, Durban. p: 40-57.

Puelo, M.A. (1980) Fennel and anise as estrogenic agent. Journal of Ethnopharmacology. 2: 337-344.

Punturee, K., Wild, C.P., Kasinrerk, W., Vinitketkumnuen, U. (2005) Immunomodulatory activities of *Centella asiatica* and *Rhinacanthus nasutus* extracts. *Asian Pacific Journal of Cancer Prevention.* 6: 396-400.

Rabie, A. (2005) Bioactivity of extracts and compounds of *Pteleopsis myrtifolia.* PhD thesis, University of Pretoria, South Africa.

Roche (2005) Cell Proliferation Kit II (XTT) Colorimetric assay (XTT based) for the non-radioactive quantification of cell proliferation and viability. Version August 2005.

Schiafella, F., Fratini, L., Mezzetti, T. & Bellavita, V. (1975) Pentacyclic triterpenoids of *Euclea natalensis*. Phytochemistry. 14: 584-585.

Schmidt, E., Lötter, M. & McCleland, W. (2002) Trees and shrubs of Mpumalanga and Kruger National Park. Jacana Johannesburg, South Africa .

Singh, G., Maurya, S., De Lampasona, M.P., Catalan, C. (2006) Chemical constituents, antifungal and antioxidative potential of *Foeniculum vulgare* volatile oil and its acetone extract. Food control. 17: 745-752.

Singh, Y. (1999) Hypoxis: yellow star of horticulture, folk remedies and conventional medicine. Veld & flora. 85:123-125.

Scott, G., Springfield, E.P., Coldrey, N. (2004) A pharmacognostical study of 26 South African plant species used as traditional medicines. Pharmaceutical biology. 42(3): 186-213.

Smith, B.J., Albrecht, C.F., Liebenberg, R.W., Kruger, P.B., Freestone, M., Gouws, L., Theron, E., Bouic, P.J., Etsebeth, S., van Jaarsveld, P.P. (1995) A phase I trial of hypoxoside as an oral prodrug for cancer therapy-absence of toxicity. S Afr Med j. 85:865-870.

Somdyala, N.I., Marassas, W.F., Venter, F.S., Vismer, H.F., Gelderblom, W.C., Swanevelder, S.A. (2003) Cancer patterns in four districts of the Transkei region-1991-1995. S Afr Med J. 93(2):144-148.

Steenkamp, V., Gouws, M.C. (2006) Cytotoxicity of six South African medicinal plant extracts used in the treatment of cancer. South African journal of botany, 72: 630-633.

Stopper, H., Schmitt, E., Kobras, K. (2005) Genotoxicity of phytoestrogens. Fundamental and molecular mechanisms of mutagenesis. 574:139-155.

Taylor, J.L.S., Rabe, T., McGaw, L.J., Jäger, A.K. & Van Staden, J. (2001) Towards the science validation of traditional medicinal plants. Plant Growth Regulation. 34: p 23-37.

Upadhyay, R.R., Hecker, E. (1975) Diterpene esters of the irritant and cocarcinogenic latex of *Euphorbia lacteal.* Phytochemistry. 14: 2514-2516.

Van der Vijver, L.M. & Gerritsma, K.W. (1973) Naphthoquinones of *Euclea* and *Diospyros* species. Phytochemistry. 12: 230-231.

Van der Vijver, L.M. & Gerritsma, K.W. (1974) Naphthoquinones of *Euclea* and *Diospyros* species. Phytochemistry.13: 2322-2323.

Van Wyk, B.E. (2005) Food plants of the world. Briza publications, Pretoria, South Africa.

Van Wyk, B.E. & Gericke, N. (2000) People's plants: A guide to useful plants of Southern Africa. Briza publications, Pretoria, South Africa.

Van Wyk, B.E, Van Oudtshoorn, B. & Gericke, N. (1997) Medicinal plants of South Africa. Briza publications, Pretoria, South Africa.

Van Wyk, B.E & Van Wyk, P. (1997) Field guide to trees of southern Africa. Struik Publishers, Cape Town, South Africa.

Vincent, P.L., Folk, W. R., Johnson, Q. (2006) TICIPS and Medicinal Plant research in South Africa and the USA. University of Missouri-Columbia, Columbia, MO, USA and University of the Western Cape, Bellville, Republic of South Africa.

Watt, J.M., Breyer-Brandwijk, M.G. (1962) The medicinal and poisonous plants of Southern and Eastern Africa. 2nd edition. Livingstone, London.

Wilt, T.J., Ishani, A., Rutks, I., MacDonald, R. (2000) Phytotherapy for benign prostatic hyperplasia. Public health Nutr. 3:459-472.

Yong, S.K., Won, G.C., Won, J.K., Woo, K.K., Myong, J.K., Won, H.K., Chang, M.K. (2002) Antimicrobial constituents of *Foeniculum vulgare*. Archives of Pharmacal Research. 25(2): 154-157.

Yoshida, M., Fuchigami, M., Nagao, T., Okabe, H., Matsunaga, K., Takata, J., Karube, Y., Tsuchihashi, R., Kinjo, J., Mihashi, K., Fujioka, T. (2005) Antiproliferative constituents from Umbelliferae plants VII. Active triterpenes and rosmarinic acid from *Centella asiatica*. *Biol. Pharm. Bull.*28(1):173-175.

Young, R.C., Ozols, R.F., Myers, C.E. (1981) The anthracycline antineoplastic drugs. The New England journal of medicine. 305: 139-153.

Zechmeister, K., Brandl, F., Hoppe, W. (1970) Structure of the new tetracyclic diterpene ingenol-triacetate with triple product methods. Tetrahedron Letters. 47: 4075-4078.

http://www.cancer.org/docroot/CDG/content/CDG_doxorubicin_hydrochloride