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Evaluating the anticancer and antioxidant activity of Southern African plants and their mechanism of action

By

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Department of Plant Sciences

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Dedication

To my husband, Wade Twilley, for all his love and support,

His guidance and motivation and all his patience throughout my studies.

I would not have been able to do it without him.

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List of Abbreviations

µg: Micrograms

µm: Micrometer

A375: Human melanoma cell line

A431: Epidermoid carcinoma cell line

A549: Human lung carcinoma

ABTS˙: 2,2-azinobis (3-ethyl-benzothiazoline-6-sulfonate)

AIDS: Acquired Immune Deficiency Syndrome

AP-1: Activation protein

ATCC: American Type Culture Collection

ATP: Adenosine Triphosphate

B16F10: Mouse skin melanoma cells

BCC: Basal Cell Carcinoma

BECN1: Beclin 1

Bp: Base pair

CANSA: The Cancer Association of South Africa

CD-36: Cluster of Differentiation 36

CDC: Centre for Disease Control

Chang: Liver cells

CHO: Chinese Hamster Ovarian cells

CO_2: Carbon dioxide

COX-1: Cyclooxygenase-1

CTL: Cytotoxic T lymphocytes

DCM: Dichloromethane

DES: Diethylstilbestrol

dH_2O: Distilled water

DISC: Death-Inducing Signaling Complexes

DLD-1: Human colorectal adenocarcinoma

DMEM: Dulbecco's modified eagle's medium

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

DPPH: 2,2-Diphenyl-2-picryl hydrazyl

ECACC: European Collection of Cell Cultures

EDTA: Ethylenediaminetetra acetic acid

EMEM: Eagles minimum essential medium

FBS: Fetal Bovine Serum

FIC: Fractional inhibitory concentration

G0 phase: Ga 0 phase where no mitosis takes place in the cell cycle

G1 Phase: Gap 1 phase where cell growth takes place

G2 phase: Gap 2 phase where cell growth takes place

Graham: Human kidney epithelial cells

GS: Growth signals

H_2O_2: Hydrogen peroxide

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HCT116: Colon carcinoma

HEK-293: Human embryonic kidney cells

HeLa: Cervical adenocarcinoma

Hep2: Larynx carcinoma cells

HepG2: Liver carcinoma cells

HIV: Human Immunodeficiency Virus

HPV: Human Papillomavirus

IAPs: Inhibitor of Apoptosis proteins

IC_{50}: Fifty percent inhibitory concentration

IFN: Interferon

IGF: Insulin-like growth factor

IGF-R: Insulin-like growth factor receptor

IL: Interleukin

IL-R: Interleukin receptor

Jak2: Janus kinase 2

KB: Human nasopharyngeal carcinoma

LC_{50}/LD_{50}: Fifty percent leathal dose

LEEP: Loop electrosurgical excision procedure

LNCaP: Human prostate cancer

M phase: Mitosis phase

MCF-7: Breast cancer cells

MEL2: Human melanoma cell line

MeOH: Methanol

ml: Mililitres

MRC: Medical Research Council

NCI: National Cancer Institute

NF-κB: Nuclear factor kappa B

NK: Natural Killer cells

$NO^{\cdot}$: Nitric oxide

NSCLC-N6: Huma non-small cell bronchopulmonary carcinoma

$O_2^{\cdot}$: Superoxide free radical

$OH^{\cdot}$: Hydroxyl free radical

$ONOO^{\cdot}$: Peroxynitrite

P-388: Murine lymphocytic leukemia

PARP: Poly-ADP-ribose polymerase

PBMCs: Peripheral blood mononuclear cells

PI3K: Phosphatidylinositol-3-kinase

PMA: Phorbol-12-myristate 13-acetate

PMS: N-Methylphenazonium methyl sulphate

pRb: Rentinobalstoma protein

PS: Phosphotidylserine

Raw 264.7: Mouse macrophages

RNA: Ribonucleic acid

ROOH: Lipid hydroperoxide

ROS: Reactive oxygen species

RSA: Republic of South Africa

S phase: Synthesis phase

SA: South Africa

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SCC: Squamous Cell Carcinoma

SF-268: Glioblastoma cells

SI: Selectivity Index

SMAC: Second mitochondrial activator of caspases

STATs: Signal transducers and activators of transcription

T24: Bladder cancer cells

TGI: Total growth inhibition

Th1: Type 1 T Helper

TK10: Renal cancer

TMB: tetramethylbenzidine

TNF: Tumour Necrosis Factor

TNF-R: Tumour Necrosis Factor receptor

Tyk2: Tyrosinase kinase 2

USA: United States of America

US: United States

U373: Human glioblastoma

U937: Histiocytic lymphoma cells

UACC62: Melanoma cells

UK: United Kingdom

UV: Ultraviolet

Vero: African green monkey kidney cells

WHO: World Health Orginization

WS1: Human fibroblasts

XTT: Sodium 3’-[1-(phenyl amino-carbonyl)-3,4-tetrazolium]-bis-[4-methoxy-6-nitro] benzene sulfonic acid hydrate

ZR-75-1: Human breast cancer

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Abstract

Ethanol extracts of twenty Southern African plants were prepared and tested for their *in vitro* cytotoxic activity against various cancerous and non-cancerous cell lines. The cancerous cell lines included human melanoma (A375), epidermoid carcinoma (A431) and adenocarcinoma cervical cancer (HeLa) and the non-cancerous cell line was human embryonic kidney cells (HEK-293). The plants studied were selected based on their traditional usage for the treatment of cancer and various skin disorders which could lead to the development of skin and cervical cancer.

The twenty plant extracts were also tested for their chemo-preventive activity by using the DPPH (2,2-Diphenyl-2-picryl hydrazyl) assay and the nitric oxide (NO) scavenging assay. All the extracts showed dose-dependent curves which were used to calculate the fifty percent inhibitory concentrations (IC_{50}). The *Syzygium jambos* extract showed the most promising radical scavenging capacity (DPPH) with activity even greater than that of the positive control, Vitamin C, with an IC_{50} value of 1.17±0.26µg/ml. *Rapanea melanophloeos* showed the greatest potential in the nitric oxide scavenging assay with activity also greater than that of Vitamin C with an IC_{50} value of 63.73±0.4µg/ml.

The cytotoxicity of the plant extracts was measured using the Sodium 3'-[1-(phenyl amino-carbonyl)-3,4-tetrazolium]-bis-[4-methoxy-6-nitro] benzene sulfonic acid hydrate (XTT) colorimetric assay at concentrations ranging between 3.125µg/ml to 400µg/ml. The *Helichrysum odoratissimum* extract showed the highest cytotoxic activity with pronounced effects against the A431 cell line. The dose-dependent studies revealed an IC_{50} value of 15.5±0.2µg/ml on the A431 cell line and relative toxicity on the non-cancerous cells with an IC_{50} value of 37.1±4.8µg/ml.

Synergistic studies on the active plant extracts were also performed to determine whether a greater toxicity could be obtained against the A431 cell line. The *H. odoratissimum* extract and the *S. jambos* extract were tested in combination at various concentrations and ratios. At the most active ratio (9:1) an IC_{50} value of 19.53±0.4µg/ml was obtained which was determined to be non-interactive. As a result of the extracts being non-interactive all morphological and cytokine studies were performed on *H. odoratissimum* only.

Light microscopy, where haematoxylin and eosin staining was used to evaluate morphological changes in the A431 cell line when exposed to various concentrations of *H. odoratissimum* extract. Morphological changes included signs of condensed and fragmented nucleus, the formation of

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apoptotic bodies, cell shrinkage and cellular debris. These observations suggested an increase in morphological changes associated with apoptosis.

H. odoratissimum was also evaluated for its cytokine production or suppression in phorbol-12-myristate 13-acetate (PMA) stimulated U937 cells. The U937 cells were exposed to various concentrations of *H. odoratissimum* extract to determine whether there was an increase in IL-12 production or IL-8 suppression. It was noted that the extract was able to increase the production of IL-12 and decrease the production of IL-8 in U937 cells.

The antioxidant activity of the twenty plant extracts based on NO scaveniging activity has been reported for the first time in this study, whereas based on the DPPH scavenging activity, a few plant extracts have been reported earlier by other researchers. Furthermore, many of the plant extracts have been reported for the first time in this study for cytotoxicity against A431, A375 and HeLa cells. *H. odoratissimum* has been reported for the first time as a possible anti-cancer agent on A431 cells and therefore, a provisional South African patent has been filed.

Future consideration would be to perform other mechanistic studies which would give more possible answers on whether *H. odoratissimum* is inhibiting specific enzymes which would inhibit cancer cell growth, such as COX-2 and SPHk1, as well as techniques such as raman and infrared spectroscopy. Furthermore would be to consider bio-assay guided fractionation of *H. odoratissimum* to determine the active compounds which are responsible for the anti-cancer activity on A431 cells.

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Chapter 1

Literature review

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1. Traditional medicine

1.1 Worldwide

The World Health Organization (WHO) has estimated that 65-80% of the world's population in developing countries depends primarily on plants for their health care needs. This is mainly due to the inaccessibility of poor income countries to modern western medicines (Tag *et al.*, 2012). Furthermore it is estimated that 35,000 to 70,000 plant species around the world have been used for their medicinal value. This value is equivalent to 14-28% of the 250,000 plant species found around the world. Evidence based on the use of plants as a source of medicine was first discovered approximately 60,000 years ago in a cave in Iraq which was inhabited by early humans (Padulosi *et al.*, 2002). These early plant medicines were taken in the form of crude extracts such as tinctures, teas, poultices and powders. A more modern way of using plants as a source of medicine involves the isolation of active compounds such as morphine, codeine and quinine (Balunas & Kinghorn, 2005).

Globally there are over 50 major drugs which have been derived from tropical plants which make medicinal plants an important source of income. In India the export ratio of medicinal plants has increased three-fold from 33,000t in the period 1992-1995, which is equivalent to US$ 46 million. In the period 2001-2002 approximately one quarter of the world's best selling drugs were derived from natural products. Currently four new plant derived drugs have been introduced into the US market (Table 1.1).

Table 1.1: Currently introduced plant-derived drugs (Balunas & Kinghorn, 2005)

Common name	Trade name	Plant source	Active compound/target area	Disease used for
Arteether	Artemotil®	*Artemisia annua* L.	Sesquiterpene lactone	Anti-malarial
Galantamine	Reminyl®	*Galanthus woronowii* Losinsk.	Acetylcholinesteras and nicotinic acetylcholinesterase receptor	Alzheimers
Nitisinone	Orfadin®	*Callistemon citrinus* Stapf.	4-hydroxyphenylpyruvate dehydrogenase enzyme	Tyrosinaemia
Tiotropium	Spiriva®	*Atropa belladonna* L.	Tiotropium bromide monhydrate	Chronic obstructive pulmonary disease

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1.2 South Africa

In South Africa traditional herbal medicine is the primary source of health care for rural areas with an estimate of approximately 27 million South Africans still relying on traditional medicine (Street *et al*., 2008). Traditional medicine is most prevalent in areas where Western health care is inaccessible or higher in cost to traditional medicines. However the main reason for the high usage of traditional medicine is due to the cultural importance (McGaw *et al*., 2005). Due to South Africa's large plant diversity it has been estimated that approximately 3000 plant species are used for their medicinal purposes (Light *et al*., 2005).

1.3 South African medicinal plants and their efficacy

Most of the South African medicinal plants are collected from the wild which negatively impacts the medicinal plant biodiversity and can lead to the contamination of water sources. Another major problem is industrial and mining waste material, which can lead to the contamination of medicinal plants. Furthermore, there is currently a large amount of polluted water and sewage sludge being dumped onto South African soils which further contaminates plant material. Another major source of contamination comes from plant materials which are contaminated with pesticides, heavy metals and other toxic substances. Possibly one of the biggest safety concerns with regard to the use of medicinal plants is that often misidentification occurs which could lead to poisoning (Street *et al*., 2008).

There are factors which also contribute to the quality of the medicinal plant. Such factors include genetic, ecological and environmental differences of where the plants are grown, which in turn affects the bioactivity of the compounds. Inconsistency of plant material is also found when the maturity of the tree is not taken into account as well as the season and geographical region. Furthermore, the drying process and storage of the collected plant has an impact on efficacy and quality (Street *et al*., 2008).

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2. Problem statement

2.1 Worldwide

Cancer is one of the leading causes of death worldwide. In developed countries cancer is the leading cause of death, whereas in developing countries it is the second leading cause. The prevalence of cancer is increasing in developing countries due to an increase in population and general aging of the population as well as unhealthy lifestyles such as smoking, physical inactivity and unhealthy diets. Statistics based on GLOBOCAN 2008 estimates that approximately 12.7 million cancer cases and 7.6 million cancer deaths occurred worldwide in 2008, of these cancer cases approximately 56% of cases and 64% of deaths occurred in the economically developing world (Fig 1.1) (Jermal *et al*., 2011).

The most frequently diagnosed cancer and the leading cause of cancer deaths around the world, in both developing and developed countries, is breast cancer in females and lung cancer in males. The second most common cancer in females in developing countries is cervical cancer, whereas in males it is stomach and liver cancer. However, in developed countries the second leading cancer in females is colorectal cancer, whereas in males it is colorectal and prostate cancer (Jermal *et al*., 2011).

Cervical cancer is most commonly diagnosed in southern Africa and Central America. Every one in ten females diagnosed with cancer is diagnosed with cervical cancer. Worldwide cervical cancer accounts for approximately 9% of female cancer deaths each year which equates to approximatly 273,000 deaths (Cancer Research UK, 2005).

Skin cancer, which includes non-melanoma and melanoma type cancers, has increased over the past ten years. Worldwide there is an estimate of 2-3 million non-melanoma skin cancer cases and 132,000 melanoma cases. It has further been estimated that one in every three cancers diagnoses is a type of skin cancer (WHO, 2012).

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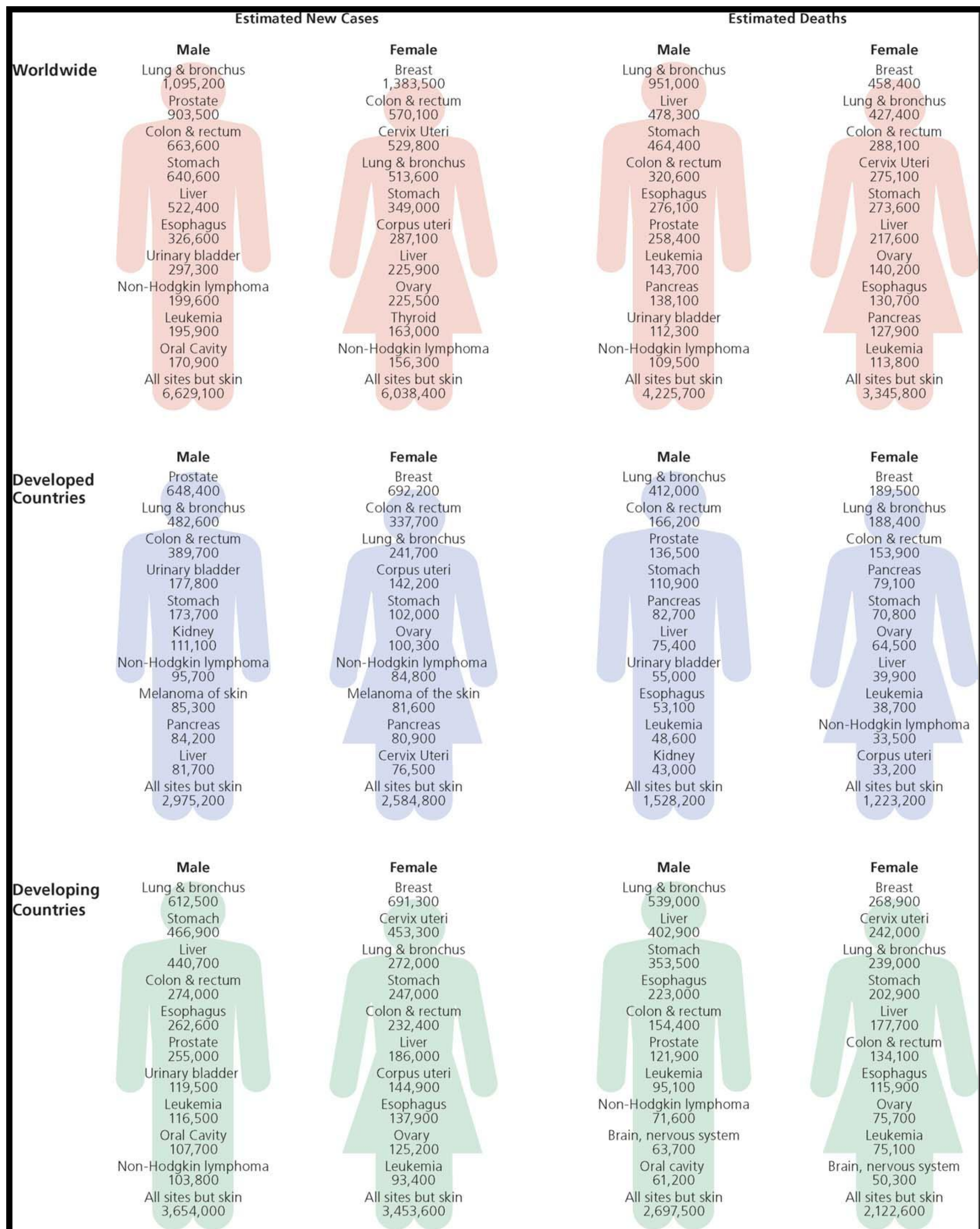

Figure 1.1: Estimated cancer cases and cancer deaths in developing and developed countries (Medical Books Online, 2012)

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2.2 South Africa

The National Cancer Registry report of 2000-2001 estimates that males have an average lifetime risk of one in six for being diagnosed with cancer, whereas females have a lifetime risk of one in eight (Fig 1.2). In South Africa, the predominant types of cancer in males are prostate (1 in 23), lung (1 in 69), oesophagus (1 in 82), colon/rectum (1 in 97) and bladder cancer (1 in 108). The predominant cancer in females is breast (1 in 25), cervical (1 in 35), uterus (1 in 144), colorectal (1 in 162) and oesophagus (1 in 196) (CANSA, 2012).

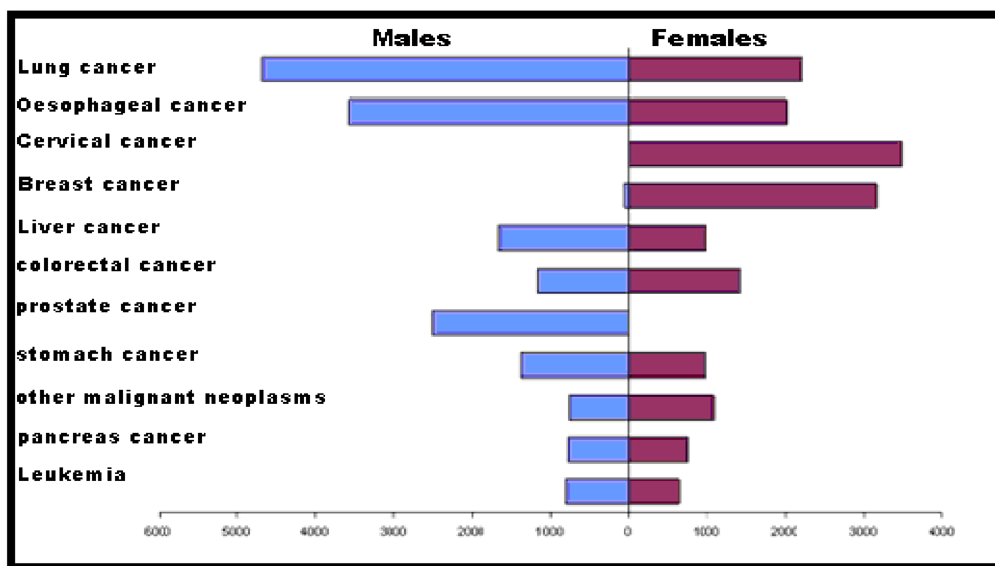

Figure 1.2: Number of deaths caused by cancer in South Africa, 2000 (MRC SA, 2010)

In South Africa skin cancer is the most common type of cancer. It consists of Basal Cell Carcinoma (BCC), Squamous Cell Carcinoma (SCC) and malignant melanoma. Each year approximately 20,000 new cases of skin cancer are reported and 700 result in death from malignant melanoma (CANSA, 2012).

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3. Cancer

3.1 Carcinogenesis

Cancer is referred to as a malignant disease that can affect many different parts of the body in many different ways depending on the area of infection. Cancer cells have the characteristic of being able to grow uncontrollably and to form abnormal cells. These rapid proliferating cells can mass together to form a tumour, called benign tumours. These tumours can surgically be removed, or they can proliferate and spread throughout the body to cause abnormal cell growth in other parts of the body. If these malignant tumours are not arrested they can be fatal (Hoffman, 2003).

Cancer is defined by its complex mechanism of differentiation and abnormal growth which is known as carcinogenesis. Carcinogenesis is the process that leads to cancer involving two different stages; initiation and promotion. Initiation is where a normal cell undergoes changes which are irreversible, whereas promotion is the stimulation of initiated cells to form cancer. This initial transformation of normal cells to abnormal cells is a result of mutations that occur in cellular genes. These mutations that lead to the development of cancer can either be inherited or can occur in somatic cells. The cellular genes play an important role in controlling and regulating the different stages and functions of the cell cycle. Mutations that occur within these cellular genes lead to the loss of suppressor genes, which is an important function in arresting abnormal cell growth. The cellular genes also play a role in the regulation of growth factors, immune supressors and hormones (Hoffman, 2003).

The cell cycle is composed of different cellular events, which lead to the division of cells (Fig 1.3). The different stages of the cell cycle include the interphase stage, where cells grow and DNA is replicated, the G1 phase where cells acquire enzymes and molecules needed for DNA replication to prepare for DNA synthesis, the S phase where chromosomal DNA is replicated, the G2 phase where cells prepare for division, and the M phase where chromosomes condense and sister chromosomes move to opposite poles for cell division to occur. There is also a G0 phase which contains metabolically active cells that do not divide. Cells in the G0 phase have the ability to re-enter the cell cycle once stimulated through external signals. Cancer cells have the ability not to respond to these signals that would normally inhibit their proliferation. It is within these various stages that the cell cycle is regulated at three different checkpoint; G1/S, G2/M and M. Mutations that occur within these control genes lead to the development of cancer due to incorrect controlling of the checkpoints which leads to the replication of damaged DNA (Klug *et al*.,2009).

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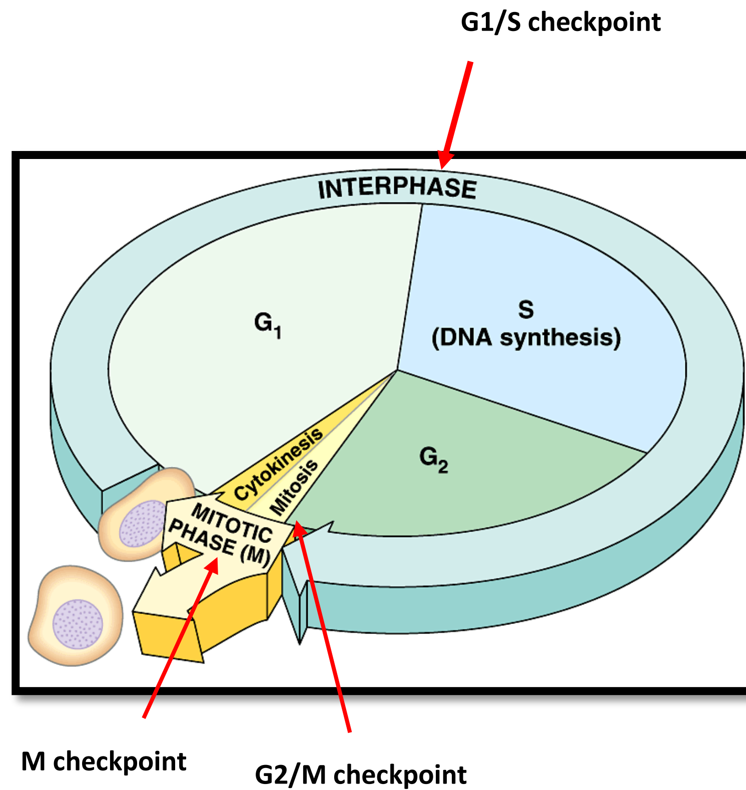

Figure 1.3: The cell cycle and its checkpoints (Kent Simmons, 2011)

3.2 Metastatic cancer

Metastatic cancer is a characteristic of cancer cells which are able to spread to distant parts of the body from the primary site of tumour formation (Fig 1.4). Tumours which are formed from these metastatic cells are known as metastatic tumours. This process by which cancer cells are spread to various parts of the body is known as metastasis. For cancer cells to spread the following steps are involved: local invasion where cancer cells invade neighbouring tissues; intravasation which involves the invasion of cancer cells in lymph vessels or blood vessels; circulation where cancer cells move through the vessels to various parts of the body; cell arrest where the cancer cells leave the bloodstream by attaching to capillary walls; tumour cell aggregation; platelet aggregation and thrombus formation. Thereafter extravasation takes place where the cancer cells invade the capillary walls and migrate into surrounding tissue. In these surrounding tissues cancer cell proliferation takes place which causes the formation of small tumours (micrometastasis). Finally this aggregation of cancer cells stimulates the growth of new blood vessels, which is known as angiogenesis. This formation of new blood vessels facilitates the supply of nutrients and oxygen to the growing tumour (National Cancer Institute (a), 2011).

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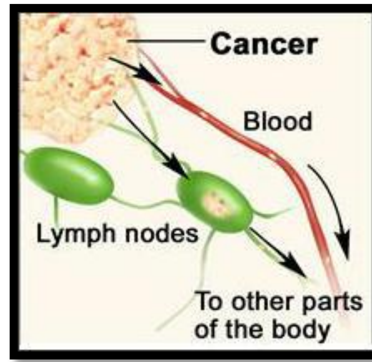

Figure 1.4: Metastasis is the ability of cancer cells to invade various parts of the body through the lymph and blood vessels (National Cancer Institute (a), 2011)

3.3 Characteristics of cancer cells

Research has indicated that the formation of tumours is a multistep process, which includes changes in genetic material causing normal human cells to become cancerous. Cancerous cells undergo six essential alterations such as: self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis as well as tissue invasion and metastasis (Hanahan & Weinberg, 2000).

3.3.1 Self-Sufficiency in Growth Signals

Normal cells move from the G0 stage (quiescent) in the cell cycle to an active proliferative state via a mitogenic growth signal (GS). The transmission of these signals into the cell occurs via transmembrane receptors that bind specific classes of signaling molecules. A normal cell can only proliferate in the presence of these stimulatory molecules. Many oncogenes in cancer cells act by copying these normal growth signals. Tumour cells differ from normal cells in that they do not solely depend on exogenous growth signals as they have the ability of generating their own growth signals. The ability of tumour cells to have acquired GS autonomy can be due to three molecular strategies. The strategies can either involve the change in extracellular growth signals, change of transcellular transducers of those signals or the alteration of intracellular mechanisms that translate those signals into action (Hanahan & Weinberg, 2000).

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3.3.2 Antigrowth Signal Insensitivity

In normal tissue there are a number of antiproliferative signals which are regulated to maintain cellular quiescence and homeostasis. The signals are normally inhibitors which are found in the extracellular matrix and on the surfaces of nearby cells. The growth-inhibitory signals are activated by transmembrane cell surface receptors which in turn activate intracellular signaling pathways. These antigrowth signals can then block proliferation by forcing cells into the G0 stage where they remain metabolically active but are unable to divide. For cancer cells to survive they need to evade these anti-proliferative signals (Hanahan & Weinberg, 2000).

Normal cells respond to anti-proliferative signals in association with the cell cycle, specifically the G1 phase and the components found within this stage. During the G1 phase, cells decide, based on external signals, whether to proliferate or whether to be sent to the G0 stage. Most of the anti-proliferative signals are processed through the retinoblastoma protein (pRb) as well as the p107 and p130 molecules. When in a hypo-phosphorylated state, the pRb molecule blocks proliferation by altering the function of E2F transcription factor, which is an essential factor that controls the expression of genes important for the progression from G1 to S phase (Weinberg, 1995). The alteration of the pRb pathway allows cell proliferation to take place and therefore the cells become insensitive to antigrowth signals (Hanahan & Weinberg, 2000).

3.3.3 Evading Apoptosis

The ability of tumor cells to expand in number is due to the rate of cell proliferation and the rate of cell death. A major pathway of cell death is apoptosis, which is a form of programmed cell death. There are many types of cancers that have acquired resistance towards apoptosis. In past observations it was noted that apoptosis is present in almost all cell types in the latent form. When apoptosis is triggered there are a number of steps that take place such as the disruption of cellular membranes, the breakdown of cytoplasmic and nuclear skeletons, the extrusion of the cytosol, degradation of the chromosomes and the fragmentation of the nucleus. The apoptotic pathway can be divided into two classes of components, the effectors and sensors. The sensors monitor the extracellular and intracellular environment to report any abnormalities and therefore decide whether a cell should live or die. These extracellular and intracellular signals furthermore regulate the effectors of apoptotic death, which include cell surface receptors which bind either death factors or survival factors (Hanahan & Weinberg, 2000). The survival signals are regulated by the IGF-1/IGF-2

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molecules through the IGF-1R and IL-3 receptors and through the IL-3R cognate receptor (Lotem & Sachs, 1996). The death signals are regulated through the binding of the FAS ligand to the FAS receptor as well as the TNFα ligand binding to the TNF-R1 receptor (Ashkenazi & Dixit, 1999). One of the most common ways of cancer cells to acquire resistance to apoptosis is through the mutation of p53 tumor suppressor gene. The inactivation of the p53 protein is observed in more than 50% of human cancers. This inactivation results in the loss of the DNA damage sensor which is responsible for inducing the apoptotic effector cascade (Hanahan & Weinberg, 2000).

3.3.4 Potential to Replicate Limitlessly

Most types of mammalian cells carry a cell-autonomous program which is important in restricting the multiplication of cells. For a macroscopic tumor to form, the cell-autonomous program must be disrupted. It has been observed that after a cell population has undergone a certain amount of doublings in number their growth is inhibited, which is known as senescence. Cancer cells tend to grow indefinitely and therefore, are characterized as immortal cells. The counting of cell generations is measured by the ends of chromosomes known as telomeres. These telomeres are composed of thousands of short 6 base pair sequence repeats. During each cell cycle the replicating generations are counted by the 50-100bp loss of telomeric DNA from the ends of the chromosomes. The gradual shortening of the DNA from the telomeres is due to the inability of DNA polymerase to completely replicate the chromosomal DNA during each S phase. The gradual loss of the telomeres therefore, causes the ends of the chromosomes to be unprotected over time. The chromosomal ends then undergo end-to-end fusion which results in karyotypic disarray and therefore, death. The maintenance of these telomeres occurs in all malignant cell types, where 85-90% of the malignant cells maintain the telomeres through upregulating the expression of the telomerase enzyme. This enzyme is responsible for adding hexanucleotide repeats to the ends of the telomeres. Another mechanism of maintenance is through the recombinant-based interchromosomal exchanges of sequence information. Through these two mechanisms malignant cells appear to maintain the length of telomeres above a critical stage and therefore, results in unlimited replication of cells. In normal human cells these two mechanisms are strongly suppressed to prevent unlimited replication form occurring (Hanahan & Weinberg, 2000).

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3.3.5 Sustained Angiogenesis

The ability of a cell to function and its survival depends on the oxygen and nutrients supplied by the vascular system. This can be ensured when all cells in a tissue remain not further than 100µm from blood capillary vessels, which is in turn ensured by the coordinated growth of vessels and parenchyma. The growth of new blood vessels (angiogenesis) is highly regulated. The process of angiogenesis is balanced by positive and negative signals, which results in either the activation or inhibition of angiogenesis. These signals can be transferred either by soluble factors and their receptors or by integrins and adhesion molecules. The initiation of angiogenesis is transmitted by vascular endothelial growth factor and acidic and basic fibroblast growth factors (FGF1/2). These growth factors bind to the tyrosinase kinase receptors on the surface of endothelial cells. An inhibitor of angiogenesis is thrombospondin-1 which binds to CD36, which is also found on endothelial cells and coupled to intracellular Src-tyrosine kinases. Angiogenesis has an essential role, as antiangiogenic substances have the ability of suppressing tumor cell growth. Tumor cells activate an “angiogenic switch” which suppresses angiogenic inhibitors and activates the angiogenic inducers, which allows for further growth and survival of tumor cells (Hanahan & Weinberg, 2000).

3.3.6 Tissue Invasion and Metastasis

In the development of most types of cancers, cells detach from the primary tumor mass and spread to adjacent tissues and thereafter to various parts of the body to form new colonies, known as metastases. These metastatic colonies cause 90% of human cancer deaths. The ability of tumor cells to invade and form new colonies allows the escape from the primary tumor mass to various parts in the body where there are sufficient nutrients and space. The formation of primary tumor masses as well as newly formed metastases depends on the previously discussed hallmarks of cancer cells (Hanahan & Weinberg, 2000).

There are many different types of cancer; as mentioned earlier, cervical cancer is one of the most commonly diagnosed cancers in the southern part of Africa and skin cancer is the most common type in South Africa, consisting of non-melanoma and melanoma types. In the present study southern African plants were tested for their effectivity in decreasing the proliferation of cervical and epidermoid cancer.

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4. Skin cancer

4.1 Biology of skin

The skin is the largest organ of the body and consists of three different layers, which each performing a different function in the body. The three layers are: the epidermis, dermis and the subcutaneous layer (fat layer) (Fig 1.5).

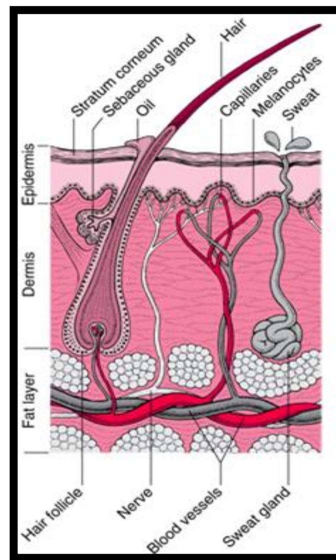

Figure 1.5: The different layers of the skin consisting of the epidermis, dermis and subcutaneous layer (The Merck Manual, 2006)

The epidermis is the tough outer layer of the skin and is relatively thin. The epidermis consists mostly of kertinocytes. These keratinocytes are produced in the basal layer of the epidermis and constantly migrate up towards the surface and replace dead skin. The outer layer of the epidermis, the stratum corneum, protects the body from invading bacteria, viruses and pathogens when it is undamaged. Furthermore the skin, with all its layers, protects the internal organs against trauma (The Merck Manual, 2006). In the basal layer of the epidermis there are cells called melanocytes, which are responsible for the production of melanin. This is a pigment which gives skin its natural colour (The Oncology Institute of Hope and Innovation a, 2007). The main function of melanin however is the ability to protect the skin from ultraviolet radiation, which is harmful to the body and can cause skin cancer through DNA damage. Furthermore, the epidermis layer contains Langerhans' cells which contribute towards the skins immune system (The Merck Manual, 2006).

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The dermis is the second layer of the skin. This is a thick layer made from collagen, elastin and fibrillin, which gives elasticity, flexibility and strength to the skin. In the dermis layer there are sweat glands, oil glands (sebaceous), nerve endings, blood vessels and hair follicles. The nerve endings are responsible for sensing pain, touch, pressure and temperature and relay this information to the brain. The sweat glands produce sweat in times of heat and stress. Furthermore the sweat helps cool the body when it evaporates off the skin. The sebaceous glands help protect hair follicles from invading pathogens and insulates against water. These glands are responsible for the secretion of sebum which is an oily substance that keeps hair soft and moist, protecting it from damaging chemicals (The Merck Manual, 2006).

The hair follicles are found throughout the body and contribute to many important roles, such as regulating body temperature enhancing sensation and protecting from injury. Furthermore, certain parts of the follicles contain stem cells which help replace damaged epidermis (The Merck Manual, 2006).

The blood vessels in the dermis play important roles such as providing nutrients to the skin and regulating body temperature. During times of heat the blood vessels will dilate allowing blood to circulate near the surface of the skin, whereas during cold times the blood vessels constrict and retain body heat (The Merck Manual, 2006).

The subcutaneous layer is the inner most layer of the skin which helps insulate the body from heat and cold. It furthermore serves as an energy storage area and its thickness varies in different regions of the body (The Merck Manual, 2006).

4.2 Types of skin cancer

Skin cancer is one of the most common forms of cancer. In most cases it is not life threatening and can be prevented if care is taken when you are exposed to the sun. There are three types of skin cancer namely basal cell carcinoma (BCC), squamous cell carcinoma (SCC) and melanoma. Basal and squamous cell carcinoma are the two most common forms and are known as the non-melanoma type skin cancers. Melanoma however is the most dangerous type of skin cancer as it has the tendency to spread rapidly throughout the body (MedicineNet.com a, 2012).

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4.2.1 Basal Cell Carcinoma

Basal Cell Carcinoma is the most common form of skin cancer and is also the least dangerous form of skin cancer as it does not have the ability to metastasize (Fig 1.6).

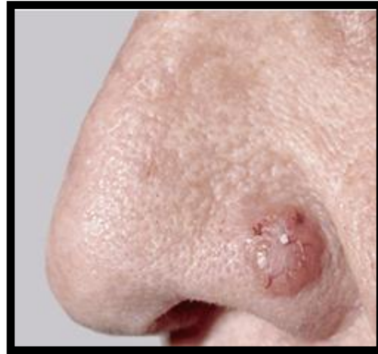

Figure 1.6: Large telangiectases forming on the nodular Basal Cell Carcinoma (MedicineNet.com a, 2012)

However, BCC can invade surrounding tissue and therefore, cause damage to it especially when it grows close to the eyes, ears or nose. BCC is characterized by the superficial blood vessels (telangiectases) that cover the surface of the carcinoma. It often forms the shape of a dome when it starts to grow and has a shiny translucent texture. BCC takes months to years to become sizable and often appear as dry patches of skin on the chest or back (MedicineNet.com a, 2012).

4.2.2 Squamous Cell Carcinoma

Squamous Cell Carcinoma is a cancer which originates in the squamous cells of the skin surface, the lining of the hollow organs of the body and in respiratory and digestive tract passages and therefore, SCC can grow in any of these areas (Fig 1.7). SCC originates as actinic keratosis which is a rough, red bump which occurs on the scalp, face, ears and hands. This early form of SCC can invade deeper into the skin and become fully developed SCC. However, appearances of thick and tender actinic keratosis can be an early diagnosis to invasive SCC. There are many types of SCC that are non-invasive. These are: keratoacanthoma, which forms a central crater in the mound; actinic cheilitis, which forms on the lips; Bowen’s disease, which appears as scaly patches on the skin and bowenoid papulosis which are warts that occur in the genital area. SCC is more dangerous than BCC as it is capable of spreading to other parts of the body (MedicineNet.com a, 2012).

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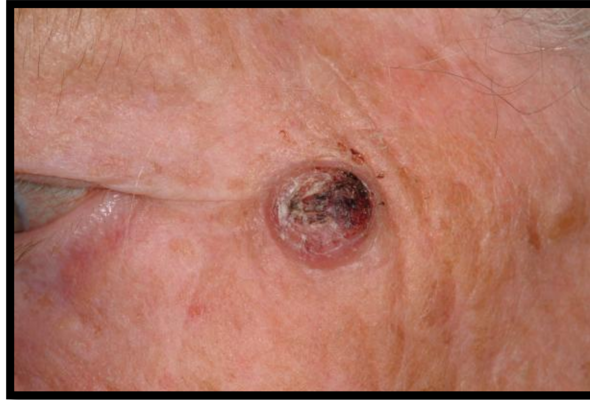

Figure 1.7: Squamous cell carcinoma appearing on sun damaged skin (From Your Doctor, 2012)

4.2.3 Melanoma

Melanoma is a type of skin cancer which forms in melanocytes. Melanocytes are skin cells which are found throughout the lower part of the epidermis (Fig 1.8). These melanocytes are responsible for the production of melanin, which is a pigment that gives skin its natural colour (The Oncology Institute of Hope and Innovation a, 2007). It is the most dangerous type of skin cancer as it has the ability to spread to various parts of the body. Often patients themselves are the first to detect melanoma as it occurs on the skin where they are able to see it, but diagnosis from a medical practitioner is still needed to confirm. The important signs to look for when a mole is present on the skin are whether it is asymmetrical, whether the border of the mole is irregular and not smooth, whether there are various colours present in the mole or the mole has changed in colour and whether the mole is growing larger in size (MedicineNet.com b, 2012).

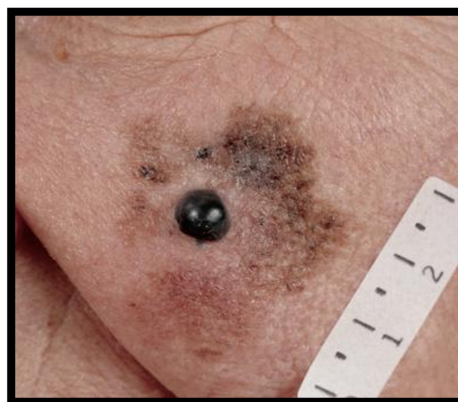

Figure 1.8: Rarer form of lentigo maligna melanoma which occurs on the face (MedicineNet.com b, 2012)

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There are three main types of melanoma; superficial spreading melanoma, which is the most common and accounts for 70% of melanoma cases. This type of melanoma occurs mainly on the legs of women and the backs of men. The mole often possesses a variety of colours, is not raised and usually takes several years to evolve. Nodular melanoma accounts for 20% of melanoma cases. They tend to evolve much faster and therefore tend to spread. Furthermore, it starts to grow as a dark blue-black to purple lump on the skin. Lentigo maligna is different from the above types in that it forms on the face due to high exposure to sun. It takes several years to evolve into a more dangerous type of melanoma. It is characterised by its large, irregular shape which could appear as a coloured freckle. There are rare forms of melanoma which can occur under the nails, on the palms and soles, in the eye and even in the body (MedicineNet.com b, 2012).

4.3 Risk factors for developing skin cancer

One of the most important factors leading to developing skin cancer is exposure to the sun. However, it often takes several years for sun damage to promote skin cancer; therefore precancerous and cancerous spots can often only develop years later in the age range of 40-50 years. Furthermore, individuals with fair-coloured skin, light coloured hair and eyes or with Caucasian ancestry have a higher risk of developing skin cancer. In the case where another type of cancer is treated with radiation there is a chance of developing basal cell carcinoma. There are often uncommon factors which could contribute to the risk of developing squamous cell carcinoma such as exposure to arsenic, hydrocarbons, heat or X-rays. Another uncommon factor includes strains of Human Papillomavirus (HPV) which causes genital warts. This can promote the growth and development of SCC in the anogenital region. A high risk factor for melanoma is the presence of close family with melanoma and the presence of many moles (more than 100) on an individual (MedicineNet.com a & b, 2012).

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4.4 Melanoma Diagnostic Indicators

4.4.1 Clark's Level

The Clark level refers to how deep the tumour has penetrated into the different layers of the skin (Fig 1.9). There are five Clark levels of invasion (The Oncology Institute of Hope and Innovation a, 2007):

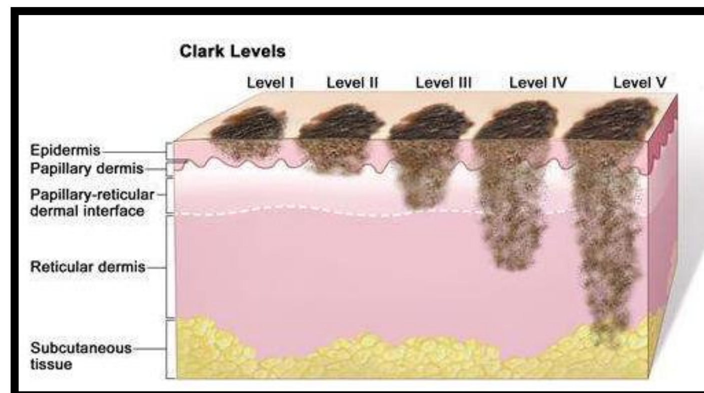

Figure 1.9: The Clark levels of Melanoma (The Oncology Institute of Hope and Innovation a, 2007)

Level I: The cancer has not spread and is only present in the epidermis of the skin, also known as "*in situ*" melanoma

Level II: The cancer has started to spread from the primary site and has entered the papillary dermis

Level III: The cancer is now spreading from the papillary dermis into the papillary-reticular dermal interface

Level IV: The cancer has made its way to the lower layer of the dermis known as the reticular dermis, but the cancer still remains in the skin

Level V: The cancer has reached the subcutaneous layer which is the first layer below the skin

4.4.2 Staging melanoma

The staging system is based on the following; the thickness of the tumour which is described by the Breslow scale, whether the tumour has broken the skin (ulceration), whether lymph nodes have been

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invaded and whether the cancer cells have spread to the other parts of the body (Fig 1.10). There are five different stages (The Oncology Institute of Hope and Innovation a, 2007).

Stage 0 ("*in situ*" melanoma): *There is a presence of abnormal melanocytes in the epidermis layer of the skin (Clark level I). The melanocytes can become cancerous and spread into tissue* (The Oncology Institute of Hope and Innovation a, 2007).

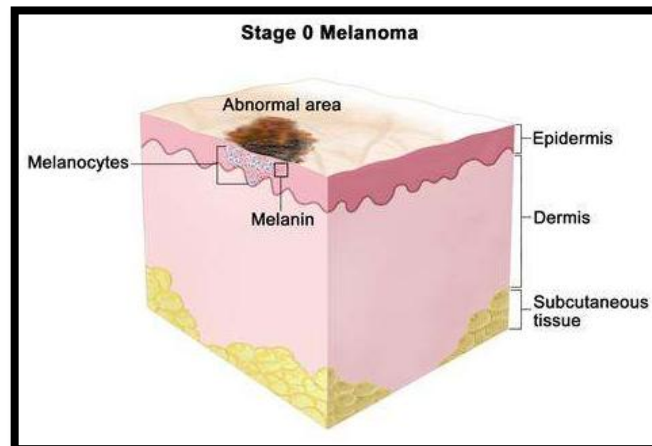

Figure 1.10: Stage 0 melanoma (The Oncology Institute of Hope and Innovation a, 2007)

Stage I melanoma: There are two types of tumours in stage I melanoma; Stage IA where the tumour is not more than 1mm thick and the skin is not broken therefore, the tumour is still present only in the epidermis and in the papillary dermis (Clark level II or III) (Fig 1.11). Stage IB can either be with ulceration where the tumour is not more than 1mm thick and therefore, the tumour has spread to the dermis or the subcutaneous layer (Clark level IV or V) or there is no ulceration and the tumour is more than 1mm thick but not more than 2mm thick *(The Oncology Institute of Hope and Innovation a, 2007).*

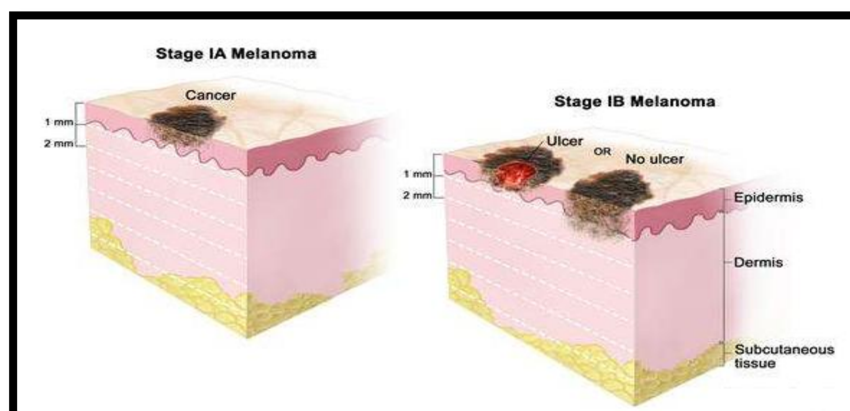

Figure 1.11: Stage IA and B melanoma (The Oncology Institute of Hope and Innovation a, 2007)

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Stage II melanoma: Stage II melanoma consists of three types of stages (Fig 1.12).

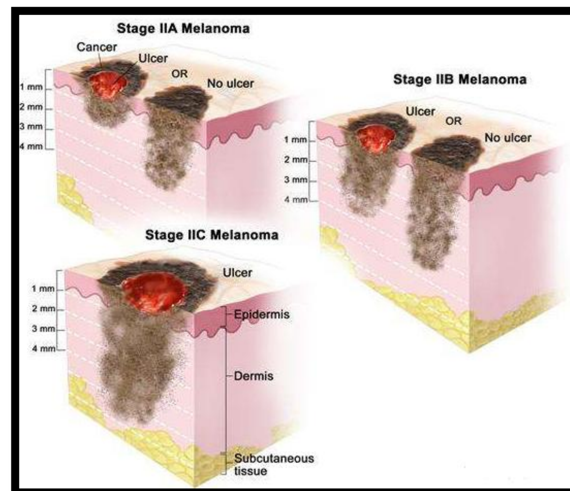

Figure 1.12: Stage IIA, B and C melanoma (The Oncology Institute of Hope and Innovation a, 2007)

Stage IIA is where the tumour is more than 1mm thick but not more than 2mm with ulceration, or more than 2mm and not more than 4mm thick without any ulceration. Stage IIB melanoma is where the tumour is either more than 2mm thick and not more than 4mm with ulceration, or more than 4mm thick with no ulceration. Stage IIC melanoma has a tumour that is more than 4mm thick and has ulceration (The Oncology Institute of Hope and Innovation a, 2007).

Stage III melanoma: In stage III melanoma the tumour may be any thickness but has spread to the lymph vessels and one or more lymph nodes (Fig 1.13) (The Oncology Institute of Hope and Innovation a, 2007).

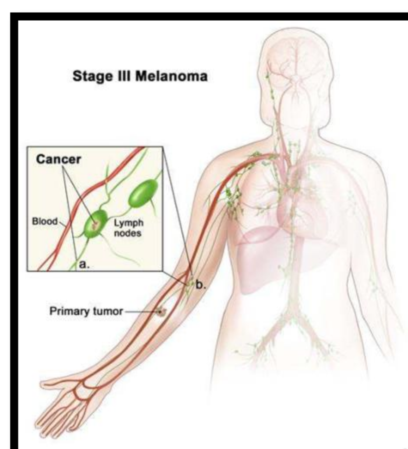

Figure 1.13: Stage III melanoma (The Oncology Institute of Hope and Innovation a, 2007)

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Stage IV melanoma: In stage IV melanoma the cancer has spread to other parts of the body (Fig 1.14). This stage of cancer is known as metastatic melanoma (The Oncology Institute of Hope and Innovation a, 2007).

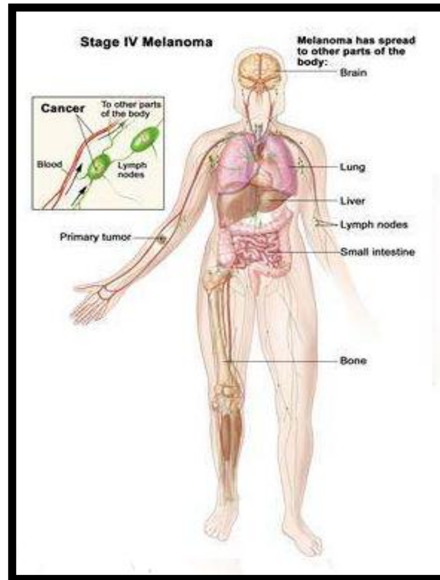

Figure 1.14: Stage IV melanoma (The Oncology Institute of Hope and Innovation a, 2007)

Recurrent melanoma: The cancer cells have recurred after the cancer was treated previously. The cancer cells can reoccur in the original site or in other parts of the body such as the lungs and liver (The Oncology Institute of Hope and Innovation b, 2007).

4.5 Prevention of skin cancer

For the prevention of all types of skin cancer the most important factor is to reduce exposure to the sun or any other harmful UV rays. Furthermore self-examination as well as physical examination by a medical practitioner is important for individuals who have a high risk of developing skin cancer (MedicineNet.com a & b, 2012).

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4.6 Conventional treatments of skin cancer

Medical practitioners have two main goals when trying to treat skin cancer; firstly to completely remove the cancer and secondly to leave as small a scar as possible. The treatment options depend mainly on the location and size of the cancer, the age of the individual, the medical history and the risk of scarring. Methods used to treat the different types of skin cancer include the following (MedicineNet.com a, 2012):

4.6.1 Curettage and desiccation

The most common method to remove basal and squamous cell carcinoma is by scooping out the carcinoma using a curette. This method is often accompanied by desiccation where an electrical current is applied to ensure that remaining cancer cells are killed. The skin often heals by itself and therefore stitches are not necessary (MedicineNet.com a, 2012).

4.6.2 Surgical excision

The tumour is cut out of the invaded area and thereafter stitched up. This is the main treatment for melanoma and in some cases the removal of nearby lymph glands is also needed (MedicineNet.com b, 2012).

4.6.3 Radiation therapy

Radiation therapy is used when skin cancer does not occur in the common areas such as the trunk, arms and legs. This type of therapy replaces surgery when skin cancer appears in places that are hard to reach (MedicineNet.com a, 2012).

4.6.4 Cryosurgery

The BCC and SCC can be treated by freezing the carcinoma using liquid nitrogen and therefore, destroying cancerous cells (MedicineNet.com a, 2012).

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4.6.5 Microscopically controlled excision

The tumour is cut from the skin in thin layers. After the removal of a small piece of tumour the layer is viewed under a microscope to see whether there are any cancer cells present. The removal of layers is continued until no more cancer cells can be seen under the microscope. This type of surgery removes as little normal tissue as possible (MedicineNet.com a, 2012).

5. Cervical cancer

5.1 Risk factors

5.1.1 HPV infection

The main risk for developing cervical cancer is caused by infection by HPV. HPV consists of approximately 100 related viruses which can cause the growth of warts on skin surfaces, genitals, anus, mouth and throat or common warts on hands, feet, lips and tongue. The HPV 6 and HPV 11 strains are responsible for causing cervical cancer and therefore, are classified as high-risk types of strains. The low risk types of strains are responsible for the common warts found on hands and feet. HPV can spread from one person to another via skin-to-skin. Therefore, contact with the body of an infected will increase the risk of developing cervical cancer (American Cancer Society, 2012).

5.1.2 Smoking

Smokers compared to non-smokers are at twice the risk of developing cervical cancer. Smoking leads to the exposure to many carcinogenic chemicals which can be absorbed into the body through the bloodstream and therefore cause damage to DNA and lead to the development of cancer (American Cancer Society, 2012).

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5.1.3 Immunosuppresion

The Human Immunodeficiency virus (HIV), which is responsible for Acquired Immune Deficiency Syndrome (AIDS), has a suppressing impact on the immune system and therefore, the body is at higher risk of developing cervical cancer. The immune system plays an important role in destroying cancer cells and therefore, reducing their proliferation. In the case where an individual is infected with HIV, the immune system is suppressed and therefore, cannot destroy cancerous cells (American Cancer Society, 2012).

5.1.4 Chlamydia infection

Chlamydia is caused by a type of bacteria which infects the reproductive system and causes pelvic inflammation and can lead to infertility. Studies have shown that women infected with Chlamydia are at higher risk of developing cervical cancer than women who are not infected with Chlamydia (American Cancer Society, 2012).

5.1.5 Diet

Women with diets consisting of a high amount of fruits and vegetables have a low risk of developing cervical cancer, whereas women who are overweight and have unhealthy diets are at higher risk (American Cancer Society, 2012).

5.1.6 Oral contraceptives

Women who take oral contraceptives have an increased risk of developing cervical cancer. The risk is increased with a longer period of taking the oral contraceptive (American Cancer Society, 2012).

5.1.7 Diethylstilbestrol (DES)

In the 1940s to 1970s, diethylstilbestrol was taken to prevent miscarriages. Daughters of mothers who took DES during their pregnancy are at risk of developing a rare kind of cervical or vaginal

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cancer. Furthermore, the daughters are also at risk of developing squamous cell carcinoma of the cervix, which is linked to HPV (American Cancer Society, 2012).

5.1.8 Family history

Women are at increased risk of developing cervical cancer if it runs in the family. Researches explain it as an inherent condition, which makes women more susceptible of being infected with HPV due to their weaker ability to fight against HPV (American Cancer Society, 2012).

5.2 Stages of cervical cancer

Diagnosing the different stages of cervical cancer is important as treatment option depends on the stage of cancer as well as the size of the tumour. The stages of the cancer are determined on the basis of where the cancer is found in the body. The different stages are as follows (The Oncology Institute of Hope and Innovation b, 2007):

Stage 0: Stage 0 is also known as *in situ* carcinoma where there are abnormal cells found in the inner most lining of the cervix. These abnormal cells have the capability of transforming into cancerous cells and spreading to nearby normal cells (The Oncology Institute of Hope and Innovation b, 2007).

Stage I: Stage I cancer is found only in the cervix and is divided into two stages depending on the amount of cancer found in the cervix. The different substages are as follows (The Oncology Institute of Hope and Innovation b, 2007):

> **Stage I A1**: In stage I A1 the cancer cells can only be viewed microscopically and are 3mm deep and not more than 7mm wide.
>
> **Stage I A2:** In stage I A2 the cancer cells are more than 3mm deep but not more than 5mm deep, and not more than 7mm wide (The Oncology Institute of Hope and Innovation b, 2007).
>
> **Stage I B:** In stage I B the cancer cells can only be seen with a microscope and are more that 5mm deep and more than 7mm wide. The cancer cells that can be viewed without a microscope are divided into stages I B1 and I B2. Stage I B1 is where the cancer is not larger

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than 4cm whereas in stage 1 B2 the cancer is larger than 4cm (The Oncology Institute of Hope and Innovation b, 2007).

Stage II: Stage II is where the cancer has spread beyond the cervix into nearby tissue towards the pelvic wall. The tumour does not invade the lower third of the vagina of the pelvic wall. There are two stages based on how far the cancer has spread (The Oncology Institute of Hope and Innovation b, 2007).

Stage II A: The cancer has spread to the cervix and to the upper parts of the vagina but not to the uterus.

Stage IIB: The cancer has spread to the cervix and to the upper parts of the vagina as well as the uterus (The Oncology Institute of Hope and Innovation b, 2007).

Stage III: The cancer has extended to the lower parts of the vagina and may have invaded the pelvic wall and caused the kidneys to stop working if the flow of urine is blocked. This stage is divided into Stages III A and III B depending on how far the cancer has spread (The Oncology Institute of Hope and Innovation b, 2007).

Stage III A: The cancer has invaded the lower part of the vagina but not the pelvic wall.

Stage III B: Cancer has spread to the pelvic wall and the tumour has blocked the ureters, which causes a blockage in the kidneys. There is a possibility that the cancer cells have also spread to the lymph nodes (The Oncology Institute of Hope and Innovation b, 2007).

Stage IV: At this stage the tumour has spread to the bladder, rectum and other parts of the body. There are two sub stages under stage IV based on where the cancer has spread to (The Oncology Institute of Hope and Innovation b, 2007).

Stage IVA: The cancer cells have spread to the bladder and possibly to the rectal wall. There is also a chance of the lymph nodes of the pelvis being invaded.

Stage IV B: Cervical cancer however, has invaded the pelvis and the lymph nodes in the pelvis and can spread to other parts of the body for example the abdomen, liver, intestinal tract and lungs (The Oncology Institute of Hope and Innovation b, 2007).

Recurrent cancer: The cancer has returned after being treated previously. After the treatment of cancer it has returned, where it can be seen in the cervix or other parts of the body (The Oncology Institute of Hope and Innovation b, 2007).

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5.3 Prevention of cervical cancer

Vaccinations against the two most common types of HPV strains are available for the prevention of cervical cancer. The vaccine is known as Gardasil and was approved by the U.S Food and Drug Administration in June 2006 to prevent early-stage cervical cancer and precancerous lesions (Pub Med Health, 2011).

Cervical cancer can furthermore be prevented by having regular check-ups including Pap smears and pelvic examinations to detect any precancerous changes. Smoking also plays a role in increasing the risk of cancer and therefore, chances of developing cervical cancer is minimised in non-smoking individuals (Pub Med Health, 2011).

The HPV can occur in the genital areas of both females and males and therefore, can be transferred to the female during intercourse. The use of protection has been shown to reduce the risk of developing cervical cancer (CDC, 2011).

5.4 Conventional treatments for cervical cancer

5.4.1 Surgery

Surgery is often used in the treatment of cervical cancer. There are a variety of surgical procedures that can be used (The Oncology Institute of Hope and Innovation b, 2007):

Conization, which is better known as a cone biopsy is based on the removal of a cone-shaped piece of tissue from the cervix. The removed piece of tissue is viewed under the microscope to view whether there are any cancer cells present.

Hysterectomy is a procedure which relies on surgically removing the uterus and cervix. There are three different procedures for removing the uterus and cervix; vaginal hysterectomy, total abdominal hysterectomy and total laparoscopic hysterectomy.

Bilateral salpingo-oophorectomy is a procedure that surgically removes both ovaries and both fallopian tubes.

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Radical hysterectomy differs from a normal hysterectomy in that it relies on the removal of the uterus, cervix and part of the vagina and in some cases the ovaries, fallopian tubes, and nearby lymph nodes.

Pelvic exenteration is the removal of the lower colon, rectum and bladder. In women the cervix, vagina, ovaries and nearby lymph nodes are also removed. Often after this procedure plastic surgery is required.

Cryosurgery, also known as cryotherapy uses liquid nitrogen to freeze and kill cancerous tissues and cancerous cells.

Laser surgery relies on a laser beam which makes cuts in the tissue so that a tumour can be removed.

Loop electrosurgical excision procedure (LEEP) makes use of an electrical current which passes through a wire loop and therefore, is able to remove abnormal tissue and cancer cells.

5.4.2 Radiation therapy

Cancer cells can be inhibited from growing or proliferating by treatment which makes use of high-energy-x-rays or other types of radiation. Radiation therapy can either be applied externally with a machine that sends the radiation to the cancer cells or internally where radioactive labelled substances are administered near the cancer cells. Whether the treatment is internal or external depends on the cancer type and stage of the cancer (The Oncology Institute of Hope and Innovation b, 2007).

5.4.3 Chemotherapy

Chemotherapy is a technique used to inhibit the growth or proliferation of cancer cells by administering a certain drug orally, intravenously or intramuscularly. This type of administration allows the drug to spread through the body via the bloodstream to reach the cancer cells. If chemotherapy is directly applied to the spinal cord, a specific organ or body cavity, the drug will only kill cancer cells in that region. Therefore, the administration of chemotherapy depends on the type of cancer as well as the area of invasion (The Oncology Institute of Hope and Innovation b, 2007).

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6. Cervical cancer linked to squamous carcinoma

As mentioned previously, squamous cell carcinoma originates in the squamous cells of the skin surface and therefore, SCC can grow in many areas of the body. In cases where SCC grows on the squamous cells, which makes up the outer layer of the cervix, cervical squamous carcinoma is formed, which is the most common type of cervical cancer. The main cause of cervical squamous carcinoma is infection by HPV and lesions (Cap, 2011). There are two main types of cervical cancer, squamous carcinoma and adenocarcinoma and the less common type adenosquamous carcinoma, which has characteristic of both types of carcinoma (American Cancer Society, 2014). Furthermore, the HPV virus can infect the skin and is able to increase the risk of developing squamous cell carcinoma (National Cancer Institute (b), 2011).

Natural products, which are mainly derived from plants, have been used in the treatment of many diseases. The World Health Organization has estimated that around 80% of the world's population relies on traditional medicine for their primary health care (Shoeb, 2006). Drug discovery from medicinal plants has played a major role in the treatment of various types of cancer. In the time period 1940 to 2002, 40% of the anticancer drugs were natural or derived from natural products (Balunas & Kinghorn, 2005).

7. Anticancer agents from medicinal plants

7.1 Chemo-preventative agents (antioxidants) derived from plants

Cancer chemoprevention was first described by Sporn *et al* (1976) as a control strategy for cancer by administering a synthetic or natural compound to reverse or inhibit the process of carcinogenesis. Carcinogenesis is a process which transforms a normal cell into a cancerous cell via initiation (which causes DNA damage to cells), promotion which increases the proliferation of cancerous cells and progression where genetic alterations are made to the cells. The chemoprevention strategies target each of these steps to: repair DNA, scavenge free radicals, detoxify, metabolise carcinogens, suppress proliferation, induce differentiation, increase immune response, reduce inflammation,

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increase cell death (apoptosis), alter gene expression and reduce angiogenesis (Balunas & Kinghorn, 2005).

There are currently many plant-derived chemo-preventive agents which are undergoing clinical trials by the U.S National Cancer Institute (NCI). These include curcumin for Phase I colon cancer, genistein for Phase I breast and endometrial cancer, soy iso-flavones for Phase II prostate cancer, indole-3-carbinol for Phase I breast recurrent cancer, phenethyl isothiocyanate for Phase I lung cancer and green tea/epigallocatechin gallate for Phase II breast cancer and Phase II recurrent bladder cancer. All these plant-derived agents act via mechanisms which target initiation, promotion and progression (Balunas & Kinghorn, 2005).

Through a Program Project funded by the NCI entitled “Natural Inhibitors of Carcinogens”, over 30 different types of active compounds were isolated. The most interesting compounds isolated included: Resveratrol which was isolated from *Cassia quinquangulata* Rich. This compound was found to inhibit cyclooxygenase-1 (COX-1) enzyme and in a mouse model was found to inhibit tumorigenesis. Ixocarpalactone A was isolated from *Physalis philadelphica* Lam., more commonly known as tomatillo. The leaf extract was active in inducing the quinine reductase enzyme, which is responsible for the metabolism of carcinogens. Isoliquiritigenin isolated from *Dipteryx odorata* Willd. also showed activity in inducing the quinine reductase enzyme (Balunas & Kinghorn, 2005).

7.2 Anticancer agents derived from plants in clinical use

The vinca alkaloids, vinblastine and vincristine were isolated from the Madagascar periwinkle, *Catharanthus roseus* G, Don. (Apocynaceae) and were the first plant derived agents to undergo clinical trials for the treatment of cancer (Fig 1.15). These vinca alkaloids are mainly used in combination with other chemotherapeutic drugs for the treatment of leukemias, lymphomas, advanced testicular cancer, breast and lung cancer and Kaposi’s carcinoma (Shoeb, 2006). The vinca alkaloids act by blocking mitosis together with metaphase arrest, which is result of binding to tubulin and causing depolymerisation (Balunas & Kinghorn, 2005).

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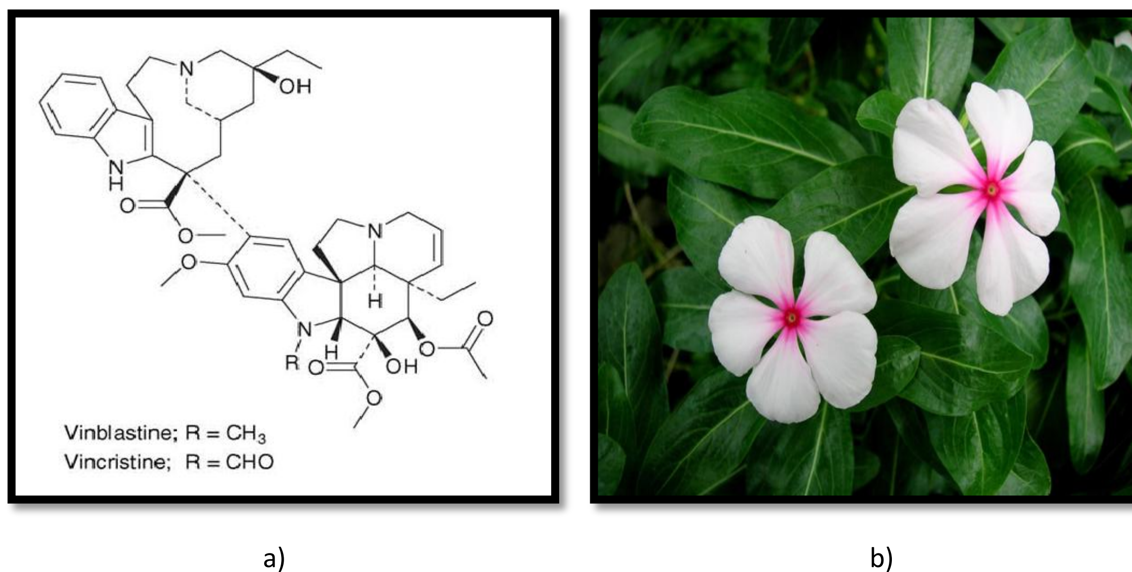

Figure 1.15: Vinca alkaloids, Vincristine and Vinblastine isolated from the b) Madagascar periwinkle (Cragg & Newman, 2005)

A great success that has come from natural products is the discovery of paclitaxel (Taxol®) which was isolated from the bark of the Pacific Yew, *Taxus brevifolia* Nutt. (Taxaceae) (Fig1.16). The structure of paclitaxel was elucidated in 1971 and was introduced to the US market in the 1990s. It is active against ovarian cancer, advanced breast cancer as well as small and non-small cell lung cancer (Shoeb, 2006). Taxenes act by binding to tubulin without causing depolymerisation or by interfering with tubulin assembly (Balunas & Kinghorn, 2005).

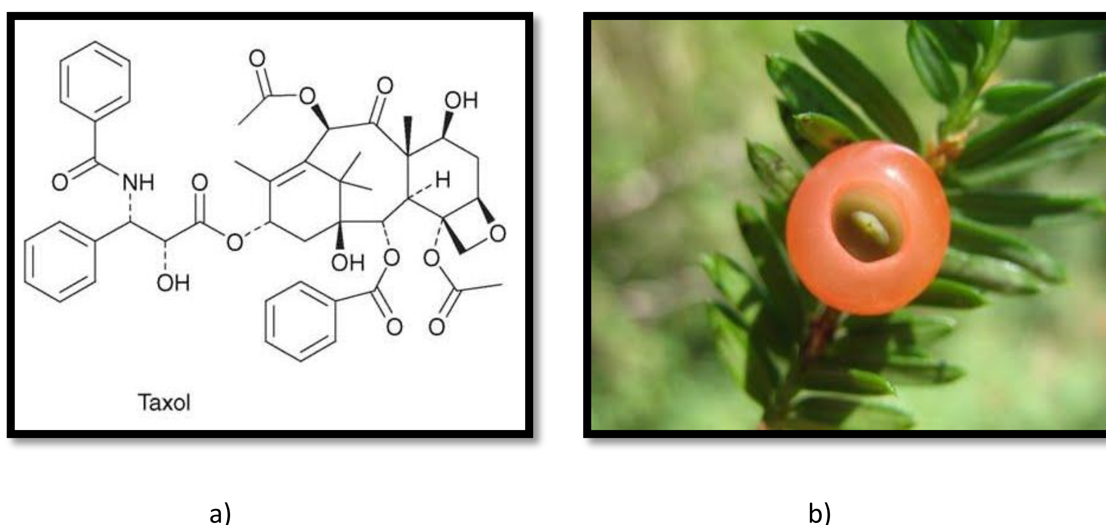

Figure 1.16: a) Plant derived taxene, Taxol® isolated from b) *Taxus brevifolia* (Cragg & Newman, 2005)

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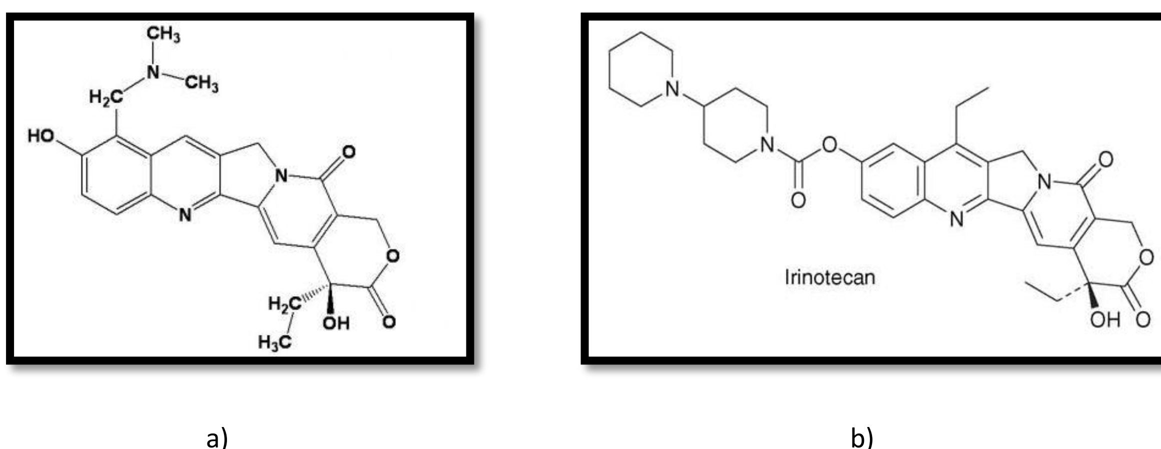
'Camptothecin' is another good example of a plant derived anticancer agent (Fig 1.17). It was isolated from the Chinese ornamental tree *Camptotheca acuminate* Decne (Nyssaceae). It was advanced to clinical trials in the 1970's but later was unsuccessful due to its high bladder toxicity. However, topotecan and irinotecan, which are semi-synthetic derivatives of 'camptothecin', have successfully been used in the treatment of ovarian and small lung cancers, and colo-rectal cancer, respectively (Shoeb, 2006). 'Camptothecin' and its derivatives act by selectively inhibiting topoisomerase I, which is important in the cleavage and reassembly of DNA (Balunas & Kinghorn, 2005).



a) b)

Figure 1. 17Camptothecin derived anti-cancer agents a) Topotecan and b) Irinotecan (Cragg & Newman, 2005)

'Etoposide' and 'teniposide' are derivatives from 'epipodophyllotoxin' and are used in the treatment of lymphomas and bronchial and testicular cancers (Fig 1.18). 'Epipodophyllotoxin' is an isomer of podophyllotoxin which is isolated from the roots of *Podophyllum* species, *Podophyllum peltatum* Linnaeus and *Podophyllum emodi* Wallich (berberidaceae) (Shoeb, 2006). 'Epipodophyllotoxin' acts by binding with tubulin and causing breakage in the DNA strands during the G2 phase of the cell cycle by irreversibly binding to DNA topoisomerase II (Balunas & Kinghorn, 2005).

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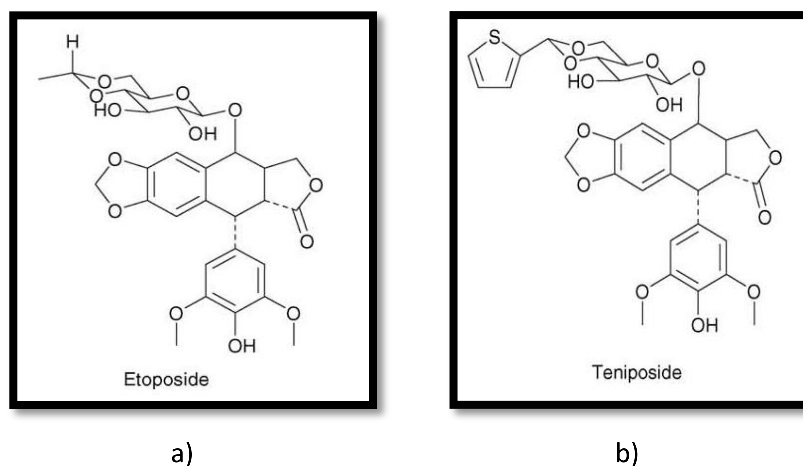

Figure 1.18: Semi-synthetic derivatives of epipodophyllotoxin a) etoposide and b) teniposide (Cragg & Newman, 2005)

Another plant-derived agent currently used in clinical trials is 'homoharringtonine', which was isolated from the Chinese tree *Cephalotaxus harringtonia* var. *drupacea* (Sieb and Zucc.) (Cephalotaxaceae) (Fig 1.19).

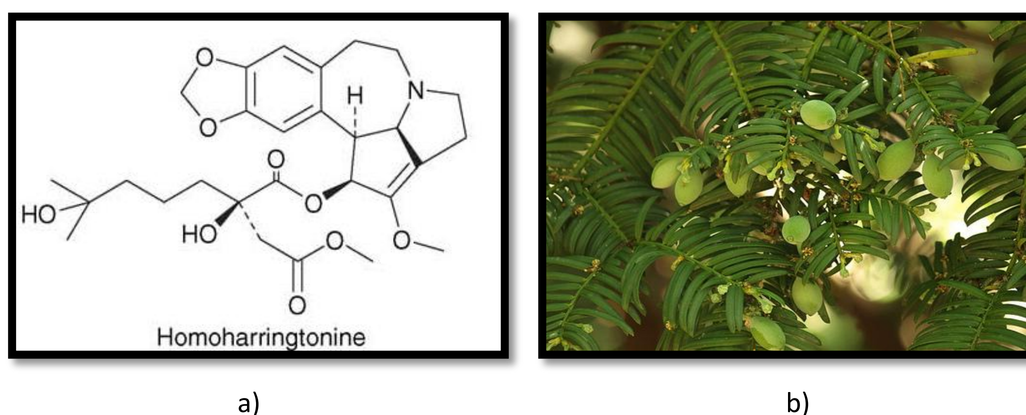

Figure 1.19: a) Homoharringtonine is a plant-derived anti-cancer agent from the species b) *Cephalotaxus harringtonia* (Cragg & Newman, 2005)

A combination of 'harringtonine', also derived from *Cephalotaxus harringtonia,* and 'homoharringtonine' is used in China for the treatment of acute myelogenous leukemia and chronic myelogenous leukemia (Shoeb, 2006).

In France a plant derived agent known as 'Elliptinium', which is derived from ellipticine, and isolated from a Fijian plant *Bleekeria vitensis* A.C. Sm., is currently being used for the treatment of breast cancer (Fig 1.20) (Shoeb, 2006).

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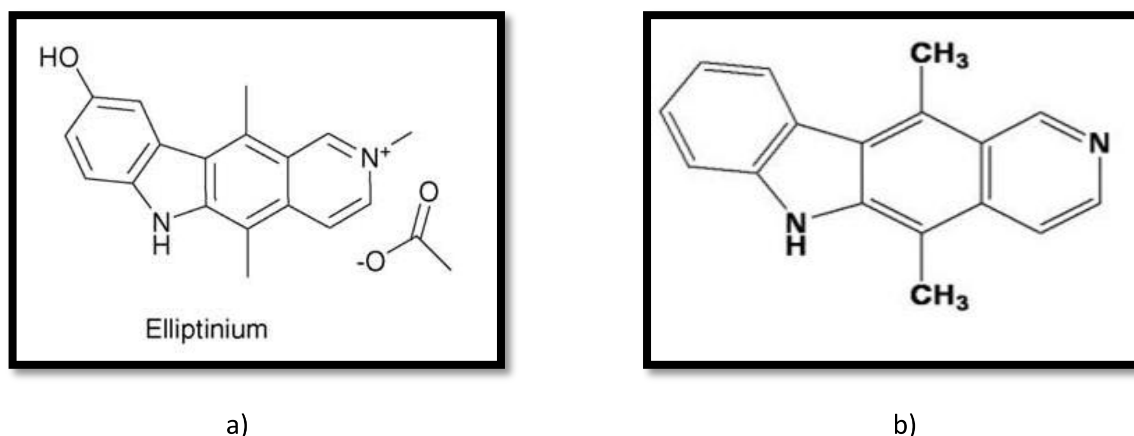

a) b)

Figure 1.20: Plant derived anti-cancer agents a) Elliptinium and b) Ellipticine isolated from *Bleekeria vitensis* (Cragg & Newman, 2005)

Silvestrol is a compound isolated from the fruits of *Aglaila sylvestre* (M. Roemer) Merrill (Meliaceae) and is used to target lung, prostate and breast cancer (Shoeb, 2006).

Schischkinnin and montamine are two alkaloid compounds isolated from the seeds of *Centaurea schischkinii* and *Centaurea Montana* respectively. Both of these compounds are being used against colon cancer cell lines (Shoeb, 2006).

7.3 Anticancer agents from plants currently undergoing clinical trials

There are many bioactive compounds that have been derived from plants but are still undergoing clinical trials or preclinical trials. Examples of such plant-derived compounds are as follows:

'Flavopiridol', a synthetic flavone, derived from rohitukine, was isolated from *Dysoxylum binectariferum* Hook. f.(Meliaceae) (Kelland, 2000). It is currently undergoing clinical trials in phase I and phase II and is being tested against tumours, including leukemia, lymphomas and solid tumours (Christian *et al*., 1997).

In Europe a synthetic agent known as 'roscovitine', derived from olomucine, isolated from *Raphanus sativus* L. (Brassicaceae) is currently undergoing phase II clinical trials (Cragg & Newman, 2005).

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A South African tree namely *Combretum caffrum* (Eckl. & Zeyh.) Kuntze was used to isolate combretastatins from the bark. The compound 'combretastatin A-4' is used against colon, lung and leukemia cancers (Shoeb, 2006).

A pentacylic triterpene known as 'betulinic acid' was isolated from the *Zizyphus* species such as *mauritiana, rugosa* and *oenoplia*. It is used for its activity against human melanoma cell lines (MEL2) by inducing apoptosis. It is currently still undergoing systemic and topical formulation development by the NCI (Shoeb, 2006).

'Pervilleine A', a compound isolated from the roots of *Erythroxylum pervillei* Baill. (Erythroxylaceae) was used in conjunction with 'vinblastine' to target a multidrug resistant oral epidermoid cancer cell line (KB-V1). It is currently undergoing preclinical development (Mi *et al*., 2001).

Natural products or natural products derived from medicinal plants are therefore, an important source for the discovery of potential new anti-cancer agents.

8. Objectives of the study

The main objective of this study was to investigate the possible anti-cancer activity of southern African plant extracts on cervical cancer, skin melanoma and epidermoid cancer. Secondly, the lead plant extracts were further investigated to determine their specific mechanism of cell death.

The specific objectives of this study were to:

- Determine the cytotoxic effect of the Southern African plants on cancerous and non-cancerous cell lines.
- Synergistic studies of lead plant extracts to determine their combined activity.
- Identify the possible anti-oxidant activity of the selected plant extracts.
- Identify the mechanism of cell death using light microscopy.
- Determine whether the selected plant induces cytokines which reject the formation of tumours and suppresses cytokines which promote cancer cell growth.

The different methods used for achieving the above mentioned objectives are briefly described.

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9. Methodology

9.1 *In vitro* cytotoxicity assay and synergistic studies

9.1.1 *In vitro* cytotoxicity

Plant extracts were tested for their *in vitro* cytotoxicity on various cancerous (A431, A375 and HeLa) and non-cancerous (HEK-293) cells using the XTT Cell proliferation Kit. The assay is based on the ability of viable cells to reduce the yellow tetrazolium salt to an orange formazan product. Therefore, the amount of formazan produced is directly proportional to the amount of viable cells (Roche, 2004). The amount of formazan was measured using a BIO-TEK Power-Wave XS multi-well plate reader.

9.1.2 Synergistic studies

Active plant extracts were tested in various combined ratios for their *in vitro* cytotototoxicity against cancerous A431 cells. The cytotoxicity was measured using the XTT method as described above. The synergistic assay was performed as described by Van vuuren (2007) with slight modifications. The assay was performed in ratios 9:1, 8:2, 7:3, 6:4, 5:5 and concentrations ranging from 400µg/ml to 3.125µg/ml. The absorbance of formazan was measured using a BIO-TEK Power-Wave XS multi-well plate reader.

9.2 Antioxidant assay

9.2.1 DPPH antioxidant assay

The plant extracts were used to determine their radical scavenging capacity using the 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH) assay. DPPH is a free radical which shows maximum absorption at 515nm. In the presence of an antioxidant the DPPH free radical (purple) is converted to a stable yellow compound (Lee *et al*., 2004). The amount of reduction of DPPH to the stable compound depends on the effectivity of the plant extract, in other words it depends on the hydrogen donating ability. Therefore the inhibition of DPPH radical absorption at 515nm by the plant extract was a measurement of the antioxidant activity (Erkan *et al*., 2008).

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9.2.2 Nitric oxide scavenging assay

Plant extracts were also used to determine their nitric oxide scavenging capacity. This method is based on the ability of sodium nitroprusside to spontaneously produce nitric oxide at a physiological pH. The nitric oxide which is produced is converted to nitric and nitrous acid when it comes into contact with oxygen and water. The amount of nitrous acid present is determined using the Griess-Illosvoy method. The Griess reagent is responsible for forming a purple azo dye and in the presence of an antioxidant the purple colour will decrease.

9.3 Microscopy

The A431 cell line was analyzed for morphological characteristics and changes in the absence of the active plant extract as well as when exposed to the active plant extract. Microscopy techniques were used to observe morphological changes associated with apoptosis, necrosis or autophagy by using light microscopy (haematoxylin and eosin). The various slides were done in triplicate to confirm the morphological changes and blind sampling was performed to exclude bias results.

9.4 Cytokine evaluation

The active plant extract was tested using U937 cells to determine whether there was an increase or decrease in cytokines. The human IL-12 and IL-8 kit was used to determine whether there was suppression of IL-8, which has been observed as causing an increase in cancer cell growth and metastasis and activation of IL-12, which has been reported to have antitumor activity in mice.

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10. Structure of thesis

Chapter 1 This chapter provides an introduction to the use of traditional medicine worldwide and in South Africa. A problem statement concerning the statistics of mortalities caused by cancer is highlighted. Furthermore an overview of cancer and more in depth cervical cancer and skin cancer is discussed. Lastly the use of medicinal plants in the treatment of cancer and currently in clinical trial has also been discussed.

Chapter 2 A concise review of the plant samples used for this study are discussed briefly. A description of the plant morphology and region in which it can be found are highlighted. Furthermore the traditional usage of the plant samples is briefly discussed.

Chapter 3 This chapter focuses on the cytotoxicity of the selected plant extracts on cervical cancer and skin cancer as well as non-cancerous cell line HEK-293. The activity of lead extracts in combination is also discussed in this chapter.

Chapter 4 The antioxidant activity of the plant extracts was determined in this chapter by the use of the DPPH assay and the Nitric oxide assay.

Chapter 5 The microscopy technique that was used to determine the mechanism of cell death by the lead plant extracts is described on A431 cells.

Chapter 6 The cytokine profile of the lead plant extracts is discussed. The discussion is based on the ability of the plant extracts to induce an increase in IL-12 response in the cells and to suppress the production of IL-8.

Chapter 7 In this chapter the final discussion and conclusion on all the results is made to give a complete overview of the findings.

Chapter 8 Appendix

Chapter 9 Publication, presentations and patents are listed in this chapter

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Chapter 2

Plant collection and extraction

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1. Introduction

The southern African plants used for this project were selected from a variety of families. They were selected based on their traditional usage for the treatment of various cancer types or skin ailments such as sores, wounds and infection, which could possibly lead to the formation of squamous carcinoma and cervical cancer. Plants were also selected based on their ability to decrease inflammation as well as increase astringent and blood cleansing properties which promotes the health of skin. A detailed description of the selected plants is as follows:

2. Plant selection

2.1 *Acacia caffra* (Thunb.) Willd

The common hook-thorn *acacia* from the Mimosaceae family is a medium-sized deciduous tree with a rounded crown which is found in the bushveld, grassland and coastal region (Fig 2.1). The branches have hooked thorns, which occur in pairs below the nodes and the leaves are fresh green and feathery, which occur in pairs. The flowers which are in the shape of elongated spikes are yellow in colour while the pods are flat and brown to red in colour (Van Wyk & Van Wyk, 1997). This plant is found in the coastal areas of KwaZulu Natal and Eastern Cape as well as some areas in the Western Cape (PlantsZAfrica, 2010).

Figure 2. 1: *Acacia caffra* with distinct yellow flowers occurring in elongated spikes (PlantZAfrca, 2010)

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The Zulu community of South Africa uses bark infusions as blood-cleansing emetics, whereas from milk and leaf infusions, enemas are made which are administered to infants who suffer from abdominal problems. The leaves can also be chewed by infants for abdominal problems (Hutchings *et al*., 1996).

2.2 *Acacia mellifera* (Vahl.) Benth

Acacia mellifera from the Fabaceae family, commonly known as the black thorn, is a low, branched tree, which has a spherical crown and is found mainly in the bushveld and semi-desert areas (Fig 2.2). The bark is black on the trunk and turns ash-grey/brown in colour on the branches. On the branches small, short, sharp hooked spines are formed in pairs. The leaves occur in pairs, each containing 1-3 leaflets. The flowers have a sweet scent and are cream to white in colour and can reach a length of 3.5cm. Pods are flat and elongated containing 2-3 seeds (Worldagroforestry, 2009). It can be found in a variety of regions such as the Northern Cape, Free State, Gauteng, North-West Province, Mpumalanga and Limpopo province (PlantZAfrica, 2010).

Figure 2. 2: *Acacia mellifera* known commonly as the black thorn because of the sharp black spines forming on branches (AfricaMuseum, 2012)

A.mellifera has widely been used in African traditional medicines against various diseases. The stem-bark of *A.mellifera* is used in Africa as treatment for pneumonia, malaria, primary infection of syphilis, sterility and stomach ache (Lalitha *et al*., 2010). A community in Kenya known as

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"Kipsigis" uses the water extract from the plant for the treatment of various skin diseases, coughs and gastrointestinal ailments (Mutai *et al*., 2009).

2.3 *Arbutus unedo* L.

Arbutus unedo also known as the "Strawberrry tree" from the Ericaceae family is an evergreen shrub which is highly branched (Fig 2.3). The bark is grey-brown in colour and when it peels off a reddish coloured bark can be seen. This shrub can reach a height of 9m and a diameter of 9m. The leaves are leathery in texture and can reach 5-10cm in length. The flowers are white or pink/blue in colour and are clustered in panicles. The fruits that are produced take approximately a year to ripen to a bright red warty fruit of about 2cm in diameter (Floridata, 2012). It is distributed in the Mediterranean and North Africa (Haouari *et al*., 2007)

Figure 2.3: *Arbutus unedo* known as the strawberry tree because of the bright red strawberry like fruits it produces (Floridata, 2012)

The leaves of *Arbutus unedo* are traditionally used as a diuretic, urinary antiseptic, anti-diarrheal, astringent, depurative against blenorrhagia and as an anti-hypertensive. The tea made from the leaves is traditionally used in Turkey as a diuretic (Pabuçcuoğlu *et al.,* 2003).

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2.4 *Buddleja saligna* Willd

Buddleja saligna from the family Buddlejaceae is commonly known as the "False olive". It is an evergreen shrub which is grey to green in colour (Fig 2.4). The shrub is commonly found in bushveld, grassland and forest, usually in woody or rocky areas. The leaves are greyish green in colour, shiny and hairless. The flowers are produced in a dense clump with small white flowers forming on the axillary and terminal panicles. The heartwood is dark and very hard (Van Wyk & Van Wyk, 1997). The false olive is widespread in South Africa from coastal to inland regions as well as parts of the Kalahari (PlantZAfrica, 2010).

Figure 2.4: *Buddleja saligna* with its prominent white clustered flowers (PlantZAfrica, 2010)

Traditionally the roots of the plant are used for its cleansing properties and the leaves are used to treat coughs and colds by the Tswana and Kwena of South Africa (PlantZAfrica, 2010). In a review on *Buddleja* species the bioactivity has been reported to have anti-inflammatory, anti-fungal and wound-healing properties (Houghton & Mensah, 1999).

2.5 *Buddleja salviifolia* (L.) Lam

Buddleja salvifolia, from the Buddlejaceae family, is commonly known as "Sagewood". It is a shrub which has a grey appearance (Fig 2.5). It is commonly found along forest margins and in high-altitude grasslands. In these regions they usually occur along watercourses and in rocky areas. The

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leaves are narrowly ovate with the bottom covered with white to brown hairs. The flowers are white to lilac or purple and are sweet in scent (Van Wyk & Van Wyk, 1997). The sagewood has a wide distribution in South Africa ranging from the Western Cape to the Eastern Cape and Freestate as well as parts of Lesotho, KwaZulu Natal, Swaziland, Northern Province, Mpumalanga and neighbouring countries such as Mozambique and Zimbabwe (PlantZAfrica, 2010).

Figure 2.5: ***Buddleja salviifolia*** **with the sweetly scented white flowers forming on the axils of the branches (PlantZAfrica, 2010)**

The Zulu communities, in South Africa, traditionally use the root decoctions to treat stomach problems and diarrhoea, whereas a tea made from the roots is used for coughs. A decoction of leaves is used as an eye wash and for colic. The Tswana communities in South Africa use fresh flower decoctions for treating sores (Hutchings *et al*., 1996).

2.6 *Clausena anisata* (Willd) Hook

Clausena anisata, from the family Rutaceae, is commonly known as “Horsewood”. It is a shrub which is often found along margins of coast and inland forest, or near streams (Fig 2.6). The leaves are dark green in colour and produce an unpleasant scent when crushed. The flowers are small and white to yellow in colour with the fruits ripening to a red, purple-black to black colour (Van Wyk & Van Wyk, 1997). *C. anisata* is distributed throughout Africa such as in Guinea, Sierra Leone, Ethiopia, Sudan and in the Cape (Prota4U (a), 2011).

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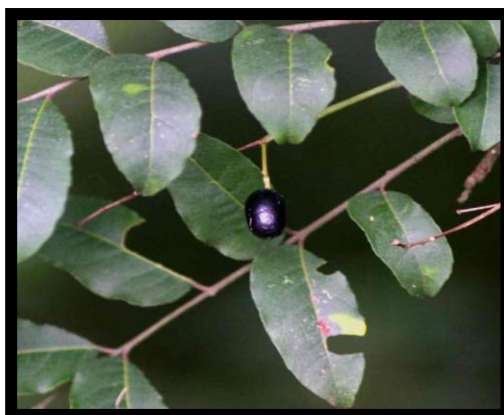

Figure 2.6: ***Clausena anisata*** **with the fruits that produce from the white to yellow in inflorescence (Flora of Zimbabwe, 2006)**

Traditionally *C. anisata* is used in Nigeria to relieve the symptoms of abdominal disturbances. A concoction of *C. anisata*, *Afraegle paniculata* and *Azadirachtha indica*, which is known as Agbo, is used as an antimalarial medicine. In Tanzania the plant is traditionally used to treat infections of the skin such as candidiasis, fungal infections and other skin disorders. In the Temeke region of Tanzania it is traditionally used as an anticonvulsant and to treat epilepsy. In South Africa however, the leaves of *C. anisata* are used against high blood pressure (MMH-MMS, 2012). In tropical Africa the leaves are crushed and applied externally to wounds, aching teeth, sores, abscesses, burns, haemorrhoids, rheumatism and other body pains. In Kenya, root decoctions are made and orally ingested to treat various skin disorders (Prota4U (a), 2011).

2.7 *Clematis brachiata* Thunb.

Clematis brachiata commonly known as “Traveller’s Joy” is from the family Ranunculacea and is a deciduous climber (Fig 2.7). The stems are slender and woody and can twine up to 5m in height. In late summer and autumn the climber bears masses of small white flowers which have a sweet scent. This plant is commonly found along roadsides twining into trees, bushes, rocks and fences. It is a perennial plant which is widespread in South Africa in the bushveld. It is found in the Free State, KwaZulu-Natal, Eastern Cape, Northern and Western Cape as well as Swaziland, Lesotho, Namibia and Botswana (PlantZAfrica, 2010).

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Figure 2.7: ***Clematis brachiata*** **flowers and buds forming amongst the green leaves (Kumbula Indigenous Nursery, 2012)**

This plant was named Traveller's Joy because of its medicinal properties that were of use to travellers, when travelling was done either on foot or on horseback. The leaves were placed in the shoes of travellers to soothe blisters, aches and pains. The Xhosa, Zulu, Sotho and Tswana communities of South Africa use the plant as a tea to ease headaches, coughs, colds, chest pains and abdominal pains. It is also used as a soothing foot wash for aching, cracking or blistering feet. The tea is cooled and used as an eye wash for tired red eyes. The stem of the plant is used as a powder which releases a strong scent that is believed to clear blocked noses, relieve sinus pains and induce sneezing. Furthermore a decoction of the roots, stems and leaves is used to inhale and relieve the symptoms of cold, malaria, sinusitis and asthma. A brew of the leaves, stems and flowers can be added to bath water to relieve aching muscles (PlantZAfrica, 2010).

2.8 *Combretum molle* R.Br. ex G. Don

Combretum molle, also known as the velvet bushwillow from the Celastraceae family, is a small to medium sized semi-deciduous to deciduous tree (Fig 2.8). In autumn the leaves are reddish to purple in colour with velvet coloured hairs. This tree is found in the bushveld in rocky areas or in the grasslands. The flowers of the tree are greenish yellow in colour which can reach up to 90mm in length. The fruit is yellow to green in colour with a red tinge and when dry the colour changes to golden reddish brown (Van Wyk & Van Wyk, 1997). It's distributed in certain areas of KwaZulu

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Natal as well as northern regions of South Africa and neighbouring countries such as Namibia, Zimbabwe and Mozambique (PlantZAfrica, 2010).

Figure 2.8: ***Combretum molle*** **producing greenish-yellow flowers on the end of the branches (PlantZAfrica, 2010)**

The Zulu communities of South Africa use fresh or dry leaves and apply it to wounds. Synergistically the leaves and roots are used as an antidote for snake bites and as a dressing for the snake bite wounds. Infusions of the inner bark are used as an enema or taken orally to treat stomach pains. In Ghana, the leaves are used as wound dressings as well as for stomach pains, colic and fevers. Boiling the leaves and inhaling the steam is used to treat headaches (Hutchings *et al*., 1996).

2.9 *Dissotis princeps* (Kunth.) Triana

Dissotis princeps is commonly known as the “wild tibouchina” from the Melastomataceae family (Fig 2.9). It is a soft herbaceous shrub which can grow up to 1.5m to 3m in length. The whole plant is covered with short hairs. The leaves of the plant are large and egg shaped which are dark green above and paler below with older leaves turning red. The flowers are produced on terminal panicles which are large and usually purple or white in colour. The fruit of the plant is a capsule that develops inside the persistent calyx, which releases small seeds. The wild tibouchina occurs in KwaZulu Natal, Swaziland and Limpopo as well as Botswana, Namibia, Mozambique, Zimbabwe, Zambia and Malawi (PlantZAfrica, 2010).

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Figure 2.9: ***Dissotis princeps*** **producing purple coloured flowers on the terminal panicles (PlantZAfrica, 2010)**

In certain tribes the roots of *Dissotis princeps* are eaten because of its aphrodisiac effect. Traditionally the plant is eaten as a vegetable during times of famine. Medicinally the leaves, stems and roots are used to make a brew and taken to prevent symptoms caused by drinking beer which is made from the season's new mealies. Furthermore the leaves are also used to treat dysentery and diarrhoea (PlantZAfrica, 2010). There have been reports on the use of other *Dissotis* species for the treatment of skin diseases and malaria (Ndjateu *et al*., 2014).

2.10 *Erythrophleum lasianthum* Corbishley

Erthrophleum lasianthum is a medium to large tree which is commonly known as the Swazi ordeal tree from the Caesaliniaceae family (Fig 2.10). It is commonly found in hot, dry bushveld regions as well as sand forests. The leaves are hairless and usually occur in 2-4 pinnae pairs with leaflets alternating. The flowers are greenish to yellow in colour which occur in dense spikes. The pods are flat, brown and straight containing around 5-11 seeds (Van Wyk & Van Wyk, 1997). This large tree can be found in Swaziland as well as South Africa.

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Figure 2.10: *Erythrophleum lasianthum* fruiting branches (Prota4U, 2006)

The bark is ground to a powder and used as a snuff to treat headaches, hysteria and to relieve body pains. A decoction of bark is taken orally to treat intestinal problems. An infusion of bark is used as an emetic, which cleanses the blood, and enemas. The leaves have also been reported to have medicinal uses (Hutchings *et al*., 1996).

2.11 *Euclea divinorum* Hiern

Euclea divinorum, from the Ebenaceae family, is commonly known as the "magic guarri" (Fig 2.11). This evergreen shrub or small tree can reach a height of up to 15m. The bark is grey/brown in colour with irregular longitudinal fissures. The leaves are arranged oppositely and are simple and leathery. The flowers are unisexual which are white to pale yellow in colour. The fruit is a fleshy berry which is brown/black to purple in colour with short white hairs (Prota4U, 2005). The magic guarri is distributed in KwaZulu Natal, Limpopo and Mpumalanga (SANBI (a), 2013).

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Figure 2.11: ***Euclea divinorum*** **fruit that develops from white to pale yellow flowers (Prota4U, 2005)**

Extracts and dried powder of the roots are traditionally used for the treatment of gastro-intestinal problems as well as cancers, ulcers, wounds, arthritis, miscarriages, jaundice, snake bites and gonorrhoea. The Shangaan communities of southern Africa use ground roots as a treatment for leprosy and to relieve headaches and toothaches (Prota4U, 2005). Furthermore, the frayed ends of the twigs are used as a toothbrush (Van Wyk & Van Wyk, 1997).

2.12 *Gomphocarpus fruticosus* (L.) Aiton f.

Gomphocarpus fruticosa from the Apocynaceae family is commonly known as “Milkweed”, which is widely distributed in southern African regions (Fig 2.12). It is a small perennial herbaceous shrub which can reach up to 1.5-2m in height. The leaves are arranged in an opposite pattern and are 4-12cm long. Furthermore, the leaves are leathery with fine white hairs forming on the midrib and margins. The flowers are carried in pendulous cluster and are creamy yellow in colour (Flora of Zimbabwe, 2012).

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Figure 2.12: *Gomphocarpus fruticosus* flowers which are carried in a pendulous cluster (plantZAfrica, 2010)

The dried leaves are ground to a powder and used as a snuff to treat headaches and also for the treatment of tuberculosis. The ground leaves are also used as an emetic, which strengthens the body. Generally the roots are reported to relieve abdominal pains and general aches in the body as well as certain liver problems and malaria (Van Wyk *et al*., 2009). In Namibia, a tea is prepared from the roots and used in the treatment of diabetes. A root decoction or infusion in South Africa is not just used for the treatment of diabetes but also used to treat general body pain and infertility. The dried crushed leaves in Ethiopia are used as an ointment and applied to sores whereas in Namibia the leaves are used as a tea for the treatment of skin cancer (Prota4U (b), 2011).

2.13 *Harpephyllum caffrum* Bernh.

The “wild plum” from the Anacardiaceae family is a medium to large tree which is found mainly in coastal forest (Fig 2.13). The leaves are crowded with often an odd red leaf in between. Furthermore the leaves are hairless and dark shiny green in colour. The flowers form at the end of branches and are white in colour. The fruit is edible with a bright red colour when ripe (Van Wyk & Van Wyk, 1997). The wild plum can be found in the Eastern Cape, KwaZulu Natal, Swaziland, Mozambique, Limpopo and Zimbabwe (PlantZAfrica, 2010).

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Figure 2.13: ***Harpephyllum caffrum*** **known as the wild plum because of the fruit it bears (PlantZAfrica, 2010)**

A decoction of the bark is used as a blood purifier as well as an emetic. It is also used as a face wash to treat skin problems such as acne and eczema. Powdered burnt bark is applied to fractures or sprains areas to relieve pain (Van Wyk *et al*., 2009).

2.14 *Helichrysum kraussii* Sch. Bip

Helichrysum kraussii, from the Asteraceae family, is commonly known as the "Curry bush" (Fig 2.14).

Figure 2.14: ***Helichrysum kraussii*** **is a highly aromatic shrub and therefore known more commonly as the curry bush (Lucidcentral, 2012)**

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It is an aromatic shrub which can reach a height of 1m. The flowers are small and yellow in colour. The leaves of the curry bush are dense. It is mainly found in grasslands and in the bushveld (van Wyk & Malan, 1998). The curry bush is not endemic to South Africa but can be found in various regions such as the Free State, Gauteng, KwaZulu Natal, Limpopo, Mpumalanga and the North West (SANBI (b), 2013).The dried flowers and seeds are used in a pipe and inhaled. This traditional use comes from the Karangas, a Zimbabwean tribe, as a remedy for coughs and pulmonary tuberculosis. The whole plant is burnt to ash and salt added and ingested by mouth to treat cough symptoms. The roots are ground down and mixed with salt and used to treat venereal diseases, which could lead to cervical cancer. Furthermore a decoction of the leaves is used to wash keloid scars (Lourens *et al*., 2008).

2.15 *Helichrysum odoratissimum* (L.) Sweet

Helichrysum odoratissium is a herbaceous shrub from the Asteraceae family and is commonly known as “Imphepho” (Fig 2.15). It is a strongly aromatic, branched, perennial shrub, which can reach a height of 50cm. The leaves are silvery and small and are covered with fine hairs. The flowers are yellow in colour and form in groups at the tips of the branches. The fruit is brown in colour and is granular (PlantZAfrica, 2010). This plant can be found in various provinces of South Africa such as in Limpopo, Mpumalanga, KwaZulu Natal, Free State, Eastern and Western Cape as well as in the Drakensberg, Swaziland, Lesotho, Mozambique, Zimbabwe and Malawi (PlantZAfrica, 2010).

Figure 2.15: *Helichrysum odoratissimum* is an aromatic shrub with bright yellow flowers forming on the tips of the branches (PlantZAfrica, 2010)

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H. odoratissimum has many traditional uses. The leaves and stems are burnt and the smoke inhaled, which acts as a sedative and treats insomnia. The roots are used to treat coughs and colds and the extract of the roots is taken in orally, and used as a cleanser. The ash of leafy twigs is ingested and used to relieve coughs, whereas the extract or sap of leaves and twigs is used as eye drops to treat conjunctivitis and the decoction is used for abdominal pains as well as for the treatment of female sterility, menstrual pain and eczema. An extract of the aerial parts of the plant is used to treat symptoms of dehydration. The ash of leaves is eaten to relieve vomiting whereas the tea of leaves is ingested and help for colic. The infusion of leaves can also be used to treat symptoms associated with fever (Lourens *et al*., 2008). In some cultures the flowers of the plant can be made into a paste for the treatment of acne and pimples (entheology.com, 2014).

2.16 *Leucas martinicensis* (Jacq.) R. Br

Leucas matinicensis, from the Lamiaceae family, is known as “whitewort”. It is a herbaceous, erect, annual shrub which can reach a height of 1m (Fig 2.16). The leaves are arranged oppositely with toothed margins. The flowers are small and white in colour and are found in round clusters along the stem (Flowers of India, 2012). This plant is widely distributed in various provinces of South Africa such as the Free State, Gauteng, KwaZulu Natal, Limpopo, Mpumalanga and the North West (SANBI (c), 2013).

Figure 2.16: *Leucas martinicensis* is a herbaceous annual shrub with its small white flowers (Flora of Zimbabwe, 2009)

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L. martinicensis is traditionally used for treating many diseases. A decoction of the whole plant together with *H. Spicigeriais* is believed to help relieve symptoms of malaria. Alone the decoction of the plant is used to treat urinary schistosomiasis. A decoction of the leaves or the aerial parts is said to treat inflammation, rheumatism and kidney disorders. An aqueous juice of the leaves is used to treat gonorrhoea and vomiting whereas leaf extracts made with water or milk are used to relieve the symptoms of headache and fever (Chouhan & Singh, 2011). Furthermore, the hot water extract is administered orally for the relief of gastroenteritis, cholera, malaria, syphilis, leprosy, diarrhea and dysentery (Das *et al*., 2012).

2.17 *Rapanea melanophloeos* (L.) Mez

Rapanea melanoploeos from the Myrsinaceae family is more commonly known as "Cape beech" (Fig 2.17). It is an evergreen tree which can grow to be medium to large in size and is found mainly along the damp areas of mountains and coastal forests as well as bush clumps. The thick and leathery leaves form clusters at the ends of the branches. The flowers are clustered just below the leaves and are small and green to white in colour (Van Wyk & Van Wyk, 1997). The cape beech tree is widely distributed in southern Africa and in the Cape (PlantZAfrica, 2010).

Figure 2.17: *Rapanea melanophloeos* produces green to white flowers

R. melanophloeos is used in traditional medicine in the southern and eastern Africa. The bark, fruits and leaves are used for various ailments such as stomach disorders, respiratory problems, fever,

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diabetes, cardiac disorders, muscle pains and for the treatment of wounds (Watt, 1962; Neuwinger, 2000; Midiwo *et al*., 2002).

2.18 *Syzygium jambos* (Alston.)

Syzgium jambos is a small to medium sized tree from the Myrtaceae family commonly known as "Rose apple" (Fig 2.18). The leaves are dark green in colour and are oppositely arranged. The flowers are cream to white in colour and form on branched axillary heads. The fruit is oval in shape and when ripe turns yellow. The fruit can grow up to 4.5cm in diameter (Flora of Mozambique, 2012). The rose apple is an invasive species in South Africa and is widely distributed in KwaZulu Natal and the Western Cape (Invasives, 2013).

Figure 2.18: *Syzygium jambos* known commonly as the rose apple due to the appearance of the fruit it produces (Flora of Mozambique, 2012)

In India, a tonic of *Syzygium jambos* is used to increase the functioning of the brain and liver. Furthermore, an infusion of the fruit is used as a diuretic. The flowers are used to relieve symptoms associated with fever, whereas the seeds are used against diarrhoea, dysentery and catarrh. A decoction of the leaves is applied to sore eyes and is also used as diuretic, expectorant and for the treatment of rheumatism (NTGB, 2012). In India the seeds are used to treat illnesses which are caused by bacterial, fungal and viral pathogens. It is also used for the treatment of colds, coughs, fever and skin problems such as rashes (Zheng *et al*., 2011).

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2.19 *Tabernaemontana elegans* Stapf.

Tabernaemontana elegans is commonly known as the toad tree and belongs to the Apocynaceae family (Fig 2.19). It is a shrub or small deciduous to semi-deciduous tree which can be recognized by its large, glossy leaves, fragrant white flowers and large fruits which are borne in pairs. This tree grows from 1.5m to 5m in height and in rare cases can reach heights up to 12m. The bark of the tree is corky, pale brown with longitudinal fissures. The leaves are arranged oppositely and have a leathery appearance. Many white flowers are produced on each inflorescence. The fruits are produced in pairs and have a green skin with pale warts which resembles the skin of a toad, giving rise to the name toad tree. All plant parts are hairless and produce a milky white sap. This tree is indigenous to tropical east Africa, South Africa and Swaziland. It is mostly found in the bushveld and coastal forests along riverbanks and in rocky areas (PlantZAfrica, 2010).

Figure 2.19: *Tabernaemontana elegans* known as the toad tree due to the large fruits it produces (PlantZAfrica, 2010)

The pulp of the white fruit is edible, but it used by the Zulu people of South Africa to speed up the curdling process by adding it to milk. There are a variety of traditional medicinal uses. The coagulated milky sap is as a styptic to stop bleeding, whereas a root infusion is used as a remedy for lung ailments and stomach aches. The maceration of the roots is used to treat tuberculosis and the dried powder of the inner wall of the fruit is used to treat cancer when boiled in water (PlantZAfrica, 2010).

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2.20 *Warburgia salutaris* (Bertol.f.) Chiov.

Warburgia salutaris, from the Canellaceae family, is commonly known as “the pepper-bark tree” (Fig 2.20). It is an evergreen tree which can reach heights of up to 5-10m in length. The leaves are simple and are arranged alternatively. Furthermore, the leaves are dark green and glossy with the bottom part of the leaf slightly paler. The leaves and the brown bark have a bitter taste and leave a burning sensation. The flowers are white to green in colour and can grow up to 7mm in diameter, which occur in the axils of the leaves. The flowers can occur singularly or in a small cluster and when develop turn into round, oval berries which turn purple in colour and leathery in texture. The distribution of the pepper bark tree is restricted to forests in KwaZulu Natal, Swaziland, Mpumalanga and the Northern Province (PlantZAfrica, 2010).

Figure 2.20: *Warburgia salutaris* is commonly known as the pepper bark tree because of its aromatic leaves (PlantZAfrica, 2010)

Ground bark is added to cold water, or smoked together with *Cannabis sativa* leaves, to treat dry coughs. The bark is also used in emetics or purgatives for febrile complaints and for rheumatism. Lotions made from the leaves are applied to the genital areas of Zulus to treat inflammation of the urethra. In West Africa the bark and roots are used for influenza, fevers, pains, stomach aches and gastro-intestinal problems (Hutchings *et al*., 1996). In the Sekenani valley in Kenya, the *Waburgia salutaris* is also used to treat malaria and chest problems. Plant extracts obtained from boiling the plant material is orally ingested (Bussmann *et al*., 2006). The powder of the plant is also applied to sores (Rabe & van Staden, 1997).

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3. Plant extraction

3.1 Plant collection

All the plant material was collected in southern Africa in the years 2010 to 2013. Several plants were collected from Venda, Limpopo province, whereas the remaining plants were collected from Walter Sisulu National Botanical Gardens (Roodepoort, Gauteng), with the help of the curator, Mr Andrew Hankey, as well as from the Manie van der Schijff Botanical Gardens (University of Pretoria, Gauteng), with the help of Mr Jason Sampson. The plants were identified and authenticated by Magda Nel at the H.G.W.J Schweickerdt Herbarium (PRU) of the University of Pretoria and voucher specimens were submitted. Ethanol extracts of the plants were prepared as described in section 3.2 of this chapter and some were donated by Prof Namrita Lall (as shown in Table 2.1 with *) from the University of Pretoria. A summary of the plant material is provided in table 2.1 below.

Table 2.1: Selected plants for the present study

	Plant	Plant part	PRU number
1.	*Acacia caffra* (Thunb.) Willd*	Leaves	120014
2.	*Acacia mellifera* (Vahl.) Benth*	Leaves	078373
3.	*Arbutus unedo* L.*	Leaves	120018
4.	*Buddleja saligna* Willd	Leaves	120009
5.	*Buddleja salviifolia* (L.) Lam	Leaves	120008
6.	*Calusena anisata* (Willd) Hook	Leaves and stems	96689
7.	*Clematis branchiate* Thunb*	Leaves	120016
8.	*Combretum molle* R.Br ex G. Don*	Leaves	120015
9.	*Dissotis princeps* (Kunth.) Triana	Leaves and stems	96679
10.	*Erythrophleum lasianthum* Corbishley*	Leaves	120019
11.	*Euclea divinorum* Hiern*	Leaves	120020
12.	*Gomphocarpus frticosus* (L.) Aiton f.*	Leaves	MN 1
13.	*Harpephyllum caffrum* Bernh.*	Leaves	120021
14.	*Helichrysum kraussii* Sch. Bip	Flowers, leaves and stems	96694
15.	*Helichrysum odoratissimum* (L.) Sweet	Leaves and stems	96677
16.	*Leucas martinicensis* (Jacq.) R.Br	Seeds and leaves	96690
17.	*Rapanea melanoploeos* (L.) Mez	Leaves and stems	119005
18.	*Syzygium jambos* (Alston.)*	Leaves	119053
19.	*Tabernaemontana elegans* Stapf.	Leaves and stems	96692
20	*Warburgia salutaris* (Bertol.f.) Chiov.*	Leaves	120013

* Plant extracts donated by Prof N.Lall

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3.2 Preparation of plant extracts

All plant material was collected and shade dried. The dried powder from each plant was macerated in distilled ethanol and left on a shaker for 48hrs. The filtrate of each plant was collected using a Buchner funnel. The extracts were collected after the filtrate was put under reduced pressure using a Rotavapor and placed in cold room until further use. The yields obtained from the plants are shown in Fig 2.23.

4. Results and discussion

The plant samples chosen for this study were selected from a variety of different plant families; 10% were selected from the Fabaceae, Apocynaceae and Buddlejaceae family respectively and 5% each from the rest of the plant families (Fig 2.21).

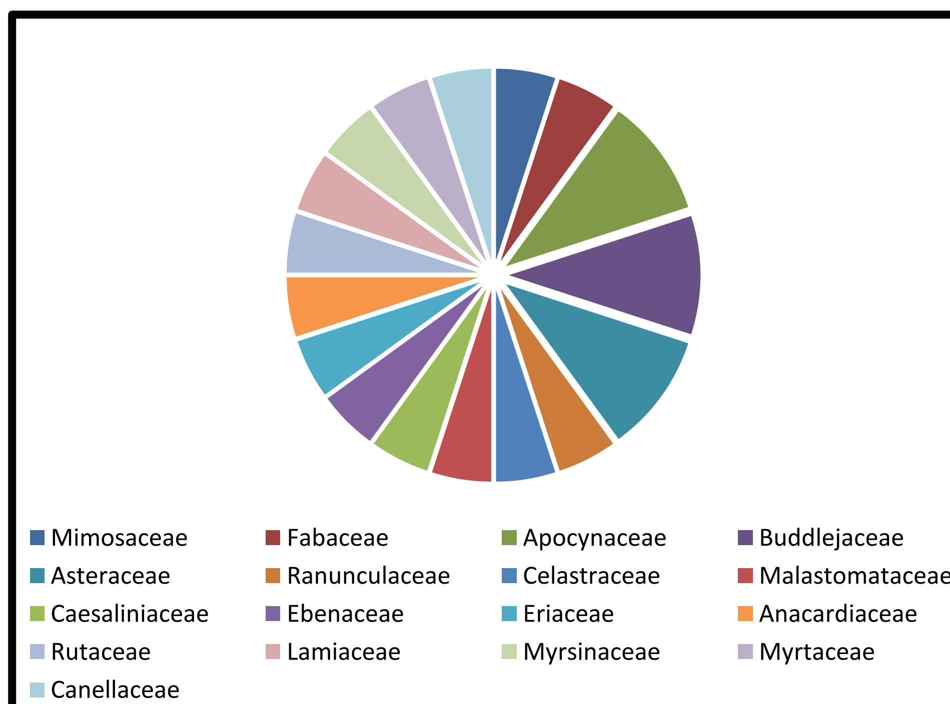

Figure 2.21: Plants selected from different plant families used in the study

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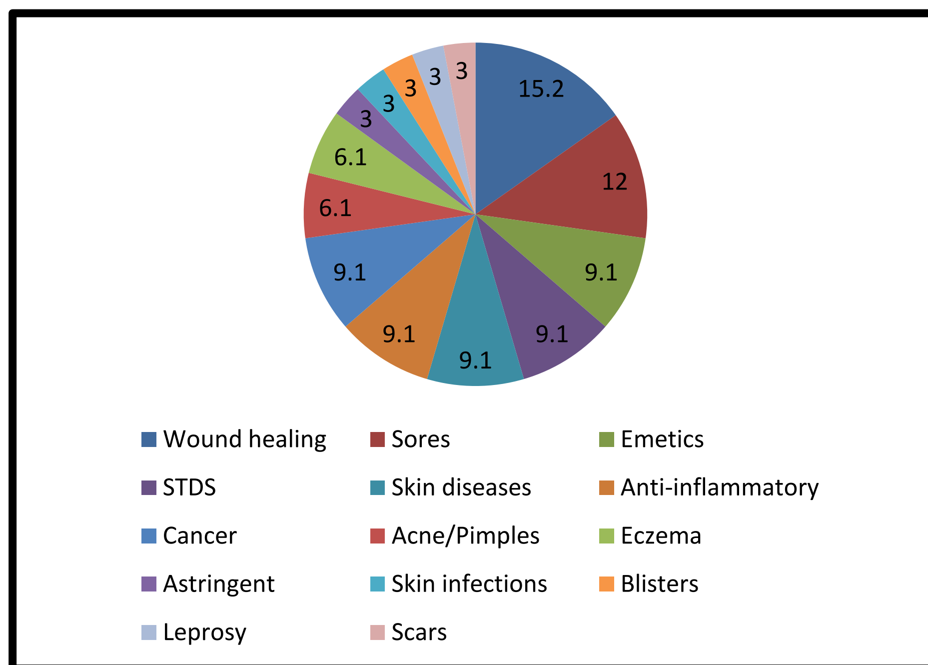

Figure 2.22: Various skin and cervical cancer related disorders treated using the selected plants

Fig 2.22 is a summary of diseases and disorders relevant to skin and cervical cancer which are treated using the twenty selected plant samples. The plants are mainly used for wound healing (15.2%) and for the treatment of sores (12%).

The extracts were prepared using ethanol as an extraction solvent, which is of medium polarity and is able to extract both non-polar compounds as well as polar compounds. Fig 2.23 depicts the weights obtained after shade drying of the different parts of the plants and the yields of the extracts.

The powdered weight and extract weights of the plant material were then used to determine the percentage yields for each sample (Fig 2.23). The percentage yields were calculated using the following formula:

$$\%\,Yield = \frac{Extract\ weight\ (g)}{Powdered\ weight\ (g)} X100$$

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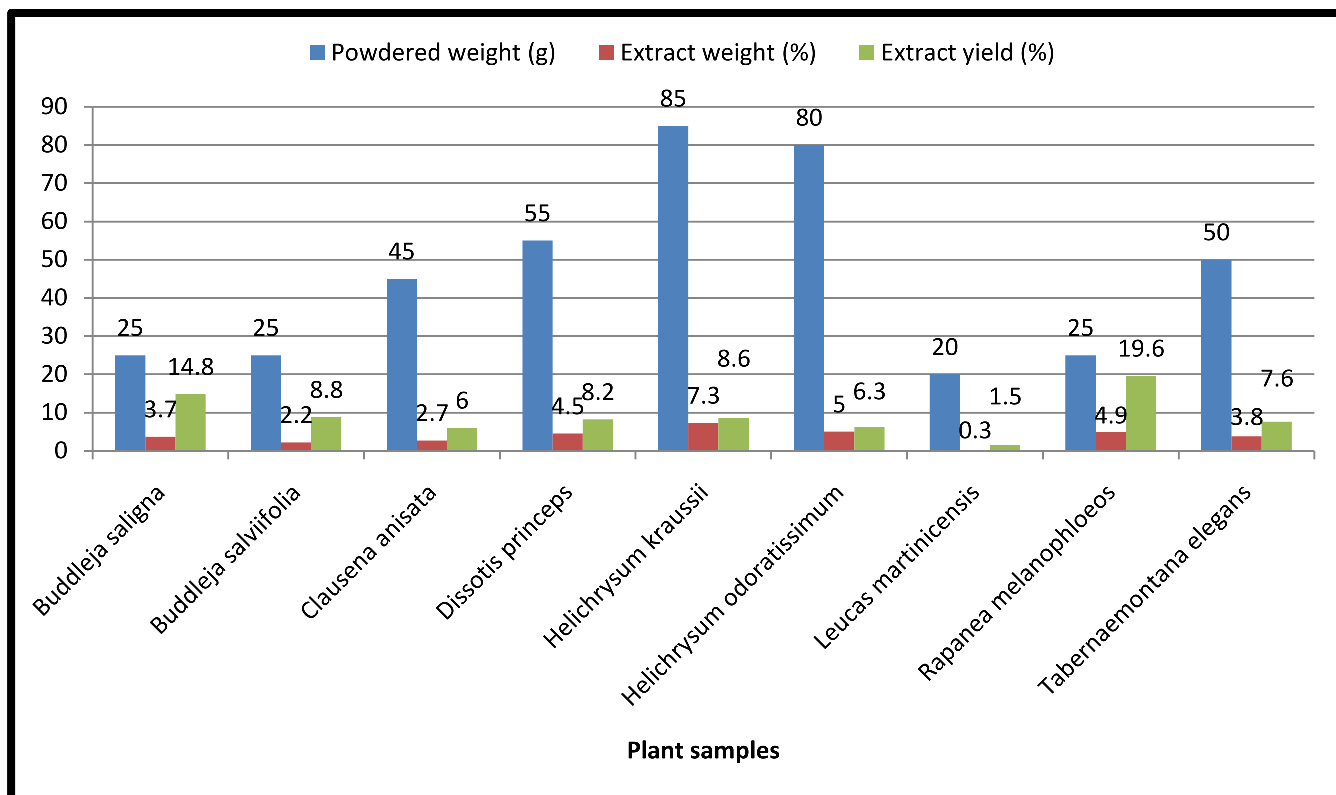

Figure 2.23: Dried weight, extract weight and percentage yield of extracts

The percentage yields differed greatly among the different plant extracts ranging from 1.5% to 19.6% with *R. melanophloeos* showing the greatest yield of 19.6% followed by *B. saligna* with a yield of 14.8% and *L. martinicensis* showing the lowest yield of 1.5%.

5. Conclusion

A range of plant families were chosen for this study, which were used mainly traditionally for the treatment of sores and wounds and which could be associated with skin and cervical cancer. All extracts were prepared successfully with ethanol as an extraction solvent. The percentage yield differed greatly among the extracts possibly due to the different amount of compounds extracted from each plant using ethanol.

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Chapter 3

Cytotoxicity of extracts against cancer cell lines

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1. Introduction

All plant extracts were tested for their cytotoxic activity using the 2,3-Bis-(2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxyanilide salt (XTT) cell viability reagent. The plant extracts were screened for their *in vitro* cytotoxicity effects on human malignant melanoma (A375), cervical epithelial carcinoma (HeLa) and epidermoid carcinoma (A431) cell lines. The plant extracts were also tested for their cytotoxic effect on non-cancerous cell lines such as human embryonic kidney cells (HEK-293). A synergistic study was performed on the A431 cells to determine whether the active plant extracts showed improved cytotoxic activity when combined.

1.1 Cell lines

The A375 cell line is an adherent cell line which was isolated from a 54-year old female with malignant melanoma (Fig 3.1). The cells were isolated from the skin of the female. These cells have 9 marker chromosomes and consist of 62 chromosomes in total (ATCC (a), 2012).

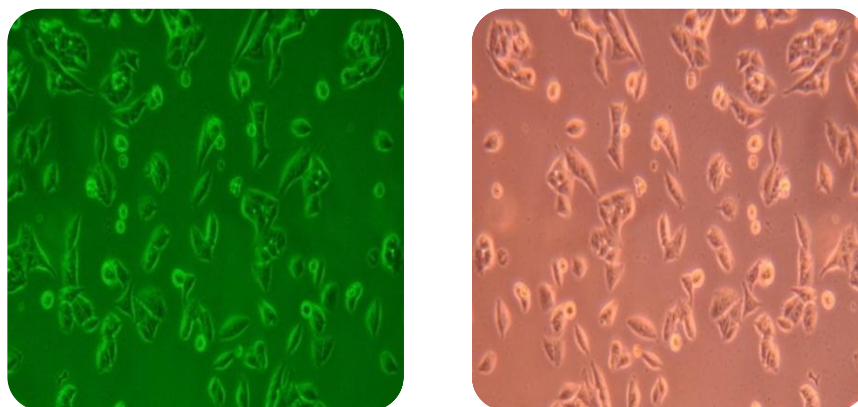

Figure 3.1: Low density A375 cells at 20X magnification

The A431 cell line is an adherent cell line which was isolated from an 85-year-old female's epidermoid carcinoma/squamous cell carcinoma (Fig 3.2). These cells have a total of 76 modal chromosomes which were found in 36% of the cells (ATCC (b), 2012).

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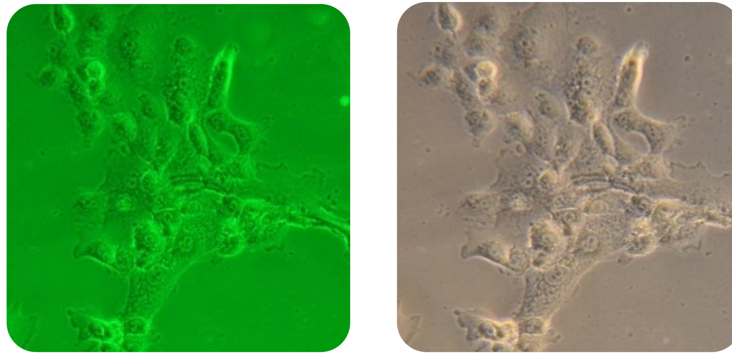

Figure 3.2: Low density A431 cells at 20X magnification

The HeLa cell line is an adherent cancerous cell line isolated from a female in the 1950's (Fig 3.3). The cells were harvested from a malignant cervical tumour from an African American named Henrietta lacks (HeLa Tranfection, 2011). In the HeLa cell line it has been reported that 4 marker chromosomes are present in the cells (ATCC (c), 2012).

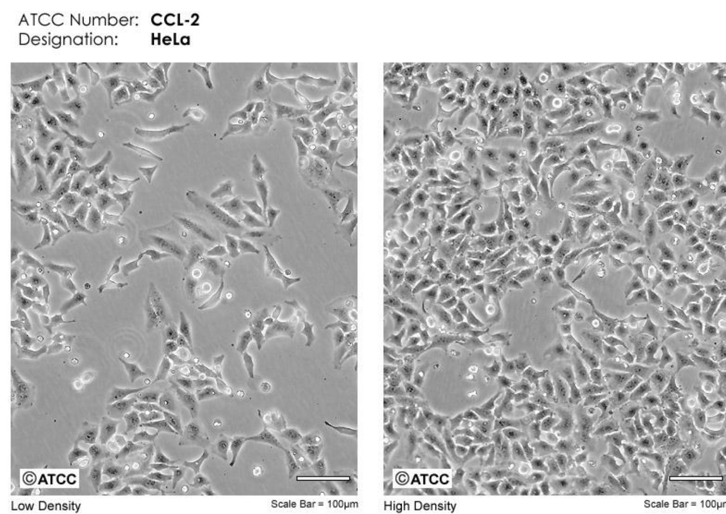

Figure 3.3: Low density and high density HeLa cells (ATCC (c), 2012)

The HEK-293 cell line is an adherent cell line which was isolated from a human fetus to obtain human embryonic kidney cells (Fig 3.4). It was found that in 30% of cells the total chromosome number was 64. Furthermore, there are 7 marker chromosomes which are found in most of the cells (ATCC (d), 2012).

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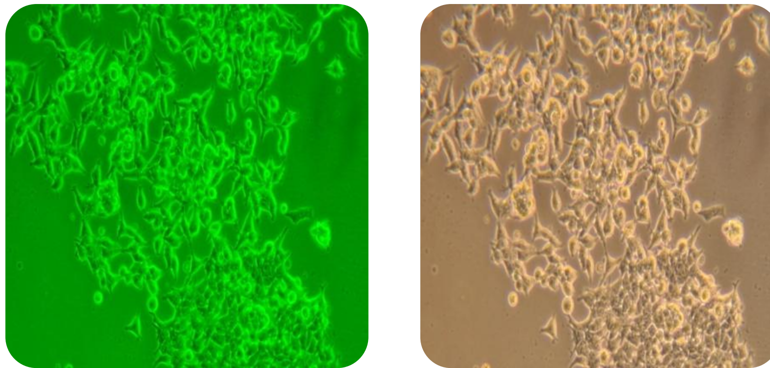

Figure 3.4: High density HEK-293 cells at 20X magnification

1.2 Positive control for cytotoxicity

Actinomycin D, also known as dactinomycin, is a known chemotherapeutic agent which damages the DNA of cancer cells. It is a used as an anti-cancer agent for a range of cancer types such as sarcomas, Wilm's tumour, testicular cancer, melanoma and chariocarcinoma (Cancer Research UK, 2012). Actinomycin D is also used as an antineoplastic antibiotic which is isolated from *Streptomyces parvulus.* It acts by intercalating between guanine-cytosine base pairs and blocking transcription by restricting the movement of RNA polymerase over the intercalated base pairs. It can also cause single-strand breakage of DNA by interacting with topoisomerase II or through a free-radical intermediate (National Cancer Institute, 2013).

1.3 XTT cell viability assay

The cytotoxicity assay was performed using the Cell Proliferation Kit II (XTT) which is used to determine the cell viability after exposure to various treatments. The XTT assay is a colorimetric assay which is based on the ability of viable cells to reduce the yellow tetrazolium salt to an orange formazan dye (Fig 3.5). The reduction of tetrazolium to formazan is due to the presence of a mitochondrial dehydrogenase enzyme present in metabolically active cells, which makes the conversion possible in living cells only. Metabolically active cells have intact mitochondrial membranes and cell membranes and therefore, contain the mitochondrial dehydrogenase enzyme. However, in cells that have been exposed to various treatments the cell membranes are disrupted and therefore, the enzyme is inactivated. Therefore, the amount of formazan produced is directly proportional to the amount of viable cells present (Roche, 2011).

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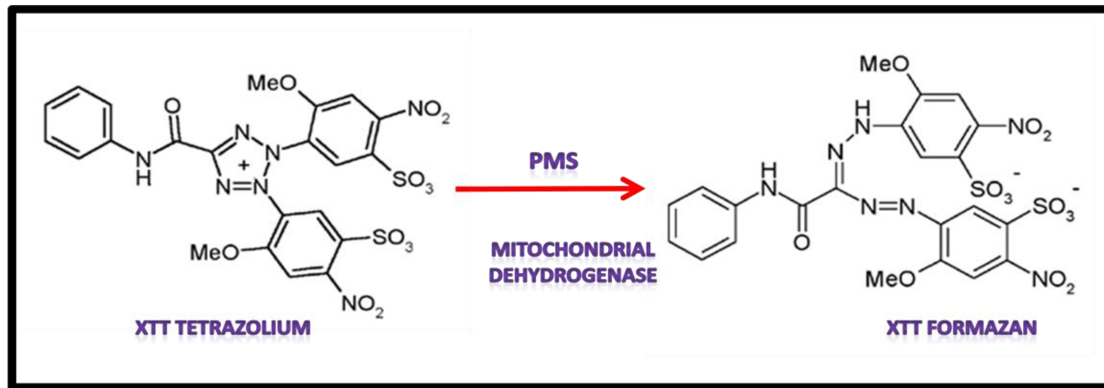

Figure 3.5: Reduction of tetrazolium salt to a formazan dye in the presence of mitochondrial dehydrogenase enzyme (AppliChem, 2010)

Included in the XTT kit is an electron coupling reagent known as N-Methylphenazonium methyl sulphate (PMS) which increases the sensitivity and efficiency of the XTT reduction (Fig 3.6).

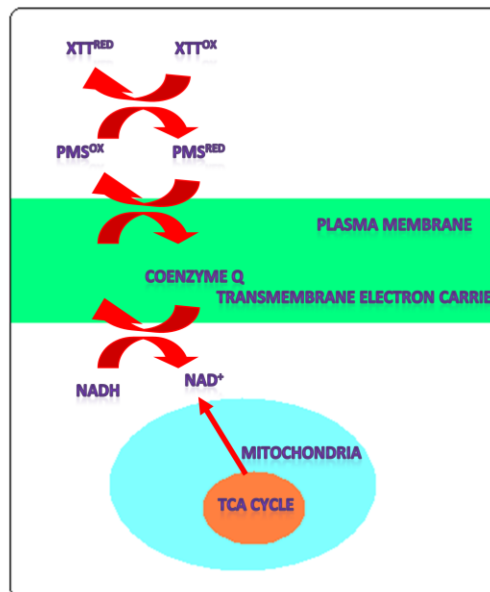

Figure 3.6: The colorimetric reduction of XTT by cellular enzymes (ATCC, 2011)

The reduction of tetrazolium to formazan has been reported to occur at the cell surface, which is facilitated by trans-membrane electron transport. The PMS activation reagent facilitates the reduction of XTT by binding to electrons occurring at the cell surface and therefore, forms a reactive intermediate which reduces the XTT (ATCC, 2011).

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2. Materials and methods

2.1 Materials

The A375 (CRL-1872), A431 (CRL-1555), HeLa (CCL-2) and HEK-293 (CRL-1573), were obtained from the American Type Tissue Collection (ATCC), MD, USA and European Collection of Cell Cultures (ECACC), England, UK. All the TPP® sterile plastic ware, cell culture flasks and fetal bovine serum (FBS) were purchased from Separations (Pty) Ltd. (Randburg, Johannesburg, RSA). The Eagles minimum essential medium (EMEM), RPMI, Dulbecco's modified eagles medium (DMEM), trypsin-EDTA and antibiotics (Penicillin-Streptomycin and fungizone) were supplied by Highveld Biological (Pty) Ltd. (Modderfontein, Johannesburg, RSA). The Cell Proliferation Kit II (XTT) used to carry out the cytotoxicity assay was purchased from Roche Diagnostics (Pty) Ltd. (Randburg, Johannesburg, RSA). All other chemicals and reagents were of analytical grade and were acquired from Sigma Chemicals Co. (St. Louis, MO, USA).

2.2 Methods

2.2.1 Cell culture

The cytotoxicity of the ethanol extracts was tested against the HeLa, A431, A375 and HEK-293 cell lines. The HeLa and A431 cell lines were maintained in culture flasks containing Eagle's Minimum Essential Medium (EMEM) supplemented with 10% heat-inactivated fetal bovine serum and 1% antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin and 250µg/ml fungizone), whereas with the A375 and HEK-293 cells, the EMEM was replaced with Dulbecco's Modified Eagles Medium (DMEM). The cells were grown at 37ºC in a humidified incubator set at 5% CO_2. Cells were sub-cultured after they formed a confluent monolayer on the flask. The cells were detached by treating them with trypsin-EDTA (0.25% trypsin containing 0.01% EDTA) for 10min at 37ºC and then adding complete medium to inhibit the reaction.

2.2.2 Counting cells using a hemocytometer

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Cells were counted in order to obtain the correct concentration for the assay. Confluent flasks were trypsinized to detach the cells from the surface of the flask. The reaction was inhibited after 10min by the addition of complete medium. The cells were centrifuged for 5min at 980rpm to form a pellet. The supernatant was discarded and the cell pellet was resuspended in enough complete medium to give a cloudy suspension (2-5ml). In an eppendorf tube a cell suspension of 1: 10 dilution was prepared in trypan blue solution (10µl cells in 90µl trypan blue). From this cell suspension 10µl was transferred to the two chambers of the hemocytometer. A hand-held tally was then used to count the number of cells under the microscope. The concentration of cells was determined using the following calculation:

$$\text{The number of cells counted averaged by the number of squares}$$

$$\text{Cell concentration (cell suspension)} = \#\text{ cells counted per square } x\ 10\ x\ 10000$$

$$= \text{cells per ml}$$

$$\text{Cell number (cell suspension)} = \text{cell concentration } x \text{ volume of cell suspension}$$

$$\text{Volume (cell suspension)} = \frac{\text{Cell concentration wanted } x \text{ volume wanted}}{\text{Concentration of cells in suspension}}$$

2.2.3 *In vitro* cytotoxicity assay

The cytotoxicity of the ethanol extracts were measured by the XTT colorimetric assay using the Cell Proliferation Kit II (Roche Diagnostics). The method described by Zheng *et al* (2001) was used to perform the assay. The cells were seeded (100µl) in a 96-well microtitre plate at a concentration of $1x10^5$cells/ml and incubated for 24h at 37ºC and 5% CO_2 to allow the cells to attach. The extracts were serial diluted and added to the plates at a concentration ranging from a 400µg/ml – 1.563µg/ml for each extract and incubated for a further 72h. The control wells included vehicle treated cells exposed to 2% DMSO and the positive control Actinomycin D with concentrations ranging between 0.5µg/ml – 0.002µg/ml. After 72hrs, XTT (50µl) was added to a final concentration of 0.3mg/ml and incubated for another 2 hours. The absorbance was read at 490nm with a reference wavelength set at 690nm using a BIO-TEK Power-Wave XS multi-well plate reader (A.D.P., Weltevreden Park, South Africa) using the KJ Junior software (Highland Park, Winooski, Vermont, USA). The assay was performed in triplicate to calculate a 50% inhibitory concentration (IC_{50}) of the cell population for each extract. The results were analysed using the GraphPad Prism 4 program (Version 4 Graph Pad Software, San Diego, CA, USA).

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2.2.4 Statistical analysis

All cytotoxicity results were analysed using GraphPad Prism 4 statistical program (Version 4 Graph Pad Software, San Diego, CA, USA). During the analysis a 95% confidence interval was chosen as well as a sigmoidal dose response (variable slope) curve fit. The analysis was limited to values between 0 and 100 and the DMSO treated control cells were used as the 100% viable parameter. The IC_{50} values were determined from the sigmoidal dose response curve fit, which in turn were used to calculate the SI values of the active plant extracts only. The selectivity index (SI) value was calculated according to the following equation:

$$SI = \frac{IC_{50}\ value\ on\ non-cencerous\ cells}{IC_{50}\ value\ on\ cancerous\ cells}$$

2.2.5 Synergistic activity

To determine the combined effect of the selected plant extracts on the A431 cells a method described by Van Vuuren (2007) was used with slight modifications. Briefly stock concentrations of 2mg/ml were prepared for both *H. odoratissimum* and *S. jambos*. The plant extracts were prepared in various ratios; 9:1, 8:2, 7:3, 6:4, 5:5, in a 96-well plate with *H. odoratissimum* always being the highest in concentration (Fig 3.7). Serial dilutions, for each ratio, were made by adding 200µl of medium to each well to prepare concentrations ranging from 1000µg/ml to 7.81µg/ml.

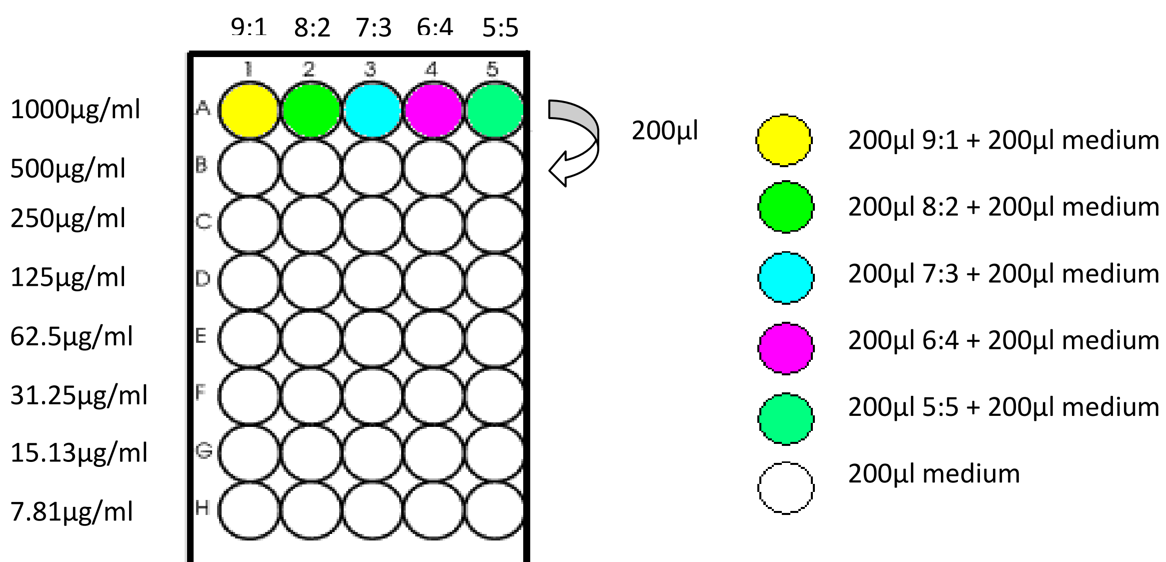

Figure 3.7: Preparation of different concentrations of plant extracts

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In a 96-well plate, A431 cells were plated at a concentration of $1X10^5$cells/well, as described in section 2.2.2 and 2.2.3 of this chapter. After cells had attached, 100µl of the above prepared serial dilutions were added to the plates with concentrations ranging between 500µg/ml to 3.4µg/ml and incubated for a further 72h at 37ºC and 5% CO_2. Thereafter the IC_{50} values were determined for each ratio using the same procedures as described in section 2.2.3 of this chapter.

Once the IC_{50} values were determined, the fractional inhibitory concentrations (FIC) were calculated, where *H. odoratissimum* is indicated as (a) and *S. jambos* as (b):

$$FIC1 = \frac{IC_{50}(a) in\ combination\ with\ (b)}{IC_{50}(a) independently}$$

$$FIC2 = \frac{IC_{50}(a) in\ combination\ with\ (b)}{IC_{50}(b) independently}$$

The ΣFIC index was used, using the FIC values, to determine the correlation between the two plant extracts and therefore, can either be synergistic (≤0.5; total effect is greater than the sum of the two extracts together), additive (>0.5-1.0; same as the sum of the two extracts together), non-interactive (>1.0-≤4.0; there is no effect when combining the extracts) or antagonistic (>4.0; the effect of the one extract is interfering with the effect of the second extract). The equation to determine ΣFIC index is as follows:

$$\Sigma FIC\ index = FIC1 + FIC2$$

3. Results and discussion

3.1 Cytotoxicity

The twenty plant extract were tested for their cytotoxic activity against the HeLa, A431 and A375 cell line (see Appendix A). Furthermore, the extracts were also tested against a non-cancerous HEK-293 cell line to determine whether the plant extracts showed any toxicity towards normal human cells. From the toxicity data, on both the cancerous and non-cancerous cell lines, the selectivity index of the lead extract was determined, which is an indication of whether the extract is more specific

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towards the cancerous cells or the non-cancerous cells. According to the US National Cancer Institute guidlines the limit of activity for a crude extract to have a cytotoxic effect after 72hrs incubation should be if the IC_{50} value is <20µg/ml (Mahavorasirikul *et al.*, 2010). Therefore, plant extracts were classified as active if their IC_{50} values complied with these guidlines and therefore, the SI values were only calculated for the active extracts. Table 3.1 depicts the toxicity data obtained on cancerous and non-cancerous cells.

Table 3. 1: Cytotoxicity of extracts on cancerous and non-cancerous cell lines

Extracts/cell line	IC_{50}[a] (µg/ml) HeLa	A431	A375	HEK-293
***Acacia caffra* (Thunb.) Willd**	185±0.4	132±0.7	>200	>200
***Acacia mellifera* (Vahl.) Benth**	54.4±1.8	61.7±0.3	180±0.8	>200
***Arbutus unedo* L.**	174±25	112±0.4	>200	>200
***Buddleja saligna* Willd**	63.6±8.1	34.4±0.2	66±5.2	72.1±3.4
***Buddleja salviifolia* (L.) Lam**	>200	>200	>200	>200
***Clausena anisata* (Willd) Hook**	108±0.1	176±18	234±4.3	295±0.3
***Clematis brachiata* Thunb.**	>200	>200	>200	>200
***Combretum molle* R.Br. ex G.Don**	100±5.3	174±6.9	>200	132±0.3
***Dissotis princeps* (Kunth.) Triana**	>200	>200	>200	>200
***Erythrophleum lasianthum* Corbishley**	>200	147±3.4	58.6±2.4	>200
***Euclea divinorum* Hiern**	107±2.4	66.6±0.6	133±1.5	177±1.2
***Gomphocarpus fruticosus* (L.) Aiton f.**	51.4±2.6	>200	>200	>200
***Harpephyllum caffrum* Bernh.**	62.5±5.0	68.3±0.9	106±3.8	135±0.1
***Helichrysum kraussii* Sch. Bip**	34.9±1.2	86±4.0	142±13	151±2.6
***Helichrysum odoratissimum* (L.) Sweet**	33.9±0.1	15.5±0.2	55.5±6.6	37.1±4.8
***Leucas martinicensis* (Jacq.) R.Br**	121±0.4	100±0.1	>200	>200
***Rapanea melanophloeos* (L.) Mez**	63.2±0.3	>200	105±4.5	152±0.2
***Syzygium jambos* (Alston.)**	56.2±2.6	54.7±0.6	198±2.8	>200
***Tabernaemontana elegans* Stapf.**	192±0.6	66.7±2.0	>200	>200
***Warburgia salutaris* (Bertol.f.) Chiov.**	>200	171±6.8	>200	>200
Actinomycin D[b]	$5x10^{-3}$±0.001	$3.9x10^{-2}$±0.001	$3.5x10^{-2}$±0.001	$3x10^{-3}$±0.001

[a] Fifty percent inhibitory concentration
[b] Positive drug control

3.1.1 *Acacia caffra* (Thunb.) Willd

A.caffra showed low toxicity in all three cell lines tested with IC_{50} values of 185±0.4µg/ml, 132±0.7µg/ml and >200µg/ml on HeLa, A431 and A375 cells respectively. On the HEK-293 cells

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low toxicity was seen with an IC_{50} value of >200µg/ml. No previous results on the cytotoxicity of *A. caffra* on cancerous and non-cancerous cells were obtained.

3.1.2 *Acacia mellifera* (Vahl.) Benth

A.mellifera showed more toxicity against the cancerous cell lines as compared to *A. caffra* exhibiting IC_{50} values of 54.4±1.8µg/ml, 61.7±0.3µg/ml and 180±0.8µg/ml on HeLa, A431 and A375 cells respectively. The cytotoxicity on the non-cancerous cells was similar to that of *A. caffra* showing low toxicity with an IC_{50} value of >200µg/ml. In a study conducted by Mutai *et al* (2004) compounds were isolated from the dichloromethane stem bark extract of *A. mellifera*. The isolated compounds were tested for activity on the NSCLC-N6 cell line, derived from a human non-small-cell bronchopulmonary carcinoma. Some compounds showed great activity while others were moderately active and other non-active. The greatest activity was seen for 28-hydroxy-lup-20-(29)-en-30-al and 28-hydroxy-3-oxo-lup-20-(29)-en-30-al with IC_{50} values of 11±0.02µg/ml and 15±0.06µg/ml respectively.

3.1.3 *Arbutus unedo* L.

A.unedo showed low toxicity on both the cancerous and non-cancerous cell lines with IC_{50} values of 174±25µg/ml, 112±0.4µg/ml, >200µg/ml, and >200µg/ml for HeLa, A431, A375 and HEK-293 cells respectively. In a similar study conducted by Andrade *et al* (2009), where the toxicity of the ethanol and acetone leaves/stems/bark extract of *A. unedo* was tested on human fibroblasts, very high toxicity was noted, which differed from the results in the present study where very low toxicity was observed. The viability resulted in four independent studies of *A. unedo* extract showed that both the ethanol and acetone extract decreased the viability of the human fibroblasts.

3.1.4 *Buddleja saligna* Willd

B. saligna showed moderate toxicity in both the cancerous cell lines and the non-cancerous cell lines. On the cancerous cell lines *B. saligna* showed the greatest toxicity on the A431 cells with an IC_{50} value of 34.4±0.2µg/ml and moderate toxicity on the HeLa and A375 cells with IC_{50} values of 63.6±8.1µg/ml and 66±5.2µg/ml respectively. Moderate toxicity with an IC_{50} value of

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72.1±3.4µg/ml was seen on the HEK-293 cell line. In a similar study compounds namely BS1, BS2, BS3 and BS4, were isolated from the hexane fraction of *B. saligna* leaves and stems. The compounds were tested for cytotoxicity on Chinese Hamster Ovarian (CHO) cells and the IC_{50} values were found to be >100µg/ml for BS1, BS2 and BS4 and 41.4µg/ml for BS3 (Bamuamba, 2005).

3.1.5 *Buddleja salviifolia* (L.) Lam

B. salviifolia showed very different results to that of *B. saligna* with low toxicity on all the cell lines with IC_{50} values of >200µg/ml for each. In a study conducted by Jonville *et al* (2011) similar results were obtained for the dichloromethane (DCM) leaf extract of *B. salviifolia* where very low toxicity was observed on cancerous and non-cancerous cell lines. The extract was tested on human fibroblasts (WS1), human colorectal adenocarcinoma (DLD-1) and human lung carcinoma (A549) with IC_{50} values all >200µg/ml. The same results were obtained for the methanol leaf extract on the cell lines with IC_{50} values all >200µg/ml, however the DCM bark extract showed an indication of slightly greater toxicity with IC_{50} value of 162µg/ml, 88µg/ml and 103µg/ml for WS1, DLD-1 and A549 respectively.

3.1.6 *Clausena anisata* (Willd) Hook

C. anisata showed low toxicity on all the cell lines with IC_{50} values of 108±0.1µg/ml, 176±18µg/ml, 234±4.3µg/ml and 295±0.3µg/ml for HeLa, A431, A375 and HEK-293 cells respectively. In a study where an acetone leaf extract of *C. anisata* was tested for cytotoxic activity on African green monkey cells (Vero) results obtained varied from the results obtained on the non-cancerous cells in the present study, with an IC_{50} value of 170µg/ml (Adamu *et al*., 2012).

3.1.7 *Clematis brachiata* Thunb.

C. brachiata showed very low toxicity on all the cell lines tested with IC_{50} values >200µg/ml for HeLa, A431, A375 and HEK-293 cells. In a study conducted by Okalebo *et al* (2005) the stem and leaf methanol extracts of *C. brachiata* were tested for toxic effects on brine shrimp larvae with fifty percent lethal dose (LD_{50}) of 365.60mg/ml and 66.5mg/ml respectively.

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3.1.8 *Combretum molle* R.Br. ex G.Don

C. molle exhibited low toxicity on all cancerous and non-cancerous cell lines with IC_{50} values of 100.4±5.3µg/ml, 174±6.6µg/ml, >200µg/ml, and 132±0.3µg/ml for HeLa, A431, A375 and HEK-293 cells respectively. In a similar study where a methanol (MeOH) extract of the leaves and roots of *C. molle* were prepared and tested for their cytotoxicity activity against HeLa cells results were comparable to the results in the present study with IC_{50} value of 72.6±1.20µg/ml and 75.6±1.67µg/ml for the leaves and roots respectively. The extracts were furthermore, tested on bladder cancer (T24) and breast cancer (MCF7) with more toxicity seen on T24 cells with IC_{50} values of 27.7±13.5µg/ml and 26.3±1.8µg/ml for the leaf and root extract respectively. The toxicity on the MCF7 cells was slightly lower than on the T24 cells with IC_{50} values of 42.6±4.9µg/ml and 49.4±6.9µg/ml for the leaf and root extract respectively (Fyhrquist *et al*., 2006).

3.1.9 *Dissotis princeps* (Kunth.) Triana

D. princeps showed very low toxicity on all the cancerous cell lines and the non-cancerous cell line. On all the cell lines the IC_{50} values were determined as >200µg/ml. No previous cytotoxicity reports for *D. Princeps* were found in literature.

3.1.10 *Erythrophleum lasianthum* Corbishley

E. lasianthum showed similar results to that of *D. princeps* with very low toxicity observed on the cancerous cell lines and the non-cancerous cells with IC_{50} values of >200µg/ml. In a similar study, the cytotoxicity of *E. lasianthum* leaf extract was tested on peripheral blood mononuclear cells (PBMCs) and was determined to have non-toxic effects at 10mg/ml for the hexane, ethyl acetate, methanol and chloroform fractions (Adebayo *et al*., 2013).

3.1.11 *Euclea divinorum* Hiern

E. divinorum showed low activity against the HeLa and A375 cells with IC_{50} values of 107±2.4µg/ml and 133±1.5µg/ml respectively. The extract however, did show moderate activity on the A431 cells with an IC_{50} value of 66.6±0.6µg/ml. On the non-cancerous cell line, low toxicity was seen with an

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IC_{50} value of 177±1.2µg/ml. Compounds isolated from the chloroform root bark extract of *E. divinorum*, which showed activity on certain cancer cell lines were identified as 3β-(5-hydroxyferuloyl)lup-20(30)-ene (compound 1) and 7-methyljuglone (compound 3). Compounds 1 showed activity on only two cell lines with IC_{50} values of 2.1µg/ml and 4.2µg/ml on (murine lymphocytic leukemia) P-388 and (human breast cancer) ZR-75-1 cell respectively. Compound 3 however, showed specificity against five various cell lines with IC_{50} values of 4.8µg/ml, 0.1µg/ml, 0.8µg/ml, 2.2µg/ml and 2.7µg/ml for (human nasopharyngeal carcinoma) KB, P-388, (human prostate cancer) LNCaP, ZR-75-1 and (human glioblastoma) U373 cells respectively (Mebe *et al*., 1998).

3.1.12 *Gomphocarpus fruticosus* (L.) Aiton f.

G. fruticosus showed moderate activity against the HeLa cell line with an IC_{50} value of 51.4±2.6µg/ml. Against the other two cancerous cell lines, low activity was noted with an IC_{50} value of >200µg/ml for both the A431 and A375 cell line. On the non-cancerous cell lines low activity was also observed with IC_{50} values of >200µg/ml. In a previous study, the total growth inhibition (TGI) values obtained for the methanol leaf/stem extract of *G. fruticosus* on (Renal cancer) TK10, MCF7 and (melanoma) UACC62 were <0.00µg/ml, 14.78µg/ml and 12.68µg/ml respectively. The DCM:MeOH root and fruit extract showed an overall lower activity on the cancer cell lines with IC_{50} values of 6.38µg/ml (TK10), 10.66µg/ml (MCF7), <0.00µg/ml (UACC62) and 6.78µg/ml (TK10), 30.20µg/ml (MCF7), 11.32µg/ml (UACC62) for the root and fruit extract respectively (Fouche *et al*., 2008).

3.1.13 *Harpephyllum caffrum* Bernh.

H. caffrum showed moderate activity on the HeLa and A431 cell lines with IC_{50} values of 62.5±5.0µg/ml and 68.3±0.9µg/ml respectively. On the A375 cells lower activity was observed with an IC_{50} value of 106±3.8µg/ml as compared to the HeLa and A431 cell lines. On the non-cancerous cells similar results were seen to that of the other extracts with an IC_{50} value of 135±0.1µg/ml. In a similar study where the ethanol leaf extract of *H. caffrum* was tested for cytotoxic activity on human hepatocellular carcinoma (HepG2), larynx carcinoma (Hep2) and colon carcinoma (HCT 116), IC_{50} value of 1.21µg/ml, 1.34µg/ml and 3.62µg/ml were obtained, which showed more activity than on the cancerous cell lines used in the present study (Shabana *et al*., 2011).

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3.1.14 *Helichrysum kraussii* Sch. Bip

H. kraussii showed relatively good activity against the HeLa cell line with an IC_{50} value of 34.9±1.2µg/ml however, it showed weaker activity against the other cancer cell lines with IC_{50} values of 86±4.0µg/ml and 142±13µg/ml for the A431 and A375 cell line respectively. On the non-cancerous cell line low toxicity was seen with an IC_{50} value of 151±2.3µg/ml. In a similar study conducted by Lourens *et al* (2011) the leaves and stems of *H. Kraussii* were extracted using a 1:1 ratio of chloroform: methanol and tested for toxicity against human kidney epithelial cells (Graham), MCF7 cells and glioblastoma (SF-268) cells. The extract showed great potential as an anticancer agent aganist MCF7 cells with an IC_{50} value of 9.0µg/ml. Moderate toxicity was also observed on the SF-268 cells with an IC_{50} value of 45.2±4.4µg/ml and slightly higher toxicity on the non-cancerous Graham cells with an IC_{50} value of 28.6±1.4µg/ml.

3.1.15 *Helichrysum odoratissimum* (L.) Sweet

Out of all the plant extracts *H. odoratissimum* showed the greatest potential as an anticancer agent against HeLa cells and A431 cells with IC_{50} values of 33.9±0.1µg/ml and 15.5±0.2µg/ml respectively. This was the only extract that showed an IC_{50} value which complied with the US National Cancer Institute guidelines and the SI value obtained for *H. odoratissimum* was determined as 2.39. On the A375 cell line, moderate activity was observed with an IC_{50} value of 55.5±6.6µg/ml. On the HEK-293 cell line, moderate to high toxicity was observed with an IC_{50} value of 37.1±4.8µg/ml. The 1:1 ratio of chloroform:methanol leaf/stem extract of *H. odoratissimum* also showed great potential on MCF7 cells with an IC_{50} value of 7.4±0.7µg/ml. On the SF-268 cells, moderate activity was observed with an IC_{50} value of 48.2±1.4µg/ml and high toxicity on the non-cancerous Graham cells with an IC_{50} value of 17.5±0.4µg/ml (Lourens *et al*., 2011). Due to the high activity of this plant extract on A431 cells and the first time the ethanol extract has been reported for its use on A431 cells, a South African provisional patent has been filed (see Appendix C).

3.1.16 *Leucas martinicensis* (Jacq.) R. Br

L. martinicensis showed low toxicity on both the cancerous and non-cancerous cell lines with IC_{50} values of 121±0.4µg/ml, 100±0.1µg/ml for HeLa and A431 respectively and >200µg/ml for A375 and HEK-293 cells. No previous results on the cytotoxicity data were obtained.

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3.1.17 *Rapanea melanophloeos* (L.) Mez

R. melanophloeos showed moderate activity against the HeLa cells with an IC_{50} value of 63.2±0.3µg/ml. On the other cancerous and the non-cancerous cells low activity was seen with IC_{50} values of >200µg/ml, 105±4.5µg/ml and 152±0.2µg/ml for A431, A375 and HEK-293 cells respectively. In a thesis written by Gwala (2011) the bark of *R. melanophloeos* was extracted using hexane, chloroform, ethyl acetate, methanol and water. The extracts were tested for their toxicity using the brine shrimp lethality test where 50% lethal dose (LC_{50}) value of 1068.7mg/ml, 3648.3mg/ml, 41.58mg/ml, 346.32mg/ml and 30.9mg/ml were obtained for hexane, chloroform, ethyl acetate, methanol and water extracts respectively. A compound was isolated from the ethyl acetate extract, which was identified as 3β-Hydroxylanosta-9, 24-dien-21-oic acid. On HEK-293 cells and HepG2 cells the compounds showed IC_{50} values of 851.5µg/ml and 796µg/ml respectively. These results were similar with the results obtained in the present study where low toxicity was observed on both cancerous and non-cancerous cells.

3.1.18 *Syzygium jambos* (Alston.)

S. jambos leaf extract showed moderate activity against the HeLa and A431 cells with IC_{50} values of 56.2±2.6µg/ml and 54.7±0.6µg/ml respectively. Low activity was noted on the A375 and HEK-293 cells with IC_{50} values of 198±2.8µg/ml and >200µg/ml respectively. In a similar study low toxicity was noted for both a hexane and methanol leaf extract on Vero cells with IC_{50} values of 150µg/ml and 600µg/ml respectively (Sirivan, 2008).

3.1.19 *Tabernaemontana elegans* Stapf.

T. elegans showed low activity on two cancerous cell lines with an IC_{50} value of 192±0.6µg/ml and >200µg/ml on HeLa and A375 cells respectively. On the A431 cell line, moderate activity was observed with an IC_{50} value of 66.7±2.0µg/ml. On the HEK-293 cell line similar results were obtained as previous extract with an IC_{50} value of >200µg/ml. The results in the present study differed from the results obtained in the study conducted by Pallant *et al* (2012), where the ethanol root extract of *T. elegans* showed activity against the HepG2 cells and THP-1 macrophages with IC_{50} values of 5.81±4.85µg/ml and 16.77±9.56µg/ml respectively.

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3.1.20 *Warburgia saultaris* (Bertol.f.) Chiov.

W. salutaris showed very low activity on the cancerous cells and low toxicity on the non-cancerous cells with IC_{50} values >200µg/ml for the HeLa, A375 and HEK-293 cells and IC_{50} values of 171.6±6.8µg/ml for the A431 cells. In a similar study where the bark of *W. salutaris* was extracted with DCM and tested for toxicity on (mouse macrophages) Raw 264.7 results were similar to that in the present where at the 50µg/ml there was no significant difference in the number of cells to the number of cells that grew in the presence of DMSO (Madikane *et al*., 2007).

3.2 Synergistic study

For the combination studies *H. odoratissimum* was selected as it showed good anti-cancer activity on the A431 cell line with an IC_{50} value of 15.5±0.2µg/ml. The *S. jambos* extract was chosen because of its moderate anti-cancer activity on A431 cells and low toxicity on HEK-293 cells with IC_{50} values of 56.2±2.6µg/ml and >200µg/ml respectively. In Table 3.2 the IC_{50} values of the different ratios on the A431 cells can be seen as well as their SI values.

Table 3.2: Fifty percent inhibitory concentrations and selectivity indexes of the different plant extract ratios

	9:1	8:2	7:3	6:4	5:5
A431 (IC_{50} in µg/ml)	19.5±0.4	28.3±0.6	28.1±0.5	30.3±0.3	31.8±0.6
SI	2.34	1.39	1.11	0.7	0.59

Once the IC_{50} values were determined the FIC values and ΣFIC indexes were calculated for each ratio and for each cell line, using the above mentioned equations, to determine whether the extracts showed any synergistic activity at those specific ratios (Table 3.3).

Table 3.3: Calculated Fractional inhibitory concentrations on the A431 cell line

	FIC 1	FIC 2	ΣFIC index	Result
9:1	1.26	0.36	1.62	Non-interactive
8:2	1.82	0.52	2.34	Non-interactive
7:3	1.81	0.51	2.32	Non-interactive
6:4	1.96	0.55	2.51	Non-interactive
5:5	2.05	0.58	2.63	Non-interactive

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After determining the ΣFIC indexes of each ratio it was determined that not one of the ratios showed any synergistic effect and that all the ratios where calculated to be non-interactive. Therefore, the different ratios were not tested on HEK-293 cells (Table 3.3). The highest activity was seen at a 9:1 ratio where *H. odoratissimum* was at its highest concentration with an IC_{50} value of 19.5±0.4µg/ml.

4. Conclusion

It was evident from the cytotoxicity studies on the cancerous cells that *H. odoratissimum* showed the greatest potential as an anti-cancer agent with an IC_{50} value of 15.5±0.2µg/ml on the A431 cell line. Furthermore, this extract showed an SI value of 2.39, which indicates that the extract is targeted more towards the cancerous A431 cell line than the non-cancerous HEK-293 cell line. The SI value was far greater than that of Actinomycin D with a value of 0.08, which suggests that Actinomycin D is more toxic towards the non-cancerous cells than to the A431 cells. Finally when combining the *H. odoratissimum* extract with *S. jambos* extract, no synergistic activity was noted and rather non-interactive results were obtained. Therefore, for the determination of possible mechanism of action of *H. odoratissimum*, the lead plant extract was tested alone and not in combination with *S. jambos*.

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Chapter 4

Antioxidant activity of selected plant extracts

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1. Introduction

All twenty plant extracts were tested for their antioxidant activity using the DPPH and nitric oxide scavenging assay. The plant extracts were tested for their ability to scavenge DPPH and nitric oxide respectively, and compared to the antioxidant activity of a positive control, Vitamin C. From the results the fifty percent inhibitory concentration (IC_{50}) values were calculated and the Vitamin C equivalents in the case of DPPH scavenging activity.

A great part of drug discovery recently has been on the development of new agents for the treatment and prevention of cancer (Houghton *et al*., 2007). The prevention of cancer, which is more formally known as "Cancer chemoprevention", has been defined as the "reverse, suppression or prevention of progression of disease from pre-invasive cancer to frank malignancy" as described by Dr Michael Sporn (Sporn *et al*., 1976). These chemo-preventive agents have been grouped together into two classes; blocking agents, which prevent cellular damage; and suppressing agents, which reverse the damage caused by carcinogens. Depending on the stage at which chemo-prevention is introduced it can be grouped into three levels; primary, secondary and tertiary. Primary chemoprevention is the use of an active constituent to prevent pre-neoplastic lesions in healthy individuals. Secondary chemoprevention is aimed at the prevention of pre-malignant lesions in individuals and lastly, tertiary chemoprevention prevents previously treated cancer patients from developing cancer a second time (Issa *et al*., 2006).

The initiation of carcinogenesis is a result of DNA damage that occurs within the cell cycle. One of the major causes of DNA damage is the presence of reactive oxygen species (ROS) and free radicals, which are produced during cell metabolism, and after exposure to various physical and chemical agents. Examples of well known reactive oxygen species include superoxide ($O_2^{\cdot}$), hydroxyl ($OH^{\cdot}$), hydrogen peroxide (H_2O_2), lipid hydroperoxide (ROOH) as well as nitrogen species such as nitric oxide ($NO^{\cdot}$) and peroxynitrite ($ONOO^{\cdot}$). There are however, antioxidant enzymes within the human body which can scavenge these ROS and can therefore, reverse the damage done to DNA and reinstate the balance between the rate of ROS production and the rate at which these ROS are detoxified. Examples of such enzymes include superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase. There are certain antioxidant molecules which can also assist in the reversal of DNA damage such as glutathione and uric acid (Issa *et al*., 2006). ROS are not only a cause of cancer but also cause other diseases and disorders such as cardiovascular disease, premature aging and neurodegenerative diseases (Ames, 1998).

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Oxidative stress in the body is caused by an excess of free radicals which causes an imbalance which is present in cancerous cells. The increase of oxidative stress causes a change in genetic material and oncogenes resulting in DNA damage which is the initial step in carcinogenesis. Reactive oxygen species are responsible for the activation of AP-1 (activation protein) and NF-κB (nuclear factor kappa B) signal transduction pathways which induces the transcriptions of genes which regulate cell growth and pathogenesis of cancer. Antioxidants can suppress the effect of cancer cell growth by inhibiting the proliferation of cancer cells, inducing apoptosis, inhibiting the invasion of tumour cells and angiogenesis as well as suppressing inflammation and stimulating the phase II detoxification enzymes (Sen & Chakraborty, 2011).

It has furthermore, been reported that inflammation plays a major role in the development of cancer, which in turn can lead to an increase in chronic inflammation which causes the production of $NO^{\cdot}$ (Schottenfeld & Beebe-Dimmer, 2006; Rollins, 2006; Ohshima *et al*., 2003). The increased production of NO due to inflammation can lead to greater tumor growth due to the increase in migration, invasion and angiogenesis (Lala & Chakraborty, 2001).

Antioxidants can be classified into two groups, primary antioxidants which are known as enzymatic antioxidants, such as the ones mentioned above, as well as secondary antioxidants, which are known as non-enzymatic antioxidants. The enzymatic antioxidants are generally produced when maintaining a proper diet and therefore, are ever present in the body and can protect the body against ROS and free radicals. The non-enzymatic antioxidants are only present in the body when they are ingested through various food sources such as Vitamin A, C and E as well as α-lipoic acid, punicalagins and resveratrol (Tre, 2011).

Worldwide there has been a trend to use natural phytochemicals such as those present in fruits and vegetables as well as in teas, herbs, spices and various medicinal plants for their antioxidant effects (Lee *et al*., 2002). The most common antioxidant present in fruits and vegetables include vitamin C, carotenoids, lycopenes and flavanoids (Steinberg, 1991). Herbs and spices also contain a variety of antioxidants such as phenolic diterpenes, flavanoids, tannins and phenolic acids (Erkan *et al*., 2008). In a study conducted by Berrington & Lall (2012) it was found that two species of *Rosemary officinalis* showed good radical scavenging activity with IC_{50} values of 3.48±0.22 and 10.84±0.12 for McConnell's Blue and Tuscan Blue respectively. Examples of polyphenolic antioxidant which are present in a variety of food sources are depicted in Table 4.1 (Issa *et al*., 2006).

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Table 4.1: Polyphenolic antioxidants present in various food sources

Food source	Bioactive compound	Mechanism of action
Red grapes and red wine	Resveratrol	Induces apoptosis and inhibits angiogenesis
Turmeric	Curcumin	Induces apoptosis and the production of antioxidant enzymes
Green tea	Epigallocatechin gallate	Induces apoptosis and inhibits cyclooxygenase-2
Chili peppers	Capsaicin	Phase I enzymes are inhibited
Ginger	[6]-Gingerol	Cell signaling pathways are modulated
Onions	Quercetin	Inhibition of cyclooxygenase-2 and phospholipase A2 enzyme
Honey	Caffeic acid phenethyl ester	Production of Phase II enzymes
Broccoli	Isothiocayanates	Production of Phase II enzymes and induction of apoptosis

To determine the radical scavenging activity of antioxidants, measurements of the inhibition of free radicals such as 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) or 2,2-azinobis (3-ethyl-benzothiazoline-6-sulfonate) (ABTS) are taken. In this study the DPPH reagent was chosen as it is a much more stable free radical than ABTS (Du Toit *et al*., 2001) (Fig 4.1).

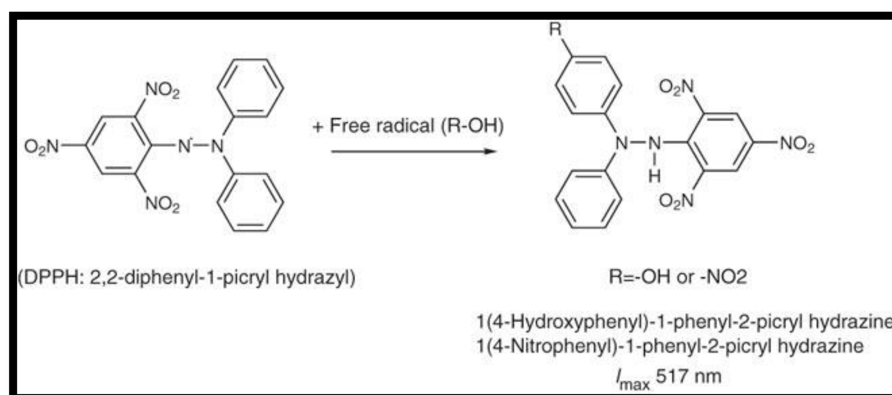

Figure 4.1: Principle of the 2,2-diphenyl-1-picryl hydrazyl (DPPH) assay (Nature protocols, 2008)

The DPPH assay is based on the interaction of the antioxidant with DPPH to convert it to the yellow compound diphenyl-picryl hydrazine. The ability to reduce DPPH therefore depends on the hydrogen donating ability of the antioxidant (Jayachitra & Krithiga, 2012).

To further determine the antioxidant activity of the samples, the nitric oxide (NO) scavenging assay was used. The nitric oxide radical inhibition assay is based on the ability of sodium nitroprusside to produce nitric oxide in an aqueous solution at a physiological pH (Fig 4.2). The nitric oxide is then converted into nitric and nitrous acids in the presence of oxygen and water. The amount of nitrous

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acid is directly proportional to the amount of purple azo dye formed in the presence of Griess reagent. Therefore, in the presence of an antioxidant the amount of nitrous acid is decreased, depending on the strength of the antioxidant and therefore, a decreased amount of purple azo dye is present (Murali *et al*., 2011). NO is an essential molecule which plays a role in regulating physiological processes such as immune response, cardiovascular dilation, blood pressure and neural signal transmission. However, nitric oxide can add to oxidative damage by reacting with superoxide to form peroxynitite anion, which can decompose into $OH^{\cdot}$ and NO_2 (Mayur *et al*., 2010).

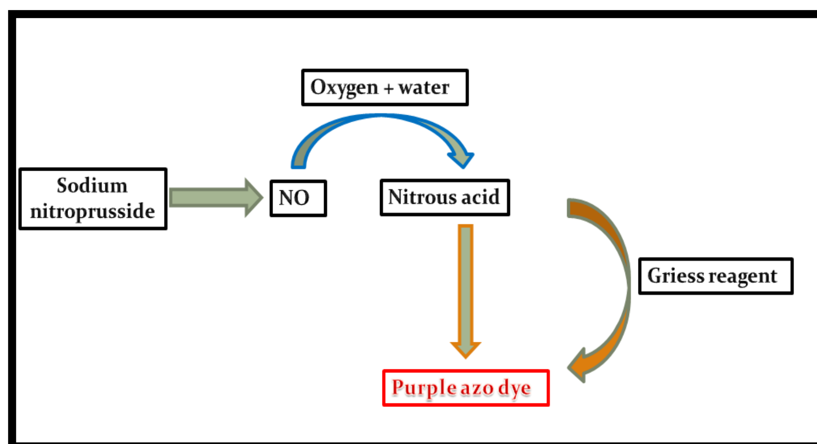

Figure 4.2: Reaction of sodium nitroprusside to nitrous acid in aqueous solution

In both the DPPH and the NO inhibition assays, Vitamin C, a well known antioxidant, was used as a positive control. Vitamin C, also known as ascorbic acid, is a lactone that is synthesized in the liver from glucose by most mammalian species. In humans however, the enzyme known as gulonolactone oxidase, which is used to produce vitamin C, is not present and therefore, vitamin C cannot be synthesized (Padayatty *et al*., 2003). Therefore, humans need to ingest vitamin C through various food sources such as cherries, papaya, melon, strawberries, and citrus fruits (Springboard, 2004). Vitamin C is commonly known for its electron donating capability which prevents compounds from being oxidized and therefore, is known as an antioxidant however, acting as a reducing agent Vitamin C itself is oxidized.

Vitamin C is converted to a free radical known as semihydroascorbate after the loss of one electron, which is relatively unreactive with a half life of 10^{-5} seconds (Fig 4.3). Therefore, when a free radical reacts with Vitamin C, the harmful free radical is reduced and the less reactive semihydroascorbate is produced in its place. After the loss of the second electron the compounds dehydroascorbic acid is formed. Both intermediates can be converted back to ascorbic acid via enzyme pathways or reducing

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compounds such as glutathione. However, in humans the reduction to ascorbic acid is not complete and therefore, there is not complete recovery of Vitamin C which could lead to the development of scurvy (Padayatty *et al*., 2003).

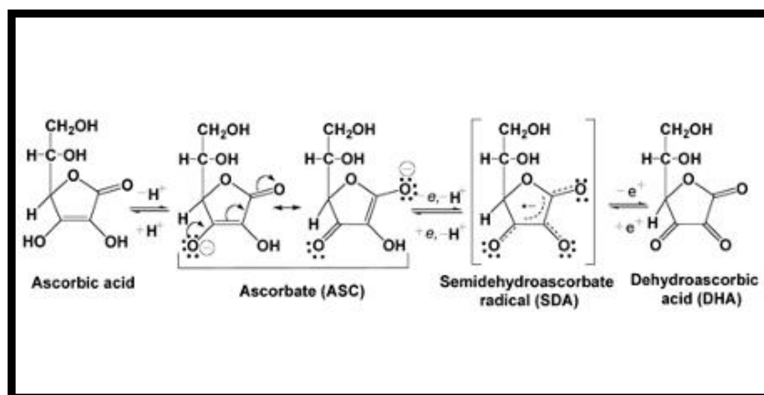

Figure 4. 3: Ascorbic acid a common antioxidant known for its electron donating capability (Farmatid, 2010)

2. Materials and methods

2.1 Materials

DPPH, ascorbic acid, sodium nitroprusside and Griess reagent were supplied by Sigma Chemicals Co. (St Louis, MO, USA), as well as all other reagents, which were analytical grade.

2.2 Methods

2.2.1 DPPH antioxidant assay

The method of Du Toit *et al* (2001) was followed to determine the radical scavenging capactity (RSC) of the plant extracts. Slight modifications to the method were made and are described briefly.

Stock solutions of Vitamin C and the extracts were prepared at concentrations of 2mg/ml and 10mg/ml respectively. Plant extracts which showed potent antioxidant activity, during the initial screening, were prepared at a stock concentration of 2mg/ml. To each well in a 96-well plate, 200µl of distilled water was added. To the rest of the wells 110µl of distilled water was added. Twenty microlitres of extract was added to the first top wells, in triplicate, followed by serial dilutions with final concentrations ranging from 3.9µg/ml to 500µg/ml for the extracts and 0.781µg/ml to 100µg/ml

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for Vitamin C (Mavundza *et al.,* 2010). Finally 90µl of 0.04M DPPH ethanolic solution was added to each well, except for the negative colour control, where distilled water was added instead. The plates were left to develop in a dark room for 30min. The RSC of the extracts was determined using a BIO-TEK Power-Wave XS multi-well reader (A.D.P., Weltevreden Park, South Africa) at a wavelength of 515nm, using KC Junior software (Highland Park, Winooski, Vermont, USA).

All plant extracts were tested in the same manner for their radical scavenging capacity and their IC_{50} values calculated using GraphPad Prism 4 software (Version 4 Graph Pad Software, San Diego, CA, USA) together with Windows Excel 2000. Lastly the Vitamin C equivalent for each extract was calculated as follows: (IC_{50} of extract X 200mg Vitamin C)/ IC_{50} of Vitamin C.

2.2.2 Nitric oxide scavenging assay

The method of Mayur *et al*. (2010) was followed to determine the nitric oxide scavenging capacity of the extracts.

Stock concentration of Vitamin C and the extracts were prepared at concentrations of 10mg/ml. Briefly, 90µl of distilled H_2O was added to the top row of a 96-well microtitre plate and 50µl to the rest of the wells in the plate. To the top wells 10µl of plant extract was added, in triplicate, followed by serial dilution with final concentration ranging from 7.81µg/ml to 1000µg/ml. Thereafter, 50µl of sodium nitroprusside was added to all the wells and the plate was incubated under light at room temperature for 90min. After the incubation period 50µl of Griess reagent was added to all the wells of the plate except for the negative colour control wells, where distilled water was added instead.

The plates were read using a BIO-TEK Power-Wave XS multi-well reader (A.D.P., Weltevreden Park, South Africa) at a wavelength of 546nm, using KC Junior software. All plant extracts were tested in the same manner for their nitric oxide scavenging capacity and their IC_{50} values calculated using GraphPad Prism 4 software together with Windows Excel 2000.

3. Results and discussion

The twenty ethanol extracts were tested for their antioxidant activity using the DPPH free radical scavenging assay and the nitric oxide scavenging assay. The DPPH assay was tested from a

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concentration of 500µg/ml to 3.90µg/ml, whereas the nitric oxide assay performed at concentrations starting from 1000µg/ml to 7.81µg/ml. In instances where the DPPH activity showed an IC_{50} below 3.90µg/ml the sample was re-tested at concentrations ranging between 100µg/ml and 0.78µg/ml. Lower IC_{50} values indicated plant extracts with greater antioxidant activity whereas high IC_{50} values were an indication of low antioxidant activity. The antioxidant activity of all twenty plant extracts based on NO scavenging activity has been reported in this study for the first time, whereas based on the DPPH scavenging activity, a few plant extracts have been reported earlier by other researchers in previous studies. A summary of the twenty plant extracts antioxidant activity is depicted in Table 4.2.

Table 4. 2: DPPH and Nitric oxide scavenging activity of plant extracts

Plant extracts	DPPH[a] IC_{50}[b]±SD in µg/ml	Vitamin C equivalents in g*	NO·[c] IC_{50}±SD in µg/ml
Acacia caffra **(Thunb.) Willd**	22.3±0.32	2.25	>1000
Acacia mellifera **(Vahl.) Benth**	129±3.15	13.03	>1000
Arbutus unedo **L.**	4.51±0.19	0.46	85.9±1.07
Buddleja saligna **Willd**	36.6±0.25	3.70	580.9±44.8
Buddleja salviifolia **(L.) Lam**	37.3±0.89	3.78	>1000
Clausena anisata **(Willd) Hook**	34.7±0.27	3.50	>1000
Clematis branchiate **Thunb.**	75.9±1.2	7.70	>1000
Combretum molle **R.Br. ex G.Don**	3.26±0.10	0.33	±1000
Dissotis princeps **(Kunth.) Triana**	2.94±0.01	0.30	±500
Erythrophleum lasianthum **Corbishley**	5.25±0.08	0.53	>1000
Euclea divinorum **Hiern**	8.30±0.02	0.84	>1000
Gomphocarpus fruticosus **(L.) Aiton f.**	32.5±1.2	3.28	±1000
Herpephyllum caffrum **Bernh.**	2.41±0.01	0.24	±250
Helichrysum kraussii **Sch. Bip**	4.66±0.05	0.47	±1000
Helichrysum odoratissimum **(L.) Sweet**	5.13±0.07	0.52	>1000
Leucas martinicensis **(Jacq.) R.Br**	34.5±0.24	3.48	>1000
Rapanea melanophloeos **(L.) Mez**	4.01±0.05	405	63.73±0.4
Syzyguim jambos **(Alston.)**	1.17±0.26	0.12	>1000
Tabernaemontana elegans **Stapf.**	157±8.3	15.86	>1000
Warburgia salutaris **(Bertol.f.) Chiov.**	464±0.01	46.87	>1000
Vitamin C[d]	1.98±0.01	-	285.9±25.88

[a] 2,2-diphenyl-1-picrylhydrazyl radical
[b] Fifty percent inhibitory concentration
[c] Nitric oxide
[d] Positive drug control
*Vitamin C equivalents calculated per 200mg Vitamin C capsule for DPPH activity

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3.1 *Acacia caffra*

A. caffra leaf extract showed relatively high antioxidant potential against DPPH free radical with an IC_{50} value of 22.3±0.32µg/ml while in the nitric oxide scavenging assay there was very low antioxidant potential with an IC_{50} value of >1000µg/ml (Fig 4.4 & 4.5). No previous reports regarding the antioxidant potential of *A. caffra* were found.

3.2 *Acacia mellifera*

A mellifera showed very low antioxidant activity in both the DPPH assay and the nitric oxide scavenging assay with IC_{50} values of 129±3.15µg/ml and >1000µg/ml respectively (Fig 4.4 & 4.5). The results obtained for the DPPH assay were much lower than those obtained for *A. caffra*. No previous reports on the antioxidant activity of *A. mellifera* were found. However, there are many reports on the antioxidant activity of *Acacia nilotica*. In a study conducted by Agrawal *et al*. (2010) a methanol extract of the stem bark of *A. nilotica* was prepared and tested for its antioxidant activity using the DPPH and nitric oxide assay. In both the assays the extract showed significant antioxidant activity with an IC_{50} value of 3.35µg/ml and 77.24µg/ml for the DPPH and nitric oxide scavenging assay respectively.

3.3 *Arbutus unedo*

A. unedo leaf extract showed potent DPPH antioxidant activity with an IC_{50} value of 4.51±0.19µg/ml. In the nitric oxide scavenging assay the ethanol extract showed an IC_{50} value of 85.9±1.07µg/ml which was much greater than that of Vitamin C with an IC_{50} value of 285.9±25.88µg/ml (Fig 4.4 & 4.5). In a similar study conducted by Orak *et al*. (2011) the methanol, ethanol and aqueous extract of *A. unedo* leaves, collected in Turkey, were evaluated for their DPPH scavenging activity. The IC_{50} values were 423.2µg/ml, 655.4µg/ml and 487.2µg/ml for the methanol, ethanol and aqueous extracts respectively which differed greatly from the ethanol extract results obtained in the present study (Orak *et al*., 2011). In a study conducted by Pavlović *et al*. (2011) the DPPH activity of ethanol extracts of *A. unedo* leaves collected from Greece and Montenegro were compared. In this study the ethanol extract obtained from Greece showed similar results as found in the present study with an IC_{50} value of 4.77±0.42µg/ml whereas the extract obtained from Montenegro showed slightly less activity with an IC_{50} value of 7.14±0.46µg/ml.

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3.4 *Buddleja saligna*

B. saligna leaf extract showed moderate antioxidant activity in both the DPPH and the nitric oxide scavenging assay with IC_{50} values of 36.6±0.25µg/ml and 580.9±44.8µg/ml respectively (Fig 4.4 & 4.5). However, in a study conducted by Adedapo *et al*. (2009) a methanol extract activity of *B. saligna* leaves was tested using DPPH, which showed a high antioxidant activity with an inhibition of 93.8% at 100µg/ml.

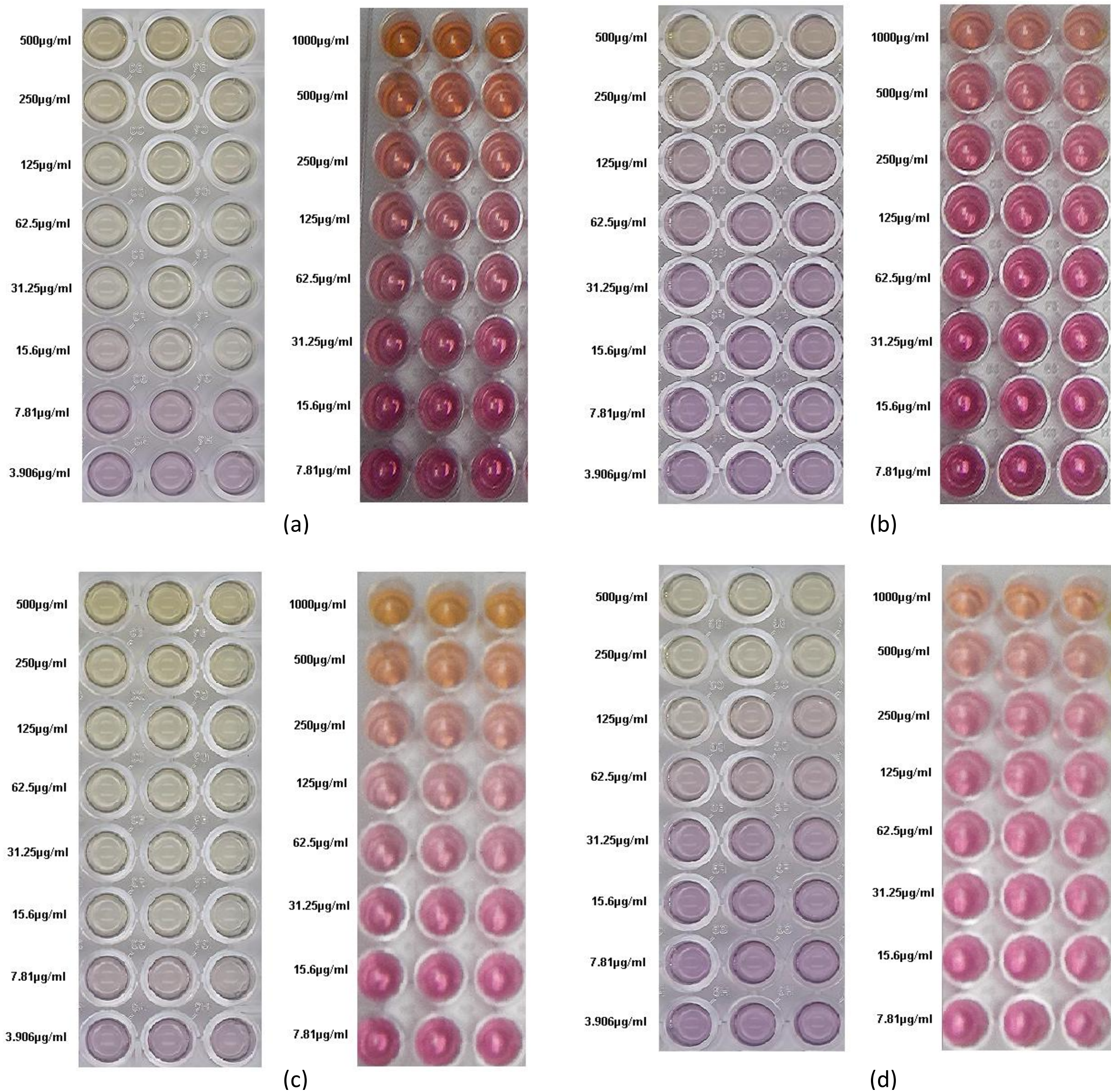

Figure 4.4: Free radical and nitric oxide colour change of ethanol extracts of (a) *Acacia caffra*, (b) *Acacia mellifera*, (c) *Arbutus unedo* and (d) *Buddleja saligna*

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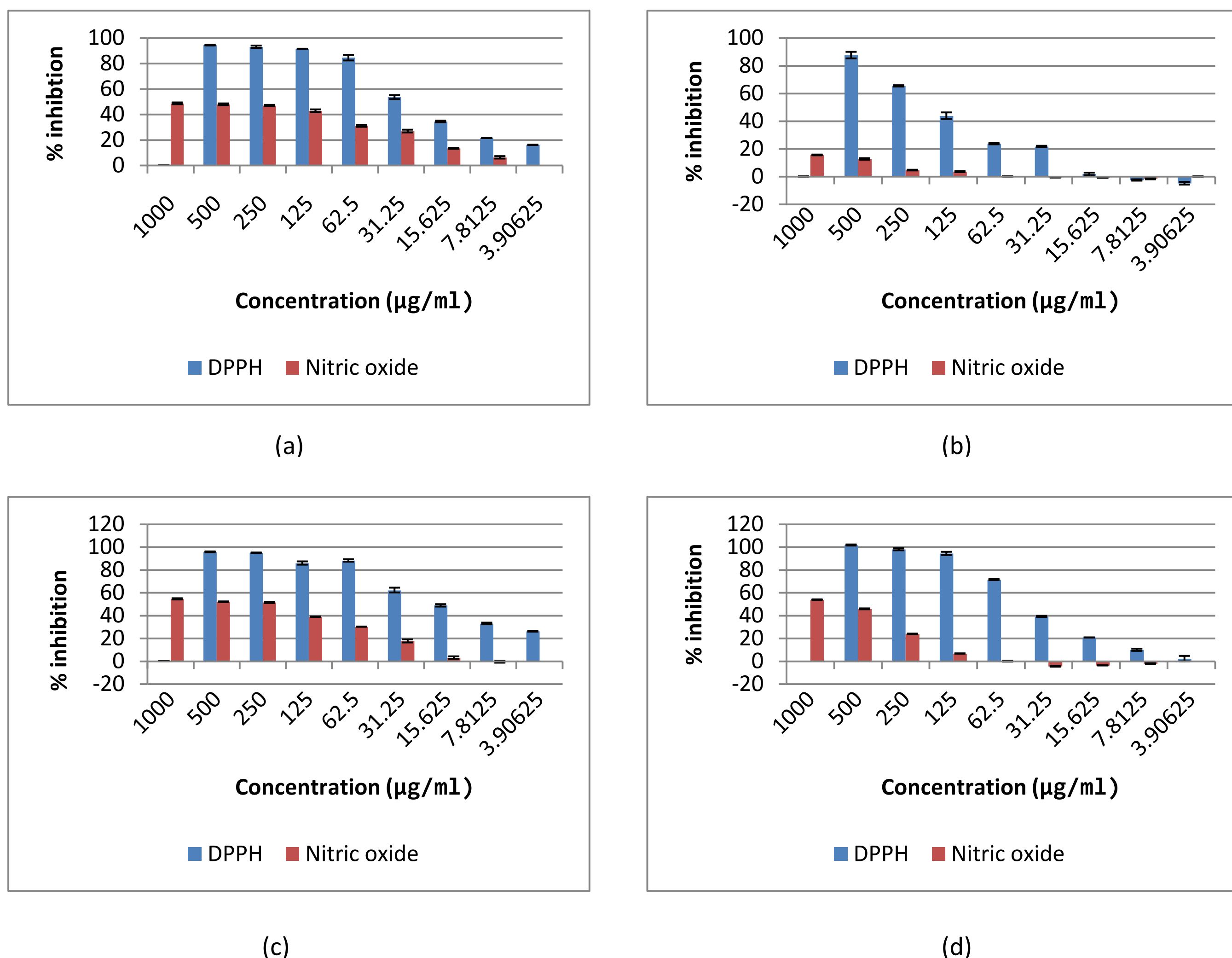

Figure 4.5: Free radical and nitric oxide activity of ethanol extracts of (a) *Acacia caffra*, (b) *Acacia mellifera*, (c) *Arbutus unedo* and (d) *Buddleja saligna*

3.5 *Buddleja salviifolia*

In the present study *B. salviifolia* showed a relatively good DPPH inhibition with an IC_{50} value of 37.3±0.89µg/ml, whereas in the nitric oxide scavenging assay a low activity was observed with an IC_{50} value of >1000µg/ml (Fig 4.6 & 4.7). Adewusi *et al.* (2011) conducted a study on the antioxidant potential of *B. salviifolia* whole plant extract to determine its activity on DPPH using two different solvents. Both extracts showed very low antioxidant activity with IC_{50} values of 0.23±0.01mg/ml and 1.60±0.51mg/ml for the dichloromethane: methanol (1:1) extract and the water extract respectively. In a similar study where a 50% methanol extract was prepared from the leaves of *B. salviifolia*, an inhibition of 94.2% at 100µg/ml was observed (Amoo *et al*., 2012).

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3.6 *Clausena anisata*

C. anisata showed relatively good radical scavenging activity in the DPPH assay with an IC_{50} value of 34.7±0.27µg/ml, whereas in the nitric oxide scavenging assay very low activity was seen with an IC_{50} value of >1000µg/ml (Fig 4.6 & 4.7). In the present study the activity in the DPPH assay was similar to the activity in the study conducted by Amoo *et al*. (2012). In this study 50% methanol extract was prepared from the leaves and stems of *C. anisata* and was subjected to antioxidant studies where an inhibition of 70.8% at 100µg/ml was observed.

3.7 *Clematis brachiata*

C. brachiata showed low antioxidant activity in both the DPPH assay and the nitric oxide scavenging assay with IC_{50} values of 75.9±1.2µg/ml and >1000µg/ml respectively (Fig 4.6 & 4.7). No other reports have been found for the antioxidant activity of *C. brachiata*.

3.8 *Combretum molle*

C. molle showed good radical scavenging activity against DPPH with an IC_{50} value of 3.26±0.1µg/ml, whereas in the nitric oxide scavenging assay a low IC_{50} value of ±1000µg/ml was observed (Fig 4.6 & 4.7). In a previous study a qualitative DPPH assay was used to determine whether the different leaf extracts of *C. molle* possessed any antioxidant compounds (Masoko & Eloff, 2007). In the study four extracts were made: acetone, hexane, DCM and methanol where moderate activity was noted for the acetone and methanol extract and no activity for the hexane and DCM extract.

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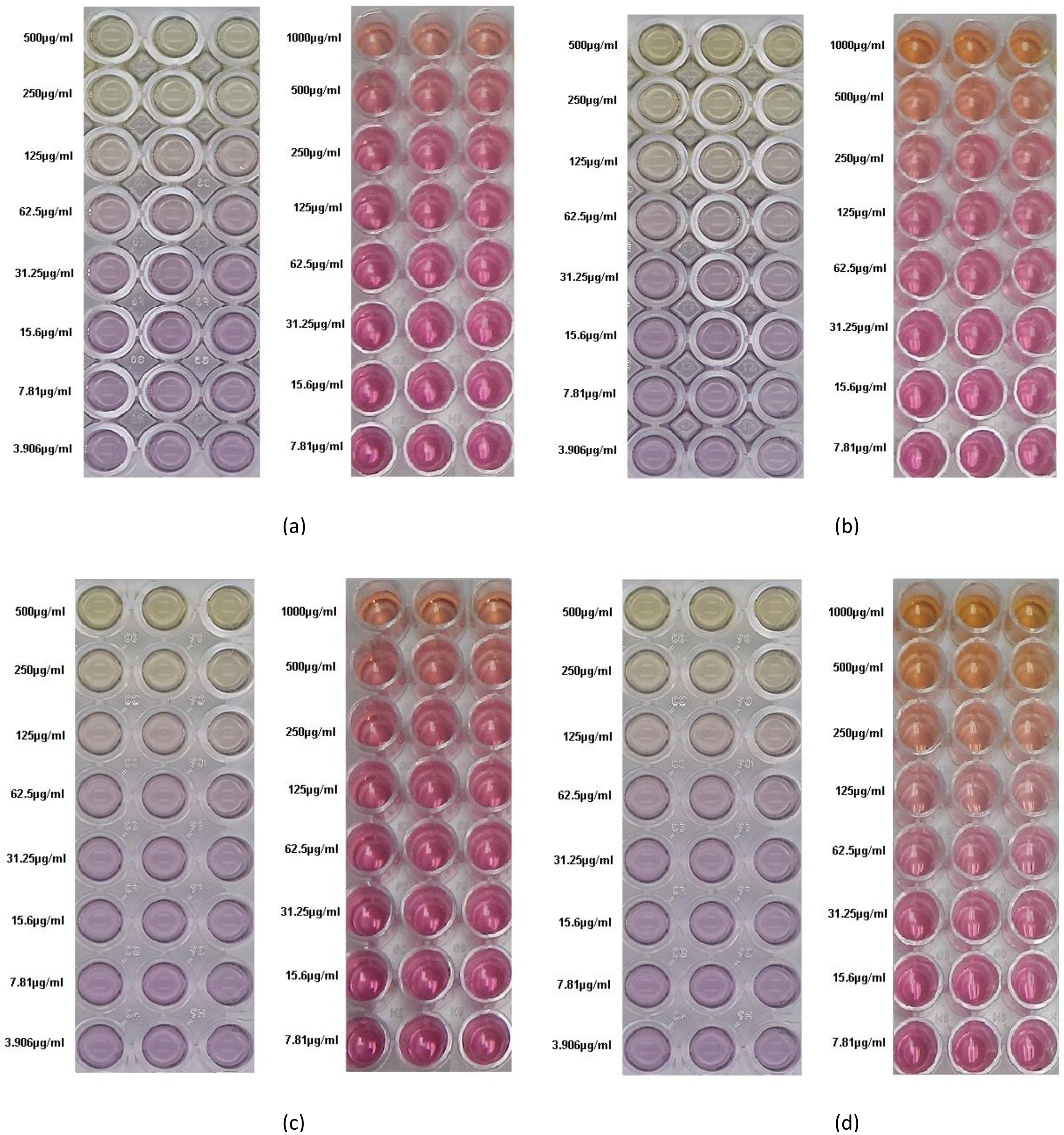

Figure 4.6: Free radical and nitric oxide colour change of ethanol extracts of (a) *Buddleja salviifolia*, (b) *Clausena anisata*, (c) *Clematis brachiata* and (d) *Combretum molle*

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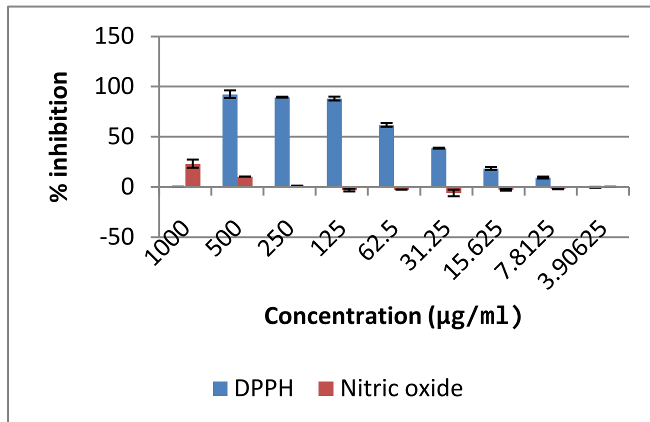
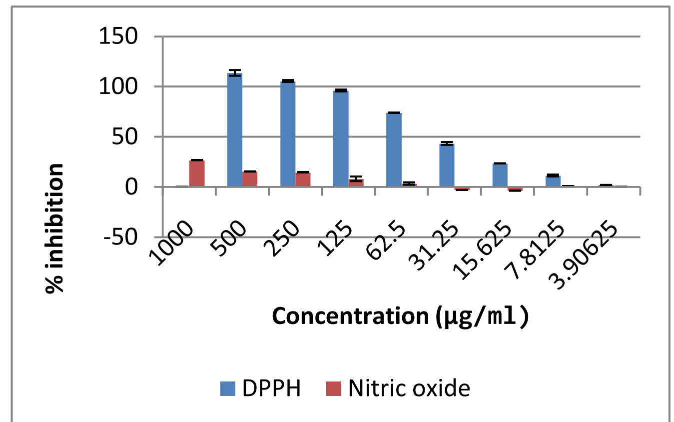


(a)



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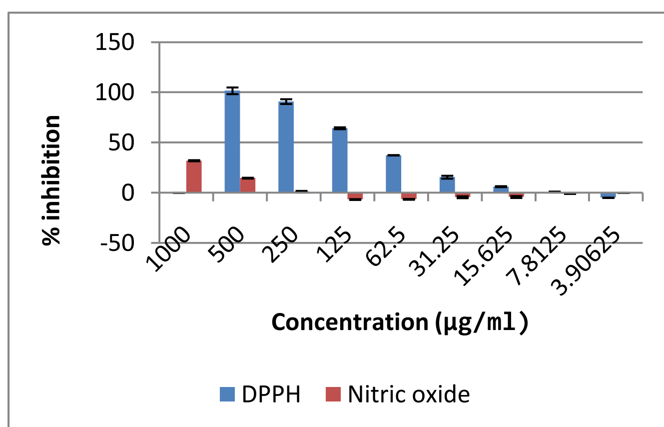

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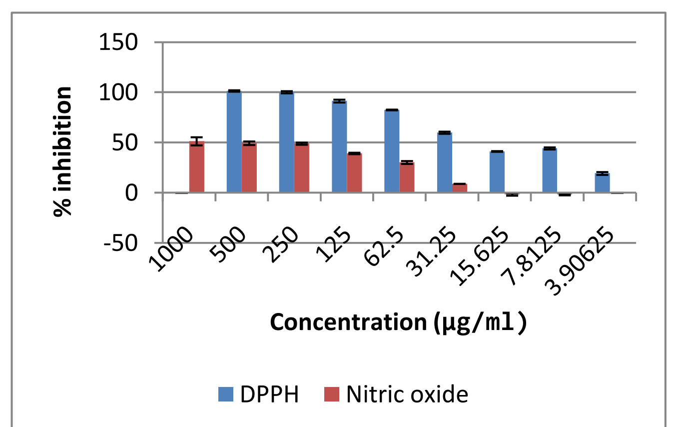

(d)

Figure 4.7: Free radical and nitric oxide activity of ethanol extracts of (a) *Buddleja salviifolia*, (b) *Clausena anisata,* (c) *Clematis brachiata* and (d) *Combretum molle*

3.9 *Dissotis princeps*

D. princeps showed the second highest antioxidant activity when compared to the other twenty extracts. It showed a high radical scavenging capacity with an IC_{50} value of 2.94±0.01µg/ml (Fig 4.8 & 4.9). In the nitric oxide scavenging assay it showed low activity with an IC_{50} value of >1000µg/ml. No previous reports on the antioxidant activity of *D. princeps* have been found.

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3.10 *Erythrophleum lasianthum*

E. lasianthum showed good radical scavenging capacity using the DPPH assay, whereas in the nitric oxide scavenging assay low antioxidant activity was observed with IC_{50} values of 5.25±0.08µg/ml and >1000µg/ml respectively (Fig 4.8 & 4.9). In a similar study where the leaf extracts of *E. lasianthum* were tested for DPPH scavenging activity, greater antioxidant activity was observed with IC_{50} values of 1.27±0.004µg/ml, 1.51±0.01µg/ml, 0.77±0.001µg/ml and 0.81±0.002µg/ml for hexane, chloroform, ethyl acetate and methanol extracts respectively (Adebayo *et al*., 2013).

3.11 *Euclea divinorum*

E. divinorum showed good antioxidant potential in the DPPH assay with an IC_{50} value of 8.30±0.02µg/ml whereas in the nitric oxide scavenging assay the activity was low with an IC_{50} value of >1000µg/ml (Fig 4.8 & 4.9). In the present study the antioxidant activity using DPPH was found to be better than that of the extracts in the study conducted by Feyissa *et al*. (2013). The maximum inhibition of the extracts was found to be 82.5%, 74.5% and 62.5% for the methanol fraction, aqueous fraction and crude methanol extract respectively at a concentration of 2000µg/ml.

3.12 *Gomphocarpus fruticosus*

In the present study *G. fruticosus* showed relative antioxidant potential with regards to the DPPH assay with an IC_{50} value of 32.5±1.2µg/ml whereas as a nitric oxide scavenger *G. fruticosus* showed low potential with an IC_{50} value of ±1000µg/ml (Fig 4.8 & 4.9). In a study conducted by Mothanal *et al*. (2009) the DPPH activity was found to be lower than that observed in the present study with 39.2% and 15.8% DPPH inhibition at 100µg/ml of methanolic and hot aqueous extracts respectively.

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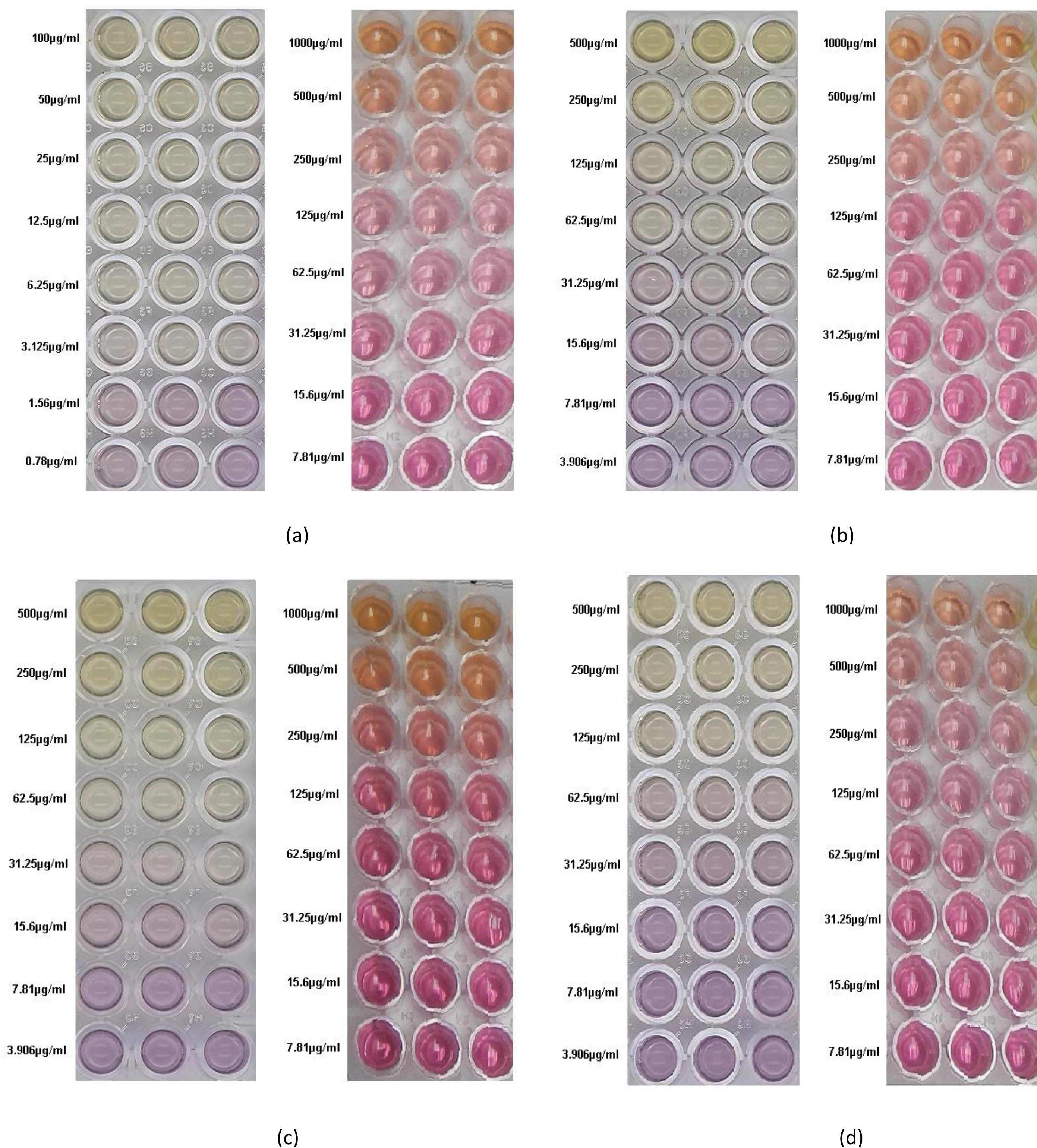

Figure 4.8: Free radical and nitric oxide colour change of ethanol extracts of (a) *Dissotis princeps*, (b) *Erythrophleum lasianthum*, (c) *Euclea divinorum* and (d) *Gomphocarpus fruticosus*

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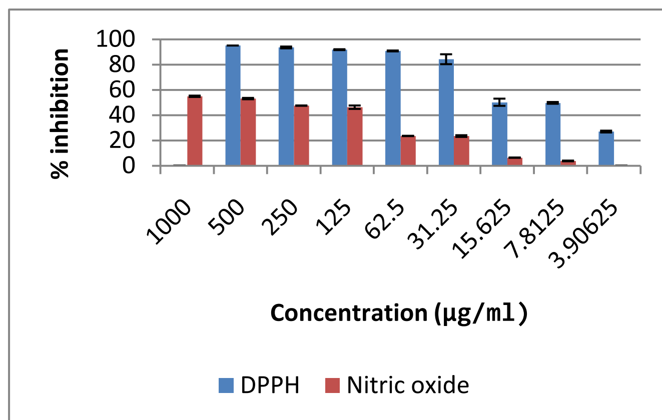
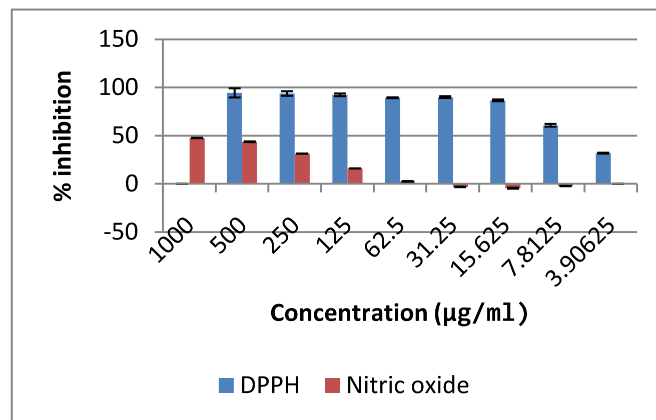
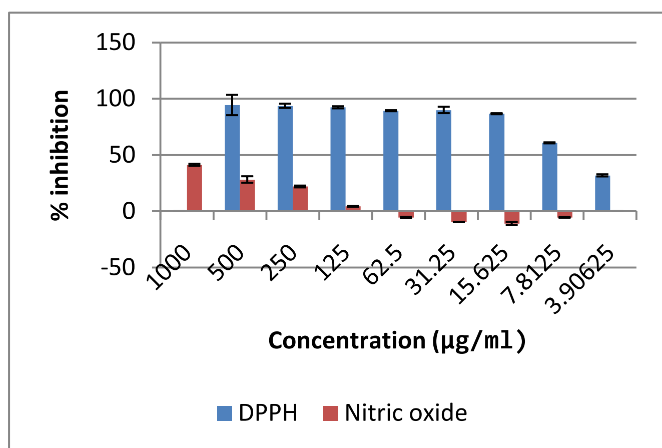


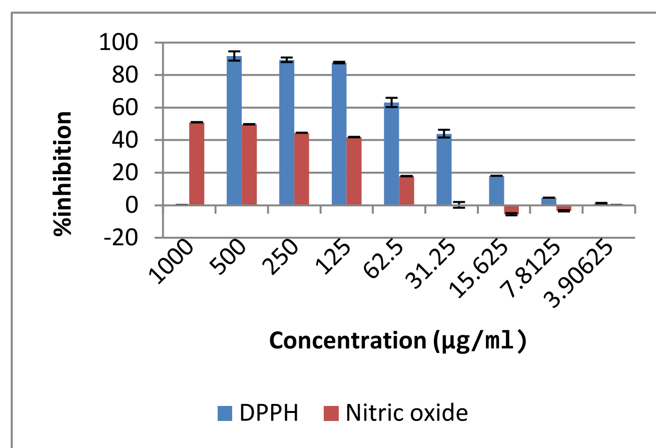
(a)



(b)



(c)



(d)

Figure 4.9: Free radical and nitric oxide activity of ethanol extracts of (a) *Dissotis princeps*, (b) *Erythrophleum lasianthum*, (c) *Euclea divinorum* and (d) *Gomphocarpus fruticosus*

3.13 *Harpephyllum caffrum*

H. caffrum showed a significant reduction in DPPH which suggests a good antioxidant activity with an IC_{50} value of 2.41±0.004µg/ml. In the nitric oxide scavenging assay *H. caffrum* also showed a significant reduction in nitric oxide with an IC_{50} value of ±250µg/ml (Fig 4.10). The results in the present study were similar to the results conducted by Moyo *et al*. (2010) where the methanolic stem bark extract showed an IC_{50} of 4.26µg/ml when used in the DPPH assay.

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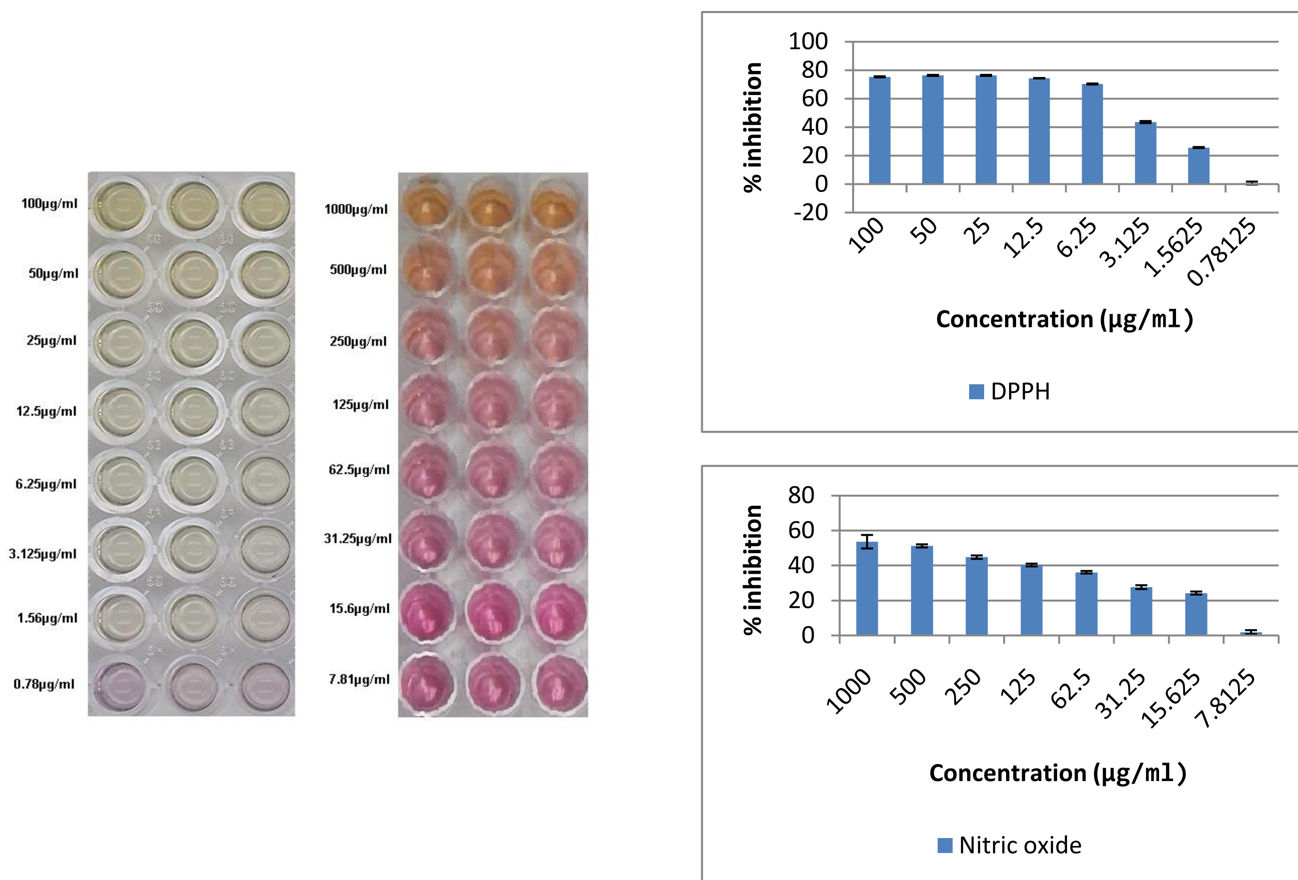

Figure 4.10: Free radical and nitric oxide scavenging activity of ethanol extract of *Harpephyllum caffrum*

3.14 *Helichrysum kraussii*

H. kraussii showed good antioxidant potential in the DPPH assay with an IC_{50} value of 4.66±0.05µg/ml. However, in the NO scavenging assay low activity was noted with an IC_{50} value of ±1000µg/ml (Fig 4.11 & 4.12). No previous reports on the antioxidant activity of *H. kraussii* were found.

3.15 *Helichrysum odoratissimum*

H. odoratissimum showed similar results to that of *H. kraussii* with good antioxidant potential on the DPPH assay with an IC_{50} value of 5.13±0.07µg/ml and a low antioxidant activity with regards to

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nitric oxide scavenging with an IC_{50} value of >1000µg/ml (Fig 4.11 & 4.12). No other reports on the antioxidant activity of *H. odoratissimum* were found.

3.16 *Leucas martinicensis*

L. martinicensis showed moderately antioxidant activity in the DPPH assay with an IC_{50} value of 34.5±0.24µg/ml. However, in the nitric oxide scavenging assay *L. martinicensis* showed low activity at the highest concentration tested with a value of >1000µg/ml (Fig 4.11 & 4.12). The results in the study were comparable to those in the study conducted by Habila *et al*. (2010) where an ethanolic extract of the whole plant possessed relatively good antioxidant activity with an inhibition of 81.48% at 250µg/ml.

3.17 *Rapanea melanophloeos*

R. melanophloeos showed strong radical scavenging capacity in both the DPPH assay and the nitric oxide scavenging assay with IC_{50} values of 4.01±0.047µg/ml and 63.73±0.4µg/ml respectively (Fig 4.13 & 4.14). The result obtained for the NO scavenging assay was the best activity obtained out of all twenty plant extracts for this assay. The results obtained in the present study varied greatly from the results obtained by Mosa *et al*. (2011) which tested various bark extracts of *R. melanophloeos* for DPPH activity. The bark extracts included hexane, chloroform, ethyl acetate, methanol and water with IC_{50} values of 3.05mg/ml, 4.32mg/ml, 4.83mg/ml, 3.31mg/ml and 3.89mg/ml respectively. These values obtained for the bark extracts were notably higher than the results obtained for the present study.

3.18 *Syzygium jambos*

S. jambos showed the most promising results when observing the antioxidant capacity in the DPPH assay with an IC_{50} value of 1.17±0.26µg/ml. The nitric oxide scavenging potential of the ethanol plant extract was very low with an IC_{50} value of >1000µg/ml (Fig 4.13 & 4.14). Due to the high DPPH activity of this plant, it was chosen for *in* vivo SPF clinical trials. It was found to have significant SPF activity due to the radical scavenging capacity of the extract. This study was also presented at the Society of Cosmetic Chemists Annual Conference (see Appendix C). In a similar

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study conducted by Islam *et al*. (2012) the ethanolic leaf extract of *S. jambos* showed slightly less antioxidant activity in the DPPH assay with an IC_{50} value of 14.10µg/ml. Similar studies were performed on the ethanolic seed extracts and bark extract of *S. jambos* which showed lower DPPH scavenging activity than the leaf extract in the present study with IC_{50} values of 95.21±1.78µg/ml and 6.75µg/ml respectively (Zheng *et al*., 2011 and Islam *et al*., 2011). In a study where compounds were isolated from the dichloromethane leaf extract three compounds showed good DPPH scavenging activity with IC_{50} values of 30µg/ml, 10.6µg/ml and 3.8µg/ml for Phloretin 4'-O-methyl ether, Myrigalone G and Myrigalone B respectively (Jayasinghe *et al*., 2007).

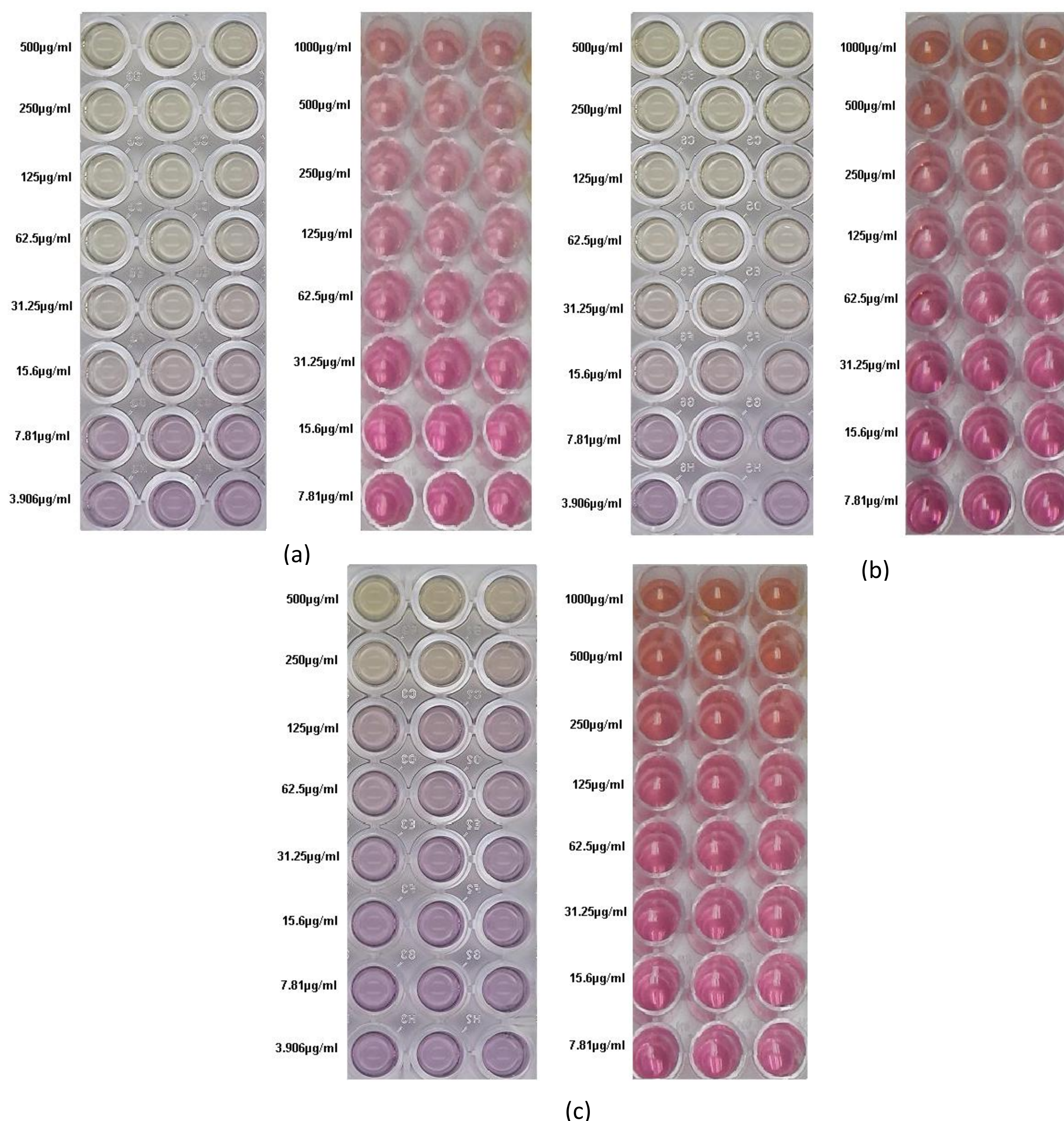

Figure 4. 11: Free radical and nitric oxide scavenging colour change of ethanol extracts of (a) *Helichrysum kraussii*, (b) *Helichrysum odoratissimum,* and (c) *Leucas martinicensis*

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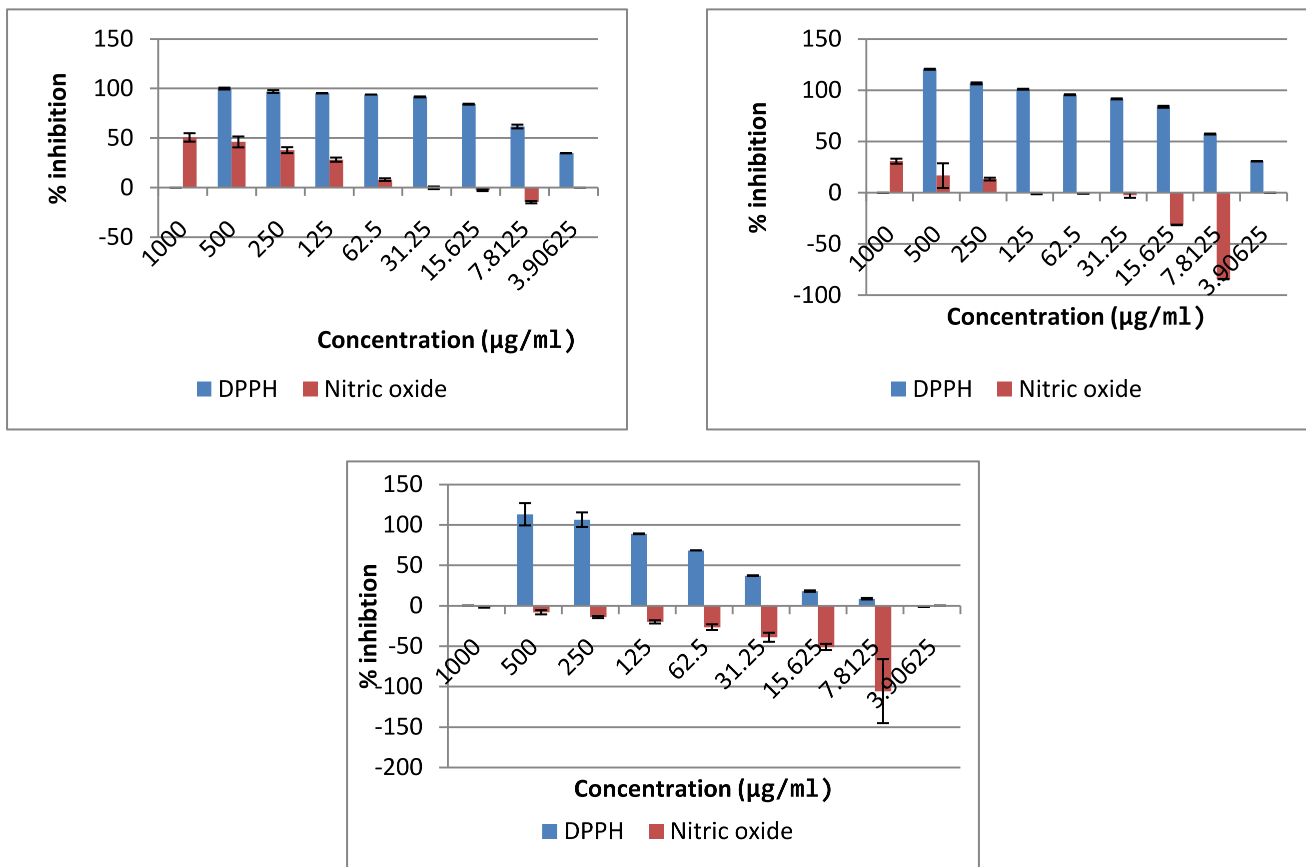

Figure 4. 12: Free radical and nitric oxide scavenging activity of ethanol extracts of (a) *Helichrysum kraussii*, (b) *Helichrysum odoratissimum*, and (c) *Leucas martinicensis*

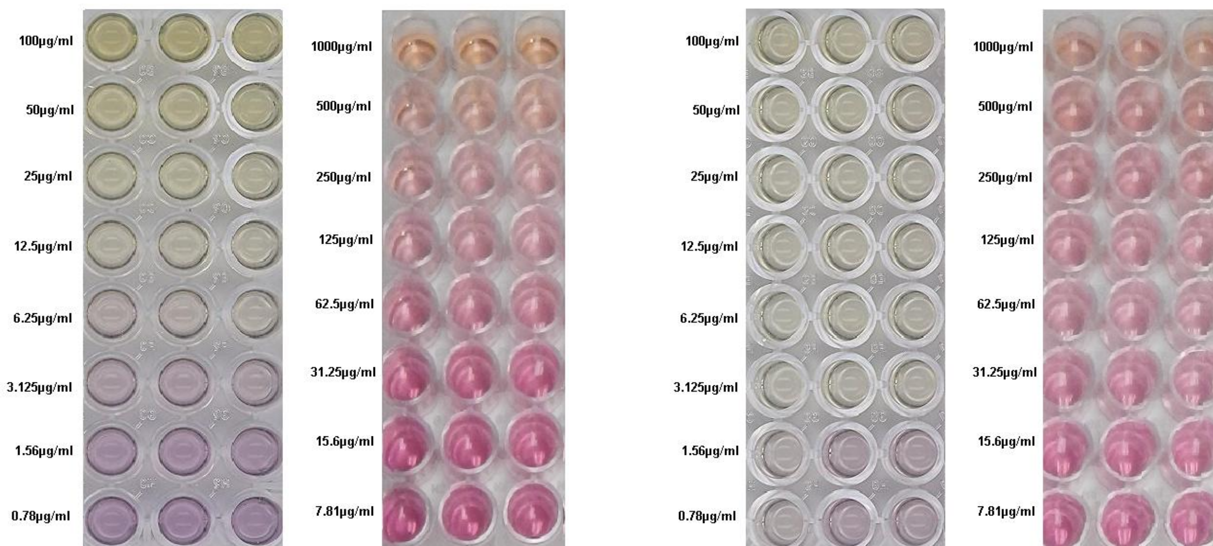

Figure 4.13: Free radical and nitric oxide scavenging colour change of ethanol extracts of (a) *Rapanea melanophloeos* and (b) *Syzygium jambos*

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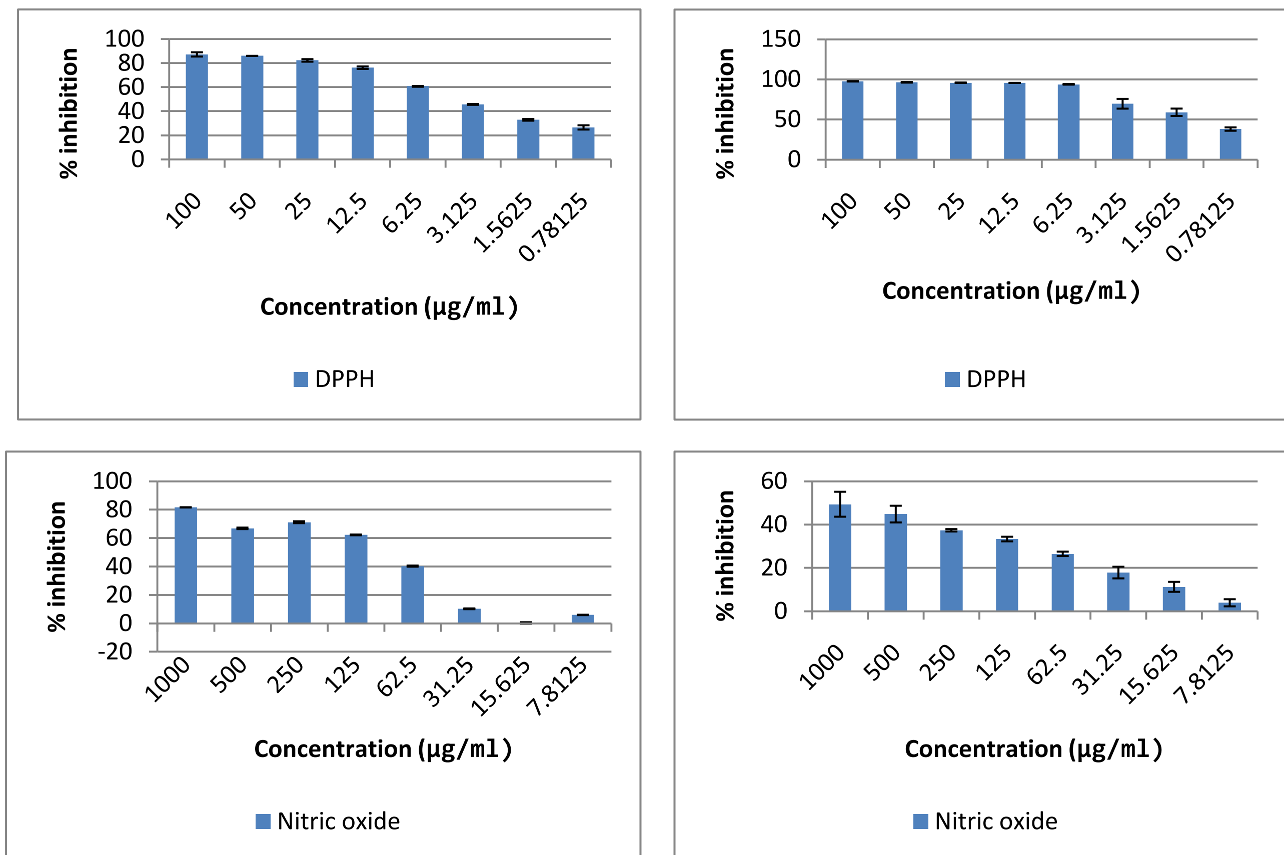

Figure 4. 14: Free radical scavenging activity of (a) *Rapanea melanophloeos* and (b) *Syzygium jambos* and nitric oxide scavenging activity of (c) *Rapanea melanophloeos* and (d) *Syzygium jambos*

3.19 *Tabernaemontana elegans*

In the present study *T. elegans* showed very poor antioxidant activity in both the DPPH assay and the nitric oxide scavenging assay with IC_{50} values of 157±8.3µg/ml and >1000µg/ml respectively (Fig 4.15 & 4.16). Regarding previous antioxidant activity on *T. elegans* no existing reports were found.

3.20 *Warburgia salutaris*

W. salutaris showed the lowest antioxidant potential in the DPPH assay and low nitric oxide scavenging activity with IC_{50} values of 464±0.005µg/ml and >1000µg/ml respectively (Fig 4.15 & 4.16). However, in a study conducted by Kuglerova *et al.* (2011) the results differed greatly with the results in the present study. In the reported study ethanolic extract of *W. salutaris* stem bark was made which showed good radical scavenging capacity with an IC_{50} value of 6.59 ± 2.84µg/ml. In a

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different study by Steenkamp *et al*. (2013) the methanolic and aqueous leaf extracts showed better activity in the DPPH assay than the results obtained in the present study with an IC_{50} value of 98.9±5.8µg/ml and 48.2±5.8µg/ml.

Figure 4.15: Free radical and nitric oxide scavenging colour change of ethanol extracts of (a) *Tabernaemontana elegans* and (b) *Warburgia salutaris*

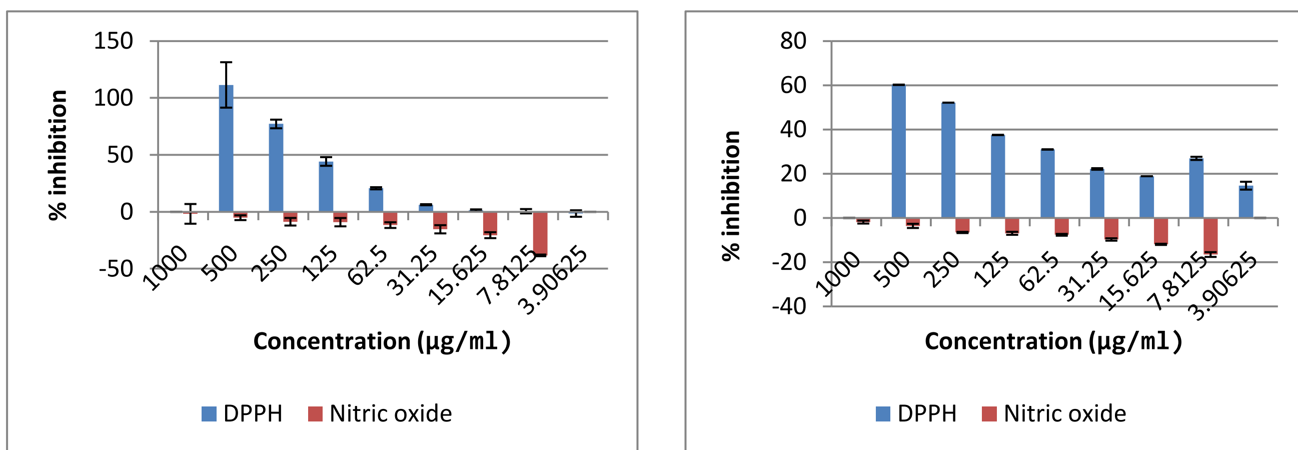

Figure 4.16: Free radical and nitric oxide scavenging activity of ethanol extracts of (a) *Tabernaemontana elegans* and (b) *Warburgia salutaris*

3.21 Vitamin C

Vitamin C was used as the positive control for both the DPPH free radical scavenging assay and the nitric oxide scavenging assay. In both assays Vitamin C showed significant antioxidant activity with

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IC_{50} values of 1.98±0.006µg/ml and 285.9±28.55µg/ml for the DPPH and nitric oxide scavenging activity respectively (Fig 4.17). In a study conducted by Amoo *et al*. (2012) similar results were obtained using the DPPH assay with an IC_{50} value of 2.1±0.005µg/ml and in a similar nitric oxide scavenging assay conducted by Mayur *et al* (2010), Vitamin C showed similar results to that found in the present study with an IC_{50} value of 220µg/ml. For the DPPH assay the Vitamin C equivalents of each plant extract was calculated using the following equation:

$$IC_{50} \text{ sample } (\mu g/ml) \text{ X } 200mg \text{ Vitamin C} / IC_{50} \text{ Vitamin C } (\mu g/ml)$$

$$= \text{Vitamin C equivalents in mg}$$

The Vitamin C equivalent determines the amount of plant extract needed to have the same radical scavenging capacity as a 200mg capsule of Vitamin C.

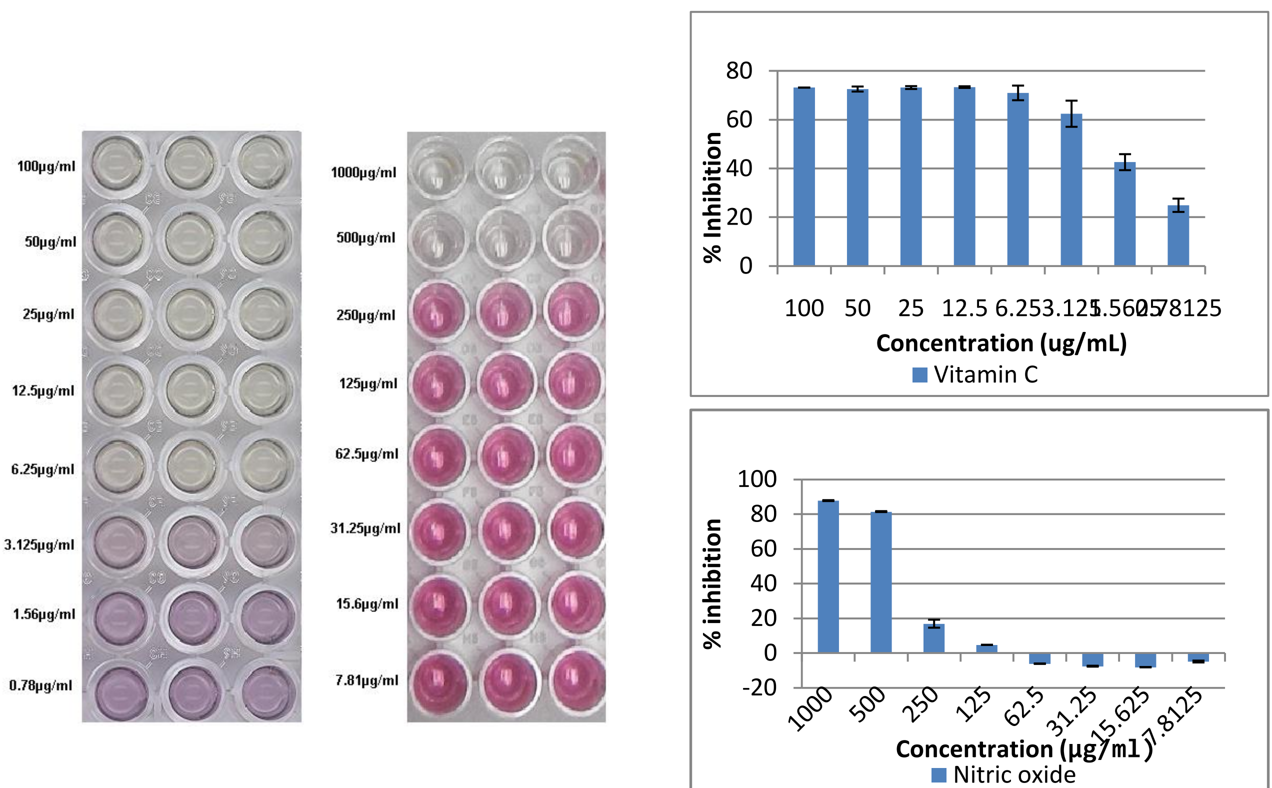

Figure 4.17: Free radical and nitric oxide scavenging activity of ethanol extract of Vitamin C

4. Conclusion

The secondary metabolites produced by plants are generally responsible for the radical scavenging capacity and antioxidant activity. Such secondary metabolites include phenolics, polyphenols and

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flavanoids which scavenge free radicals and therefore inhibit the oxidative stress caused by such free radicals (Patel *et al*., 2012).

A few plant extracts showed better antioxidant activity in the DPPH scavenging assay as compared to when used in the nitric oxide scavenging assay. In this study *Syzygium jambos* showed significant antioxidant potential with regards to DPPH scavenging capacity with an IC_{50} value of 1.17±0.26µg/ml which was even higher than that of Vitamin C. *Rapanea melanophloeos* showed the highest nitric oxide scavenging capacity with an IC_{50} value of 63.73±0.4µg/ml which was also greater than that of Vitamin C.

All plant extracts were reported for the first time based on their nitric oxide scavenging activity, whereas a few plant extracts have been reported earlier for their DPPH scavenging activity by other researchers.

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Chapter 5

Morphological changes of A431 cells in response to treatment with *Helichrysum odoratissimum*

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1. Introduction

It was evident in Chapter 3, that the *H. odoratissimum* extract showed the greatest potential as an anti-cancer agent on the A431 epidermoid carcinoma cell line with a fifty percent inhibitory concentration (IC_{50}) value of 15.5±0.2µg/ml and a selectivity index (SI) value of 2.39. Therefore this extract was selected to determine its effect on the morphology of A431 cells when exposed at various concentrations of the extract.

1.1 Cell division - mitosis

Cell division is a process which distributes genetic DNA into two daughter cells which will be identical to one another. The replication and distribution of DNA to two daughter cells is mediated via chromosomes, which is made up of DNA molecules. In a non dividing cell the chromosomes are in the form of a chromatin strands which after DNA duplication form densely coiled chromosomes. The duplicated chromosomes are made up of two identical sister chromatids which are attached via a centromere. During the cell division process the two sister chromatids separate from one another and are distributed into two new nuclei to form separate chromosomes. After the formation of the nucleus mitosis occurs, where the nucleus divides, cytokinesis occurs where the cytoplasm divides, which produces two daughter cells (Fig 5.1) (Campbell & Reece, 2005).

Mitosis is only one part of the cell cycle which is broken up into five different phases; prophase, prometaphase, metaphase, anaphase and telophase, which is completed by cytokinesis. In the G_2 phase of interphase the nucleus contains a nucleolus which is bound by a nuclear envelope. Within the nucleus there are also long fibres of chromatin which are duplicated. Furthermore, two centrosomes are present, each containing a pair of centrioles. In the prophase the chromatin becomes condensed and starts to coil to form chromosomes, which consists of two sister chromatids attached via a centromere. In this phase the mitotic spindles start to form which causes the centrsomes to move away from eachother. The nuclear evelope starts to fragment in the prometaphase causing the microtubules to spread over the whole cell and interact with the chromosomes. In the metaphase the chromosomes align at oppsite ends of the cell on the metaphase plate. Each sister chromatid in a chromosome starts to then separate from one another in the anaphase, which then forms two ends of the cell that are identical to eachother. In the telophase the two daughter nuclei and the nuclear envelopes start to form. The chromosomes on each end start to uncoil and become less condense,

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which completes the cycle of mitosis. During late telophase, cytokinesis starts to take place where the cytoplasm starts to divide to form the two daughter cells (Campbell & Reece, 2005).

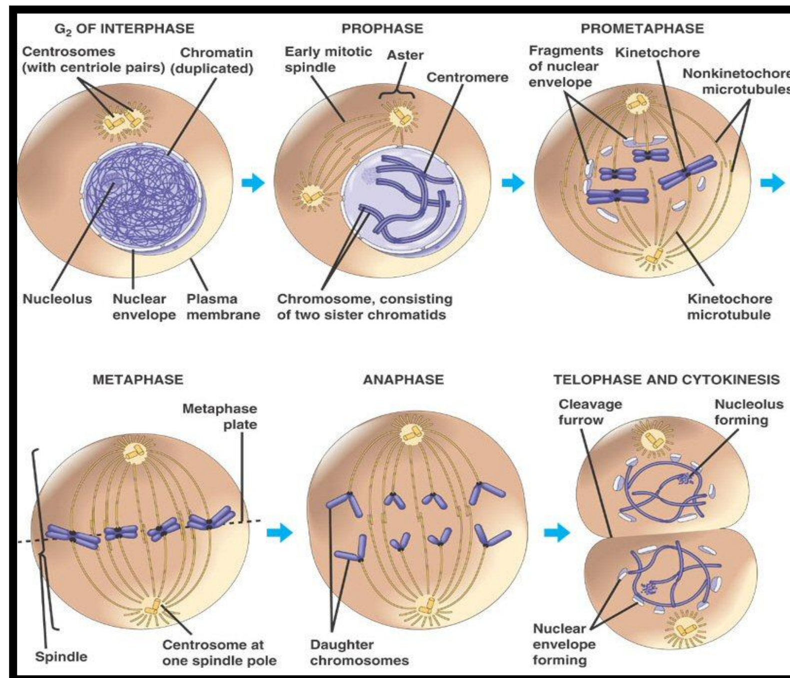

Figure 5.1: Different phases of mitosis in the cell (Campbell & Reece, 2005)

1.2 Cell death

Cell death is a process by which there is loss of membrane integrity which is irreversible. Cell death and cell proliferation is carefully regulated to keep a balance in multi-cellular organisms. If a balance is not maintained then diseases such as cancer can result (Coates *et al*., 2009). There are three known types of cell deaths namely, apoptosis, necrosis and autophagy, of which apoptosis and necrosis are more commonly known. The cell deaths differ with regard to their morphological characteristics. Apoptosis is charcterized by morphological changes in the nucleus, such as chromatin condensation and fragmentation, changes in the cytoplasm, shrinkage of the cell, plasma membrane blebbing as well as the apoptotic bodies' formation which contains nuclear and cytoplasmic material. Autophagy is charcterized by the accumulation of two autophagic vacuoles which are responsible for the degradation of unwanted material. Finally necrosis, which is distinctly different from apoptosis and autophagy, in that membrane rupturing is an early process and the cell and organelles swell (Golstein & Kroemer, 2006).

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Therefore, when cells do not receive the proper supplies and nutrients they undergo stress and this can result in cell death. The three main types of cell deaths are described in the section below.

1.2.1 Apoptosis

Apoptosis is a mechanism of cell death which is highly programmed and controlled and which functions in maintaining cellular and organelle homeostasis (Coates *et al*., 2009). Apoptosis is further described as a form of suicide due to the processes the cell undergoes in order to result in death. The cell undergoes both morphological and biochemical changes during programmed cell death. Characteristic features of apoptosis include cell shrinkage and shrinkage of the nucleus, which is opposite to the characteristics of necrosis which is characterized by swelling of the cell and organelles. The differences in the mechanism of apoptosis and necrosis are partly due to the function of the plasma membrane in these processes. In necrosis, the loss of plasma membrane integrity is an early process which allows for the influx of ions and fluid which in turn causes the swelling of the cell and organelles. In apoptosis however, the plasma membrane is not affected until later in the process where cell integrity starts to deteriorate (Hotchkiss *et al*., 2009). The main morphological changes that occur within an apoptotic cell include condensed chromatin (pyknosis), shrinking of the cytoplasm, nuclear fragmentation, and plasma membrane blebbing. This results in the breaking up of the cell into apoptotic bodies (zeiosis) which in turn are engulfed by phagocytes by a process known as phagocytosis (Fig 5.2). Phagocytosis can only occur when changes in the cell composition take place, such as externalization of phosphatidylserine (PS) as well as the rearrangement of carbohydrates which the macrophages recognize and therefore bind to the cell surface (Orrenius *et al*., 2011).

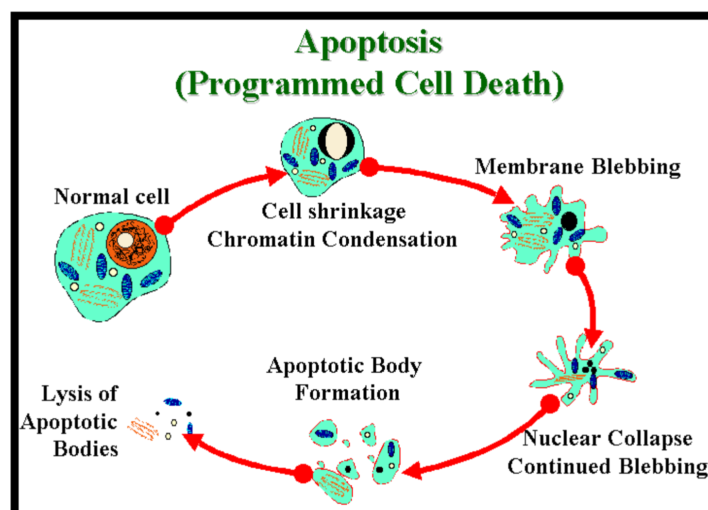

Figure 5.2: Morphological changes associated with apoptosis (DCA shop, 2013)

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Once apoptosis has been activated it is followed by membrane permeabilization, caspase activation and cell death. This process is rapid and irreversible (Mathew *et al*., 2007; Adams & Cory, 2007). Caspase activation can commit cells to either the death receptor pathway (extrinsic) or the mitochondrial-mediated pathway (intrinsic), which are the two major pathways of apoptosis (Fig 5.3). The death receptor pathway is activated by members of the tumor necrosis factor (TNF) family binding to death-receptors on the cell surface. The binding of TNF ligands (FAS, TNF, TRAIL) to these receptors causes the formation of multi-protein death-inducing signaling complexes (DISC). The aggregation of DISC can lead to conformational change which causes the activation of the catalytic activity of caspase-8. Capase-8 activates caspase-3 which cleaves target proteins and leads to the apoptosis of cells. Caspase-8 can also directly cleave Bid which inserts Bax and/or Bak into the outer layer of the mitochondrial membrane leading to the release of proteins, such as Cytochrome *c*, from the mitochondrial intermembrane space. This leads to the formation of a cytocolic apoptosome complex which activates caspase-9 in the presence of dATP and therefore ultimately leads to apoptosis (Orrenius *et al*., 2011).

The intrinsic mitochondrial apoptotic pathway is controlled and regulated by various factors. These include members of the proapoptotic and antiapoptotic BCL2 family, which regulate cytochrome *c*, as well as inhibitor of apoptosis proteins (IAPs), which inhibits caspases, second mitochondrial activator of caspases (SMAC) and Omi, which negatively regulates IAPs. The initiation of the intrinsic pathway is caused by an increase in reactive oxygen species (ROS) as well as DNA damage. The ROS are responsible for increasing the permeability of the mitochondrial membrane which causes the release of cytochrome *c* from the intermebrane space. The Diablo homologue (SMAC/DIABLO) is also released at the same time which is ultimately responsible for the activation of caspases and therefore apoptosis (Hotchkiss *et al*., 2009).

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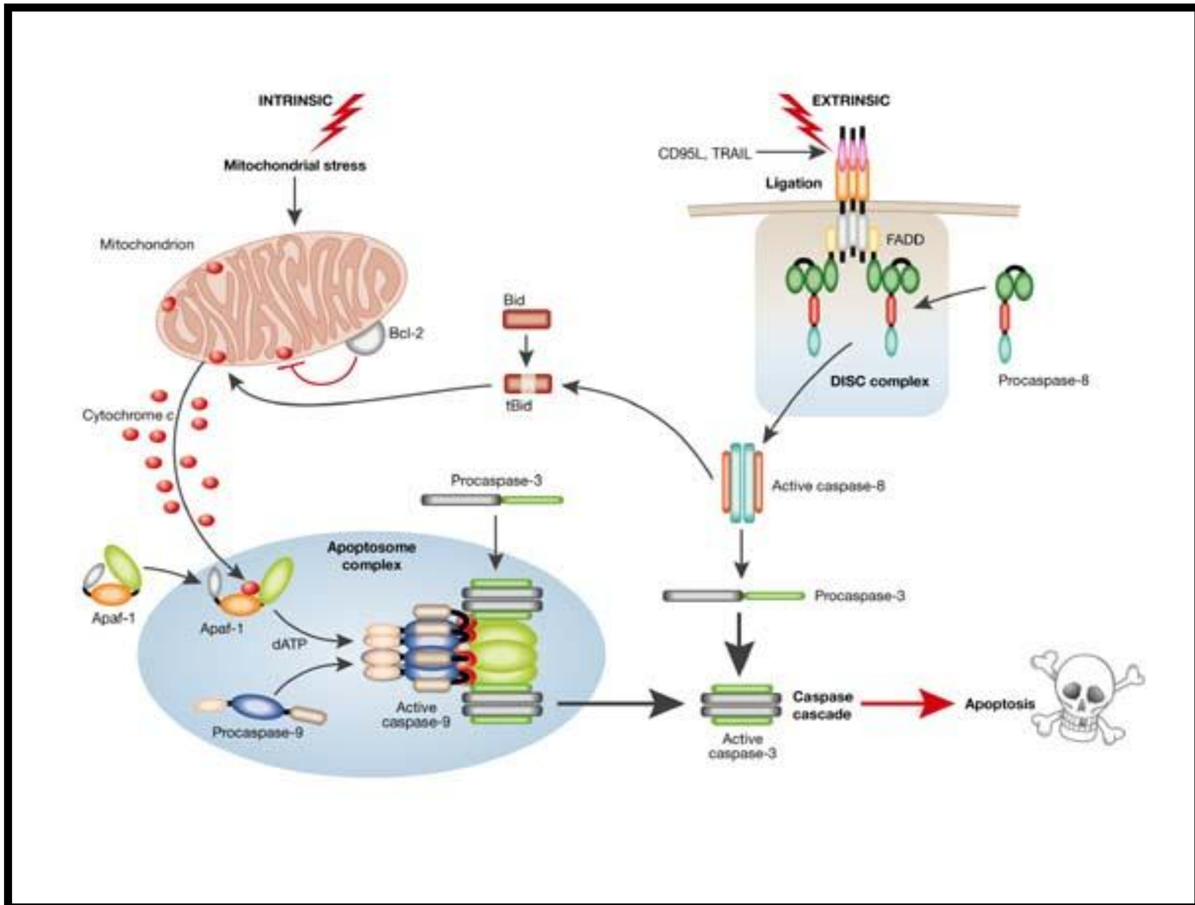

Figure 5. 3: The extrinsic and intrinsic caspase mediated pathways leading to apoptosis (MacFarlane & Williams, 2004)

1.2.2 Necrosis

Necrosis is a form of cell death which is characterised by cell and organelle swelling. The term "oncosis", which means swelling, has also been used as an alternative to the term necrosis. Necrosis is considered as non-programmed cell death as it is an accidental form of cell death, which is a result of various factors such as lethal chemicals, biological or physical events such as hyperthermia, complement attack, ischemia, metabolic poisons, hypoxia and direct cell damage (Hupperts *et al.*, 1999; Schwartzman & Cidlowski, 1993). During necrosis the plasma membrane is disrupted which causes spillage of intercellular proteins and leads to the activation of a damage response from the immune system (Fig 5.4) (Zeh & Lotze, 2005).

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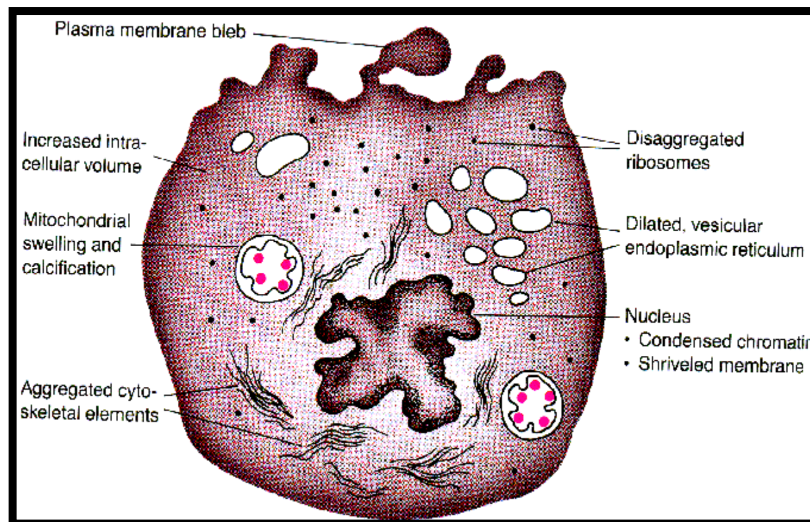

Figure 5.4: Characteristic features of necrosis (Medic, 2011)

Necrosis is mediated by factors such as ROS, calcium ions, poly-ADP-ribose polymerase (PARP), calcium-activated non-lysosomal proteases (calpains) and cathepsins. PARP is an enzyme which is responsible for the repair of DNA by using adenosine triphosphate (ATP). In the case of multiple strands of DNA damage the ATP in the cells could be depleted which leads to necrosis. Levels of calcium ions can also determine the type of cell death. An inflow of calcium ions across the plasma membrane can lead to necrosis and a release of calcium ions from the endoplasmic reticulum can lead to apoptosis (Hotchkiss *et al*., 2009).

1.2.3 Autophagy

Autophagy is a type of cell death where cells reuse their own non-essential and macromolecular components as well as their own damaged organelles. Autophagy is a type of response in the cell when there are minimal nutrients which are needed to produce metabolites necessary for cell survival. An important role of autophagy is the elimination of toxic proteins and microorganisms as well as the suppression of tumor growth (Hotchkiss *et al*., 2009). There are three forms of autophagy which are based on the use of lysosomes needed for degradation. These are known as macroautophagy, microautophagy and chaperone-mediated autophagy (Fig 5.5) (Amaravadi & Thompson, 2007). In macroautophagy a double-membraned autophagosome engulfs the material and then fuses with the lysosome to form an autophagolysosome. The material is then degraded, through an acid hydrolases, and recycled. In microautophagy an invagination of the lysosomal membrane engulfs the material which then needs to be recycled. Finally in chaperone-mediated autophagy there are heat-shock proteins which deliver the substrates that need to be recycled to the lysosomes. The

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formation of an autophagosome, which is the hallmark of autophagy, is regulated by a set of proteins such as a complex which consists of phosphatidylinositol-3-kinase (PI3K) and beclin-1 (BECN1) (Hotchkiss *et al*., 2009).

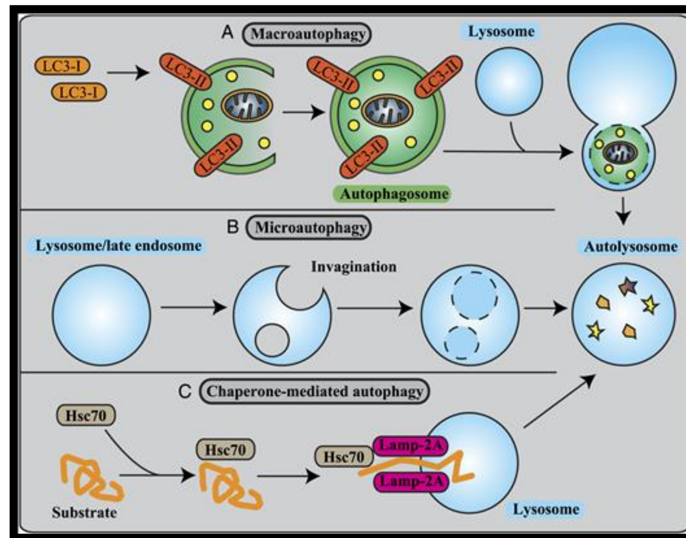

Figure 5.5: Steps involved in macroautophagy, microautophagy and chaperone-mediated autophagy (Mizumura et al., 2012)

Autophagy is also known, not only for its survival mechanism, by providing the cells with alternative sources of nutrients, but also for its protective mechanism. In addition to eliminating the presence of toxic proteins or micro-organisms, autophagy can also be useful in eliminating damaged mitochondria, which leads to apoptosis (Hotchkiss *et al*., 2009).

A summary of the morphological characteristics associated with apoptotic, necrotic and autophagy cell death is depicted in Table 5.1.

Table 5.1: Summary of the morphological and biochemical changes that occur during cell death

Apoptosis	Necrosis	Autophagy
Cell shrinkage & Chromatin condensation	Cell and organelle swelling	Partial chromatin condensation
Cellular blebbing	Plasma membrane rupturing	No DNA fragmentation
Nuclear fragmentation	Clumping of nuclear DNA	Plasma membrane blebbing
PS externilization	DNA degredation	Increased lysosome activity
Apoptopic body formation	Organelle degeneration	Plasma membrane rupture
Increased permeability of mitochondria	Increased vacuoles	Increase in autophagic vacuoles
Caspase activation	Mitochondrial swelling	Caspase independent

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After discussing the different mechanisms of cell death, the *H. odoratissimum* extract was used to determine its effect on the morphology of A431 cells and to observe which characteristic of a type of cell death occurred.

2. Materials and methods

2.1 Materials

The materials used to culture the A431 cell line were the same as mentioned in section 2.2.1 of Chapter 3. The 6-well plates were purchased from Separations (Pty) Ltd. (Randburg, Johannesburg, RSA). The Bouin's fixative, haematoxylin stain, eosin stain and xylene were all purchased from Sigma Chemicals Co. (St. Louis, MO, USA) and were all of analytical grade.

2.2 Methods

The morphological characteristics of the A431 cells were analyzed by staining the cells with haematoxylin and eosin. Morphological characteristics were analysed after exposure to 15µg/ml (IC_{50}), 30µg/ml ($2IC_{50}$) and 7.5µg/ml ($1/2IC_{50}$) of *H. odoratissimum*. The specific concentrations of extract were chosen due to the anti-proliferative activity that was observed. Exponentially growing cells were seeded at 100,000 cell per well in 6-well plates on heat-sterilized coverslips. The plates were incubated for 24h at 37ºC and 5% humidity for cell adherence to occur. Thereafter cells were exposed to 15µg/ml, 30µg/ml and 7.5µg/ml of *H. odoratissimum* extract including vehicle-treated control cells (2%DMSO); cells propagate in growth medium, as well as 0.025µg/ml actinomycin D, and incubated for a further 72h at 37°C. Cells were then fixed in Bouin's fixative for 30min and stained by standard haematoxylin and eosin staining procedures (Stander *et al*., 2009).

3. Results and discussion

An analysis of the effect of 15µg/ml, 30µg/ml and 7.5µg/ml of the *H. odoratissimum* extract on A431 cells was determined by analysing the cell morphology after 72h of exposure and compared to

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the control cells (DMSO and growth medium) and the positive control, using an electron microscope at 20X and 40X (oil) magnification (Fig 5.6-5.11). The standard procedures for haematoxylin and eosin staining were used to differentiate the cytoplasm (pink) from the nucleus (blue/purple).

The A431 showed no signs of cell death in the growth medium control, which was expected. In these slides normal cell cycle morphology was observed with interphase clearly viewed at 20X magnification (Fig 5.6 (a)) and at 40X magnification anaphase, where chromosomes migrate to different poles of the cell, as well as interphase were observed (Fig 5.6 (b)). In the vehicle treated control cells similar observation were made to that of the growth medium control cells. The cells showed signs of normal cell cycle division with interphase taking place (Fig 5.6 (c)). In Fig 5.6 (d) the same results were observed as in the growth medium control cells where cytokinesis was observed forming two daughter cells. This confirms that at a 2% DMSO concentration the cells were not affected by the solvent in which the stock solution was made.

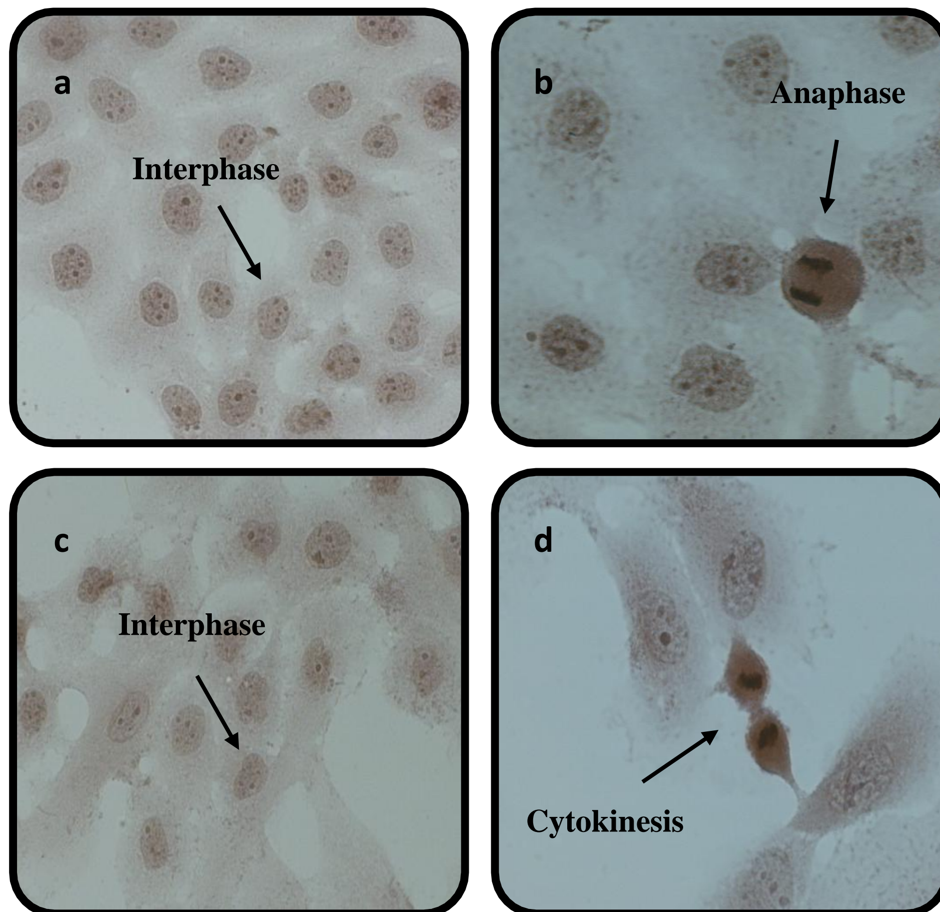

Figure 5. 6: Haematoxylin and eosin staining of A431 cells in growth medium at (a) 20X magnification and (b) 40X magnification and vehicle treated control cells at (c) 20X magnification and (d) 40X magnification, after 72h of exposure

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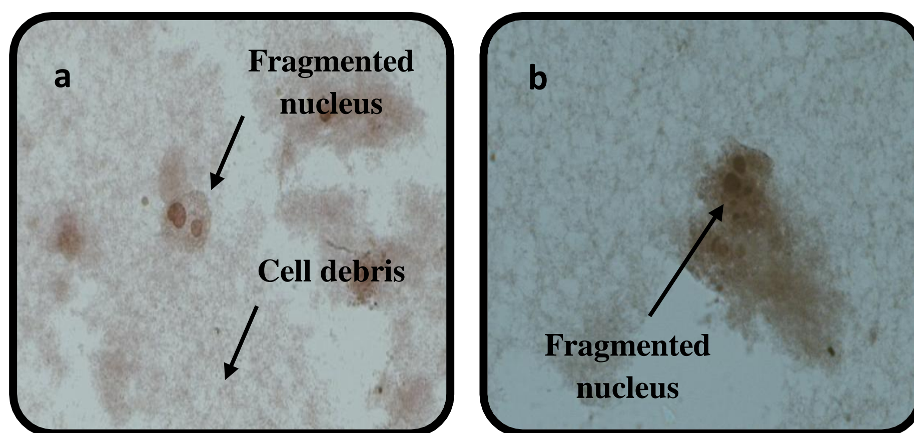

Figure 5. 7: Haematoxylin and eosin staining of A431 cells exposed to 0.025µg/ml Actinomycin D at (a) 20X magnification and (b) 40X magnification, after 72h of exposure

In the A431 cells exposed to 0.025µg/ml Actinomycin D, there was a great amount of cell death and no viable cells were observed. At this concentration characteristics of apoptosis were viewed where the nucleus had compacted and fragmented (Fig 5.7 (a)). Also a great amount of cell debris was observed and total loss of cytoplasm and loss of cell membrane integrity was seen. There were also morphological changes such as compacted and fragmented nucleus (Fig 5.7 (b)).

Cells exposed to the IC_{50} value of *H.* odoratissimum showed no normal cell proliferation and signs of cell death started to appear. Typical signs of apoptosis could be viewed such as apoptotic body formation. There was also a definite loss of cytoplasm and the shrinkage of cells and organelles (Fig 5.8 (a)). A great amount of fragmented and condensed chromatin, as well as the total loss of cytoplasm and cell membrane integrity was observed (Fig 5.8 (b)).Cells exposed to the ½ IC_{50} value of *H. odoratissimum* showed less cell death and signs of normal cell cycle division were noticeable however, there were some signs of cell death also observed. At 20X magnification cells in the interphase were observed. Signs of reduced cytoplasm and fragmented nucleus were also observed but at a lower extreme than at IC_{50} or $2IC_{50}$ (Fig 5.8 (c)). Signs of anaphase were also noted which indicated normal cell cycle functionality (Fig 5.8 (d)). Cells exposed to the $2IC_{50}$ of *H. odoratissimum* showed very low cell viability with signs of apoptotic cell death. In Fig 5.8 (e) a large amount of cell debris was observed; condensed chromatin and a fragmented nucleus were also seen. At the 40X magnification characteristic apoptotic bodies as well as cell shrinkage were seen (Fig 5.8 (f)). In a study conducted by Stander *et al*. (2009), a water extract was prepared using the leaves and twigs of *Sutherlandia futescens*. The influence of the extract on the morphology of MCF-7 cancer cells was observed at 5mg/ml and 10mg/ml. It was evident that the cell density decreased when treated at these concentrations and signs of cytoplasmic shrinking, hypercondensed chromatin,

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membrane blebbing and apoptotic bodies were evident. These finding were similar to those found in our study, where characteristic signs of apoptosis were observed.

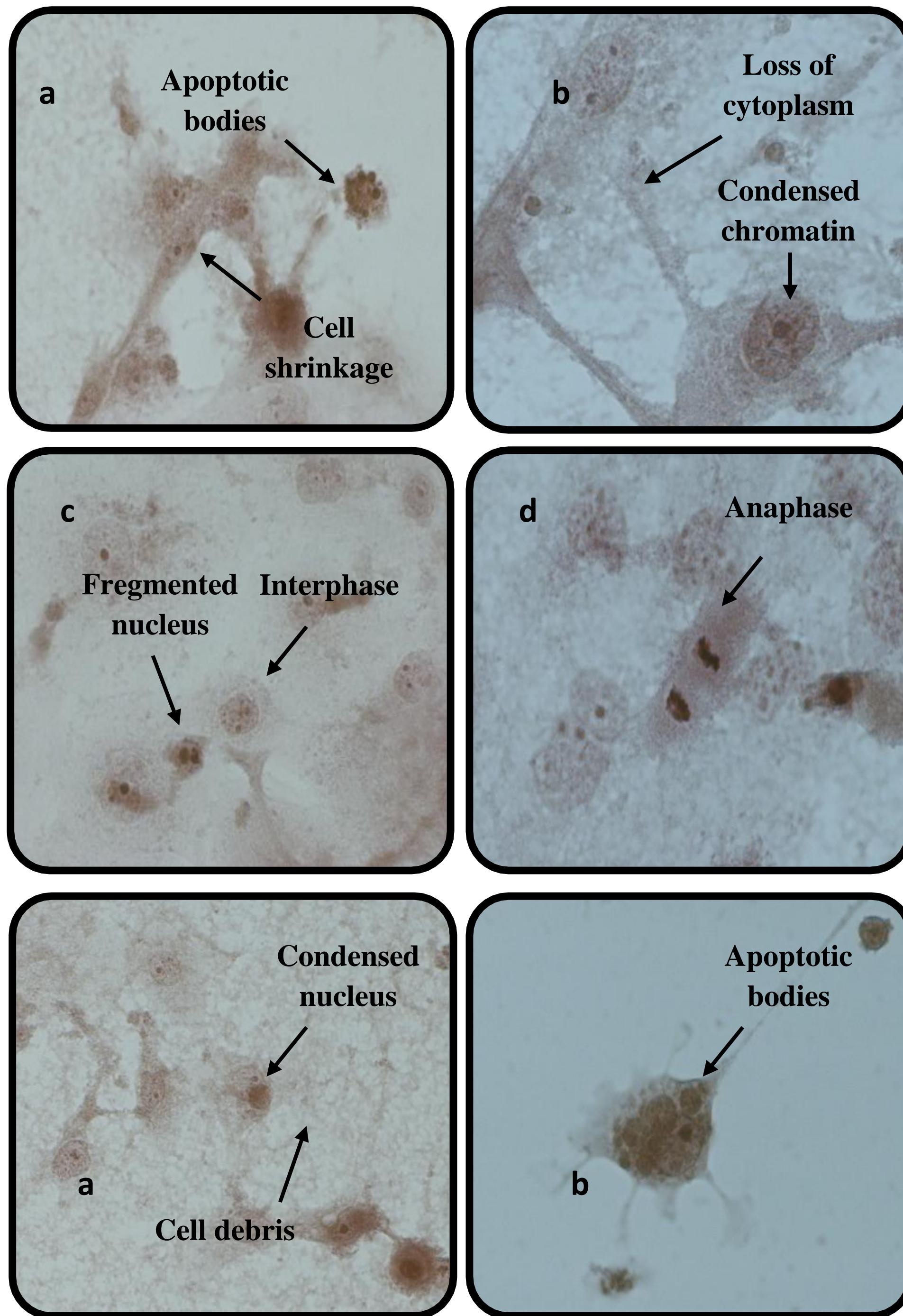

Figure 5.8: Haematoxylin and eosin staining of A431 cells exposed to 15µg/ml *Helichrysum odoratissimum* at (a) 20X magnification and (b) 40X magnification, after exposure to 7.5µg/ml *Helichrysum odoratissimum* at (c) 20X magnification and (d) 40X magnification, and after exposure to 30µg/ml *Helichrysum odoratissimum* at (e) 20X magnification and (f) 40X magnification, after 72h of exposure

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4. Conclusion

When observing the A431 cell line under a light microscopy using Haematoxylin and eosin staining it was found that various morphological changes had occurred. It can be concluded that when the A431 cell lines were exposed to various concentrations of *H. odoratissimum* extract there was an increase in morphological features which correlated to apoptosis. However at lower concentrations of the extract (7.5µg/ml) the signs of cell death were less and there was a higher presence of viable cells; and finally at the vehicle-treated control and the growth medium control a high cell viability with signs of normal cell cycle proliferation was observed.

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Chapter 6

Effects of *Helichrysum odoratissimum* on IL-12 and IL-8 cytokine production

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1. Introduction

H. odoratissimum showed the greatest activity as a possible anti-cancer agent on the A431 cell line with a fifty percent inhibitory concentration (IC_{50}) of 15.5µg/ml. Furthermore, the characteristic features of apoptosis were evidently seen upon examining the morphological changes the A431 cells underwent after being exposed to various concentrations of *H. odoratissimum*. Therefore, *H. odoratissimum* was also selected to determine whether various concentrations were able to increase the production of IL-12 and decrease the production of IL-8 in U937 cells.

1.1 Basic biology and the role of IL-12 in cancer

Interleukin 12 (IL-12) is a heterodimeric cytokine which has stimulatory effects on T-helper cells, cytotoxic T lymphocytes (CTL) and natural killer cells (NK). IL-12 has a molecular mass of 70kDA consisting of subunit p40 (40kDa) and p35 (35kDa) which are linked through disulfide bonds (Robertson & Ritz, 1996).

The IL-12 cytokine is produced by monocytes, macrophages, dendritic cells, neutrophils and in some cases by B cells, which in turn can cause an immunostimulatory effect on the helper T cells, CTL and NK cells (Fig 6.1). The effect of stimulation on these cells includes increased proliferation of T cells and NK cells, increased cytotoxicity of CTL, production of IFN-γ (in NK and T cells) as well as mediating immunity against intracellular pathogens through polarization of $CD4^+$ T cells towards Th1(Watford *et al*., 2003).

One major role of IL-12 is the ability to induce IFN-γ in NK cells and T cells, which is important for increasing the bactericidal activity of phagocytic cells, which in turn boosts the innate immune response. IL-12 is also important in that it stimulates further production of IL-12 in dendritic cells, which increases the maturation of skin Langerhans cells and therefore, increases immunity in the skin. Another role, as mentioned above, is the ability of IL-12 to increase the cytotoxic activity of NK cells and T cells. Perhaps one of the major functions of IL-12 is the ability to regulate the adaptive immune response. IL-12 is one of the main cytokines that are able to regulate the differentiation of Th1 cells, which in turn produces IFN-γ which increases the response against intracellular pathogens. Furthermore, with the production of IL-12 and IFN-γ there is an antagonizing effect towards the Th2 differentiation (Watford *et al*., 2003).

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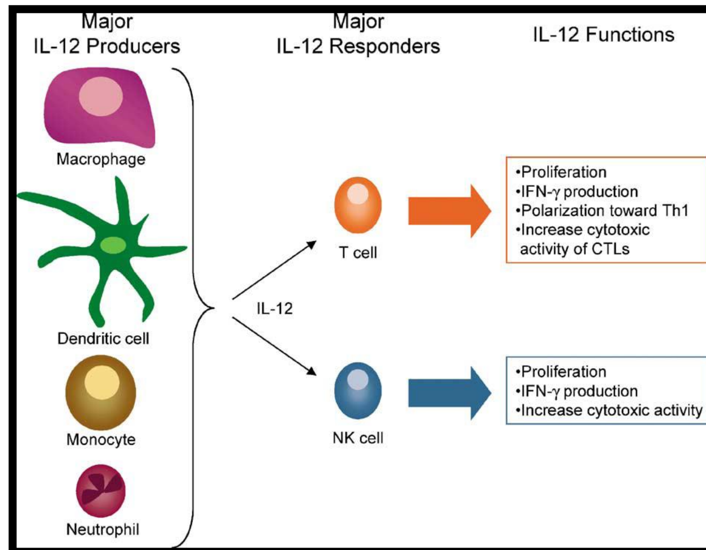

Figure 6.1: The major producers and responders of IL-12 (Watford *et al*., 2003)

The IL-12 receptor (IL-12R) is made up of two subunits; β1 and β2, which are structurally similar to the type I cytokine receptor superfamily (Fig 6.2).

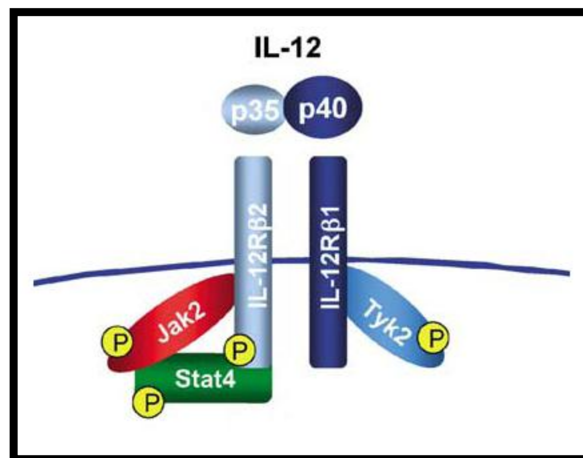

Figure 6.2: Mechanism of IL-12 signalling pathway (Watford *et al*., 2003)

The IL-12R is found on activated NK cells, dendritic cells and activated T cells. For high-affinity binding to IL-12R there needs to be a co-expression of both subunits, as affinity of IL-12 for a single subunit is low. As mentioned before IL-12 consists of two subunits, p40 which interacts mainly with the β1 subunit of the receptor, and p35 which predominantly interacts with the β2 subunit. The β2 subunit is responsible for the signal transduction chain reaction, whereas the β1 subunit is responsible for ligand binding (Presky *et al*., 1996). The IL-12 has a major role in activating signal transducers and activators of transcription (STAT's) in the signalling pathway. This is important to

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increase the differentiation of $CD4^+$ T cells into helper T cells (Th1), increase cytotoxicity of NK cells and proliferation of T cells (Watford *et al*., 2004).

IL-12 activates these functions by binding to its receptors and thereby activating the Janus family kinases namely Tyrosinase kinase 2 (Tyk2) and Janus kinase 2 (Jak2). The two subunits of the receptors are responsible for different pathways. The IL-12β1 receptor binds to Tyk2, whereas the IL-12β2 binds to Jak2. Jak2 is responsible for phophorylating the tyrosine residues of STAT3 and STAT4, which in turn is responsible for expression of IFN-γ (Jacobson *et al*., 1995; Thierfelder *et al*., 1996 & Kusaba *et al*., 2005). The STAT4 is also responsible for the transcription of IL-12Rβ2 and IL-18Rβ1 which leads to an increased production of IL-12 and differentiation of Th1 cells. The cell adhesion during T cell differentiation is further increased by ligand formation. Furthermore, IL-12, through STAT4, is responsible for increasing the expression of IL-2R which ultimately leads to an increase in T cell proliferation and leads to an increase in NK cytotoxicity. Jak2 is also responsible for the phosphorylation of STAT5 which causes an increase in cell proliferation (Ahn *et al*., 1998). IL-12 therefore, plays an important role in regulating the inflammatory response, innate immune response and the adaptive immune response.

IL-12 has been used in numerous studies to illustrate its potential as an antitumor agent. A range of studies have been performed which suggests that IL-12 acts by inhibiting tumour growth and inhibiting metastastis of tumours (Brunda *et al*., 1993). It has also been noted that the antitumor activity of IL-12 can be linked to its ability to activate IFN-γ, which exhibits antitumor activity as well as inhibits angiogenesis (Nastala *et al*., 1994; Kerbel & Hawley, 1995; Voest *et al*., 1995).

The antitumor activity of IL-12 is a complex mechanism which makes use of both the innate and adaptive immunity (Fig 6.3). IL-12 can directly stimulate NK and T cells to produce IFN-γ and TNFα, which in turn have a cytotoxic effect on invading tumour cells and cause cancer cell death. IFN-γ and TNFα can also produce CXCR3 ligands, which inhibit tumour angiogenesis through various mechanisms that disrupt the growth of vessel. Not only does IFN-γ have cytotoxic effects on tumour cells, but it can also induce the production of oxygen and nitrogen metabolites which can negatively affect cancer cells. During the adaptive immune response IL-12 can further induce a Th1 response and induce the production of CTL and antibodies which recruits the phagocytes and NK cells to the site of the tumour cells (Colombo & Trinchieri, 2002).

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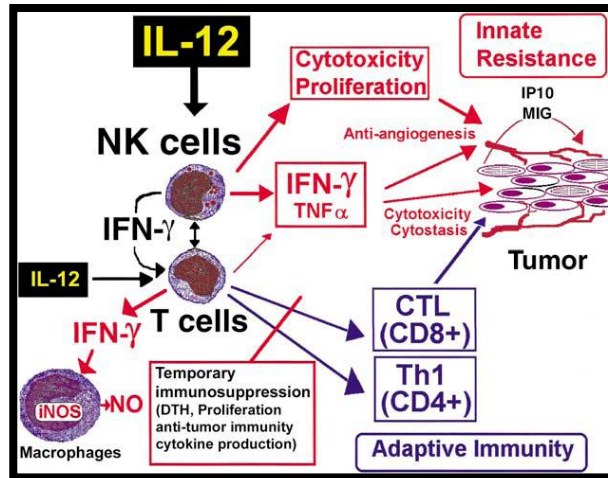

Figure 6.3: The antitumor activity of IL-12 is mediated via both the innate and adaptive immune system (Colombo & Trinchieri, 2002)

There have been many clinical trials on the use of IL-12 as an antitumor agent but to this date they have shown minimal effect. However, IL-12 still remains a promising cytokine for preclinical and clinical investigation as an antitumor agent.

1.2 Basic biology and role of IL-8 in cancer

Interleukin 8 (IL-8) is a cytokine of the CXC chemokine family, where C indicates the NH-2 end cysteines and X indicates the amino acid which links the two. IL-8 plays an important role in tumour progression and metastasis. The possible mechanism by which IL-8 plays a role in tumour progression is by regulating angiogenesis, cancer cell growth as well as cancer cell survival, tissue invasion and metastasis (Yuan,*et al*. 2005,; Kuai *et al*., 2012).

The role of IL-8 in tumour angiogenesis was first discovered by Smith *et al* (1994) where an over-expression of IL-8 was noted in bronchogenic cancer cells. In other studies it was found that there was also an over-expression of IL-8 in various cancer cells such as hepatoma, melanoma, transitional cell carcinoma and astrocytoma (Yuan *et al.,* 2005). Furthermore, in solid human cancers it was found that IL-8 was again over-expressed in cancers such as head and neck squamous cell carcinoma, colorectal cancer, glioblastoma, melanoma and lung cancer as well as pancreatic cancer (Eisma *et al*., 1999; Ueda *et al*., 1994; Desbaillets *et al*., 1997; Luca *et al*., 1997; Yatsunami *et al*., 1997; Xie, 2001).

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In a recent study conducted by Kuai *et al* (2012) experiments were conducted to determine whether IL-8 was involved in the proliferation, adhesion, migration and invasion of human gastric cancer cells. After performing Transwell and wound-healing assays it was found that IL-8 was involved in migration and after performing cell invasion assays it was found that IL-8 was involved in cell invasion.

It is therefore evident that IL-8 does play a major role in the progression of various cancer cells by invasion, migration, adhesion, proliferation as well as increased angiogenesis and also shows great potential as a target for cancer treatment.

In this study monocytic U937 cells were used and differentiated into macrophages using phorbol-12-myristate 13-acetate (PMA). *H. odoratissimum* was then added to the macrophages to determine whether the extract was able to increase the expression of IL-12 or decrease the expression of IL-8 in the differentiated U937 cells.

1.3 U937 cells, PHA and pentoxifylline

The U937 cells are monocytic cells, which are known as histiocytic lymphoma cell line (Fig 6.4). These cells can be differentiated into macrophage type cells upon exposure to phorbol esters such as PMA (Garcia *et al.*, 1999).

Phytohemagglutinin (PHA) is a plant derived mitogen that is able to stimulate T cells (NeuroScience, 2010). In a study conducted by Sullivan *et al.* (2000) peripheral blood mononuclear cells (PBMCs) were stimulated with 10µg/ml PHA and it was found that it was successful in stimulating the production of IL-2, IL-4, IL-6 and TNFα.

Pentoxifylline is a 3,7-dimethyl-1-(5-oxohexyl)-xanthine which is known to inhibit phosphodiesterase and is used for the treatment of various disorders such as vascular diseases and degraded microcirculation. It furthermore, has the ability to increase platelet aggregation and inhibit LPS-induced leukocyte adhesion (Poulakis *et al.*, 1999). Pentoxifylline is also known for its ability to induce certain cytokines and inhibit certain cytokines. In a study conducted by Schandené *et al.* (1992) found that at high concentrations of pentoxifylline IL-6 production was increased in PBMC's, whereas TNFα production was inhibited.

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2. Materials and methods

2.1 Materials

The U937 (CRL-1593.2) cell line was obtained from the ATCC, MD, USA. All the TPP® sterile plastic ware, cell culture flasks, FBS and gentamicin were purchased from Separations (Pty) Ltd. (Randburg, Johannesburg, RSA). The RPMI 1640 was supplied by Highveld Biological (Pty) Ltd. (Modderfontein, Johannesburg, RSA). The Cell Proliferation Kit II (XTT) used to carry out the cytotoxicity assay was purchased from Roche Diagnostics (Pty) Ltd. (Randburg, Johannesburg, RSA). The reagent set B, BD Falcon™ ELISA plates, human IL-12 (p40) and human IL-8 ELISA kits were purchased from BD Biosciences, San Diego, CA, USA. The PMA, PHA and all other chemicals and reagents were of analytical grade and were acquired from Sigma Chemicals Co. (St. Louis, MO, USA).

2.2 Methods

2.2.1 Cell culture of U937 cells

The U937 suspension cells were maintained in culture flasks containing RPMI 1640 medium supplemented with 10% heat-inactivated FBS and 1%gentamicin. The cells were grown at a temperature 37°C and 5% CO_2.The cells were sub-cultured every two days until there were enough cells to reach a concentration of $2.5X10^5$ cell per well in a 24-well plate. To plate the cells in a 24-well plate the same procedures were followed as in Chapter 3, section 2.2.2. However while preparing the relevant dilutions for plating the cell, PMA was added to reach a final concentration of 0.1µg/ml in order for the cells to differentiate from monocytes to macrophages. To determine the cell viability the XTT Cell Proliferation Kit II was used in the same manner as Chapter 3, section 2.2.3.

2.2.2 Collection of supernatant

The U937 cells were plated at a concentration of $2.5X10^5$ cells per well in a 24-well plate in the same manner as mentioned above. The cells were then incubated for 24h at 37°C at 5% CO_2 to allow the

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monocytes to differentiate into macrophages. The *H. odoratissimum* extract was prepared at stock concentration of 2mg/ml and tested at a concentration of 20µg/ml (IC_{50} value obtained on U937 cytotoxicity), 15µg/ml (IC_{50} value of *H. odoratissimum* on A431 cells), 10µg/ml and 5µg/ml. Pentoxifylline and PHA were prepared at stock concentrations of 2mg/ml and 1mg/ml and tested at 20µg/ml and 5µg/ml respectively. Lastly included was a DMSO vehicle control at 1%, as well as a medium only control (Fig 6.4). The plate was then incubated for a further 20hrs. Thereafter the plate was centrifuged at 980rpm for 5min and the supernatant was collected in 96-well plate and placed in a -70°C freezer until further use. All the samples were tested in triplicate.

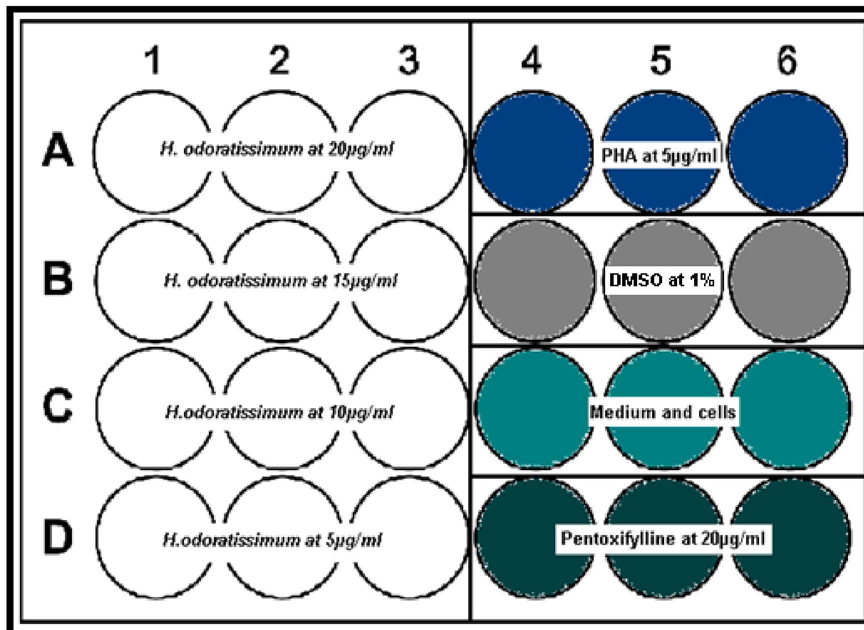

Figure 6.4: Layout of 24-well plate including cells and samples

2.2.3 Cytokine detection assay

To determine whether the plant extract was able to induce IL-12 secretion and inhibit IL-8 secretion a procedure was used as recommended by the manufacturers' protocol. Briefly, a 96-well plate was coated with 100µl capture antibody (see Appendix B) and incubated overnight at 4ºC. After the incubation period the wells were washed 3 times with wash buffer (see Appendix B) and thereafter blotted on absorbent paper to remove excess wash buffer and incubated with 200µl assay diluents for 1h at room temperature. The wells were then washed as previously described. The standards (see Appendix B) and samples were added (100µl) to the appropriate wells and incubated for a further 2hrs at room temperature. Once incubation was complete the wells were again washed as previously mentioned but with a total of five washes. Thereafter 100µl of working detector (detection antibody

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and enzyme reagent) was added to each well and incubated for 1h at room temperature. After 1h the wells were washed a final of seven washes and thereafter 100µl of substrate solution was added and incubated for 30min in the dark. After 30min 50µl of stop solution was added and the plates were read at a wavelength set at 450nm and a reference wavelength of 570nm using an ELISA plate reader.

3. Results and discussion

3.1 Cytotoxicity

The cytotoxicity of *H. odoratissimum*, PHA and pentoxifylline was determined after 72hrs incubation period. The cytotoxicity was performed to determine the IC_{50} values at which the extract could be tested when evaluating the cytokine profile and to ensure that the concentrations of PHA and pentoxifylline, used to determine the cytokine profile, was not toxic to the U937 cells. The cytotoxicity results on U937 cells are depicted in Table 6.1 (see Appendix B).

Table 6.1: Cytotoxicity of *H. odoratissimum*, PHA and pentoxifylline on U937 cells

Sample	IC_{50} in µg/ml
H. odoratissimum	20.3±3.1µg/ml
PHA	318±7.8µg/ml
Pentoxifylline	170±6.4µg/ml

As seen from the cytotoxicity results, *H. odoratissimum* showed high toxicity with an IC_{50} value of 20.3±3.1µg/ml. Therefore, the concentrations chosen for the cytokine assay started from 20µg/ml and included ½IC_{50} at 10µg/ml, ¼IC_{50} at 5µg/ml as well as the IC_{50} of *H. odoratissimum* on A431 cells at 15µg/ml. The PHA and pentoxifylline controls showed low toxicity on U937 cells with IC_{50} values of 318±7.8µg/ml and 170±6.4µg/ml respectively. Therefore, the concentrations at which PHA (5µg/ml) and pentoxifylline (20µg/ml) were tested for cytokine evaluation did not have a negative effect on the cell viability.

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3.2 Cytokine assay

The differentiated U937 cells were exposed to various concentrations of *H. odoratissimum* (20µg/ml, 15µg/ml, 10µg/ml and 5µg/ml) to determine whether the plant extract was able to induce production of IL-12 and suppress the production of IL-8. In Table 6.2 is a summary of the results obtained for IL-12 and IL-8 production in U937 cells.

Table 6.2: Production of IL-12 and IL-8 in U937 cells and cell viability

Samples	IL-12 (pg/ml)	IL-8 (pg/ml)	Cell viability±SD in %
***H. odoratissimum* at 20µg/ml**	4.09±2.2	70.1±0.12	74±1.6%
***H. odoratissimum* at 15µg/ml**	5.65±2.8	73.8±1.9	101±1.9%
***H. odoratissimum* at 10µg/ml**	7.55±3.7	95.3±1.9	105±6.3%
***H. odoratissimum* at 5µg/ml**	12.4±7.0	103±6.1	128±1.6%
PHA at 5µg/ml	9.49±3.8	107±5.6	101±0.3%
DMSO at 1%	4.60±1.4	44.8±4.9	100%
Pentoxifylline at 20µg/ml	1.37±0.9	51.2±7.2	106±2.2%
Medium and cells	4.71±0.5	123±3.0	117±0.3%

H. odoratissimum was able to enhance the production of IL-12 when compared to the production of IL-12 in the medium control. The medium control produced a concentration of 4.71pg/ml, whereas *H. odoratissimum* produced 12.4pg/ml at a concentration of 5µg/ml. As the concentration of *H. odoratissimum* increased, the production of IL-12 decreased as can be seen in Table 6.2. At 20µg/ml *H. odoratssimum*, the production of IL-12 was lower than that of the medium. This could be due to the lowered viability of the cells at this concentration, which was 74%, whereas for medium the viability was 117%. This could also explain the increase in production at the lowest concentration of *H. odoratissimum* which showed a cell viability of 128%. Furthermore, at 1% DMSO the production of IL-12 was similar to that of the medium control, which suggests that DMSO did not have any effect on IL-12 production. The PHA control showed an increase in IL-12 production of 9.49pg/ml at a cell viability of 101%. No other results on PHA stimulated U937 cells were found. Pentoxifylline showed a great inhibition of IL-12 with a concentration of 1.37pg/ml and a cell viability of 106%, which suggests the viability of the cells did not affect the production of IL-12. The results obtained for pentoxifylline corresponded with the results obtained in the study performed by Moller *et al*, 1997. In the related study, pentoxifylline was tested in a dose-dependent manner on LPS stimulated PBMCs and it was found that as the concentration of pentoxifylline increased the production of IL-12 decreased.

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During the evaluation of IL-8 production a similar pattern was noted to that of IL-12 production; the higher the concentration of *H. odoratissimum* the lower the production of IL-8 as noted in Table 6.2. At a concentration of 20µg/ml *H. odoratissimum*, the IL-8 production was at its lowest of 70.1pg/ml with a cell viability of 74%. Furthermore, at 5µg/ml of *H. odoratissimum* the production of IL-8 was higher than the above mentioned with a production of 103pg/ml. However, it can also be noted that at all the concentrations of the extract the production of IL-8 was inhibited when compared to the medium control, which showed a production of 123pg/ml. Further noted was the inhibition that occurred at the PHA and pentoxifylline control. PHA showed slight inhibition with an IL-8 production of 107pg/ml, which is not in accordance with what is described by BD FastImmune, 2013, which states that IL-8 is produced by monocytes in response to PHA. Pentoxifylline showed an inhibition of less than half the IL-8 production in the medium control with a production of only 51.2pg/ml. However, what was interesting to note was the high inhibition of IL-8 in the presence of DMSO at 44.8pg/ml. This inhibition by DMSO could also explain the slight inhibition of the extracts at the different concentrations as the extracts were dissolved in DMSO. These findings correlate with the results found in a study conducted by DeForge *et al*., 1992. In this study whole blood was stimulated using LPS and exposed to 1% DMSO to determine whether DMSO inhibited the release of IL-8. It was found that DMSO was able to inhibit LPS-dependent IL-8 in a dose-dependent manner ranging from 0.008%-5% DMSO concentration.

In a study conducted by Karimi *et al*. (2014), isolated dendritic cells were exposed to various concentrations of a 70% ethanol root extract of *Cichorium intybus*. It was observed that at a concentration of 0.1µg/ml and 1µg/ml, the extract was able to increase the production of IL-12 when compared to that of the DMSO control (88.58±23.87pg/ml), to 280.6±26.58pg/ml and 195.5±16.88pg/ml respectively. This was similar to the results in the present study where at a lower concentration of extract a higher production of IL-12 was observed.

4. Conclusion

The *H. odoratissimum* was successful in producing IL-12 in PMA stimulated U937 cells when compared to the medium control. Furthermore, it was also noted that *H. odoratissimum* was able to inhibit the production of IL-8 when compared to the medium control; however, this could also be partly due to the inhibiting effect of DMSO on IL-8. Both IL-12 and IL-8 results were similar in that

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they showed results in a dose dependent manner, where the highest concentration of the extract showed lower production of the cytokines and the lower concentration of the extract showed higher production of the cytokines. Furthermore, at the active concentration of *H. odoratissimum* on the A431 cells (15µg/ml) the extract was able to both induce IL-12 production and inhibit IL-8 production. Further studies would investigate the effect IL-12 and IL-8 have on migration, adhesion and proliferation of A431 cells.

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Chapter 7

Final conclusion and recommendation for further work

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1. Conclusion

In this study twenty southern African plants were selected based on their traditional usage for the treatment of various cancers and skin diseases. These plants were chosen to determine whether they possessed any potential anticancer activity against human melanoma (A375), cervical cancer (HeLa) and epidermoid carcinoma (A431). To determine the efficacy of the plant extracts to have anticancer activity the antioxidant potential of the plant extracts was determined, using the DPPH and nitric oxide scavenging assay, as well as their cytotoxicity against the cancerous cells and a non-cancerous cell line, HEK-293.

After performing the XTT assay to determine the cytotoxic effects of the plant extracts, it was evident that only one plant extract showed great potential as an anti-cancer agent as it showed an IC_{50} value <20µg/ml after 72hrs incubation, which complies with the guidelines of the US National Cancer Institute (Mahavorasirikul *et al*., 2010). *Helichrysum odoratissimum* showed an IC_{50} value of 15.5±0.2µg/ml against A431 cells and an IC_{50} value of 37.1±4.8µg/ml against HEK-293 cells. From this it was determined that *H. odoratissimum* with an SI value of 2.39 was the best candidate for further investigation.

To further determine the chemo-preventive properties of the extracts, a DPPH radical scavenging and nitric oxide scavenging assay were performed. Two extracts showed potent antioxidant activity, *Rapanea melanophloeos* showed an IC_{50} value of 63.73±0.4µg/ml in the nitric oxide assay whereas *Syzygium jambos* showed an IC_{50} value of 1.17±0.26µg/ml in the DPPH assay. Both these extracts showed greater potential as antioxidants than Vitamin C. The antioxidant activity can be attributed to secondary metabolites such as phenolics, polyphenols and flavanoids which inhibit oxidative stress (Patel *et al*., 2012). *H. odoratissimum* also showed a good radical scavenging capacity with an IC_{50} value of 5.13±0.07µg/ml.

Due to the good anti-cancer activity of *H. odoratissimum* on A431 cells the extract was further used to determine the mechanism of action. Mechanistic studies included light microscopy (haematoxylin and eosin staining) and cytokine evaluation. During light microscopy characteristic features of apoptosis were observed such as apoptotic bodies, hypercondensed chromatin, cellular debris and reduced cytoplasm. In the cytokine evaluation experiment IL-12 was increased after exposure to *H. odoratissimum* whereas IL-8 decreased. IL-12 has been linked to a decrease in cancer growth whereas IL-8 has been linked to an increase in cancer growth (Brunda *et al*., 1993; Yuan,*et al*. 2005,; Kuai *et al*., 2012).

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Future consideration would be to perform other mechanistic studies which would give more possible answers on whether *H. odoratissimum* is inhibiting specific enzymes which would inhibit cancer cell growth, such as COX-2 and SPHk1, as well as techniques such as raman and infrared spectroscopy. Furthermore would be to consider bio-assay guided fractionation of *H. odoratissimum* to determine the active compounds which are responsible for the anti-cancer activity on A431 cells.

2. Review of conclusions

- Ethanol extracts of twenty southern African plants were prepared and tested for cytotoxic and antioxidant activity.
- The extract *H. odoratissimum* showed the greatest anti-cancer activity with an IC_{50} of 15.5µg/ml on A431 cells.
- Synergistic studies performed with *H. odoratissimum* and *S. jambos* on A431 cells showed non-interactive results.
- *R. melanophloeos* and *S.jambos* displayed potent antioxidant activity with IC_{50} values of 63.73±0.4µg/ml and 1.17±0.26µg/ml in the nitric oxide and DPPH scavenging assay respectively.
- During light microscopy A431 cells showed characteristic features of apoptosis when exposed to various concentrations of *H. odoratissimum* extract.
- *H. odoratissimum* was further able to increase the production of IL-12 and inhibit the production of IL-8 in U937 cells.
- This is the first report of *H. odoratissimum* ethanol extract showing potent activity on A431 cells.

3. Future recommendations

- Bio-assay guided fractionation of *H. odoratissimum* to determine the active compounds responsible for the anti-cancer activity of A431 cells.

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- Perform real-time cell cycle analysis, using xCELLigence, to determine the kinetic profile of the active compounds and to possibly detect the mechanism of action.
- Perform enzyme inhibition studies, such as COX-2 and SPHK1, which could lead to the inhibition of cancer cell growth

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Appendix A

Cytotoxicity graphs

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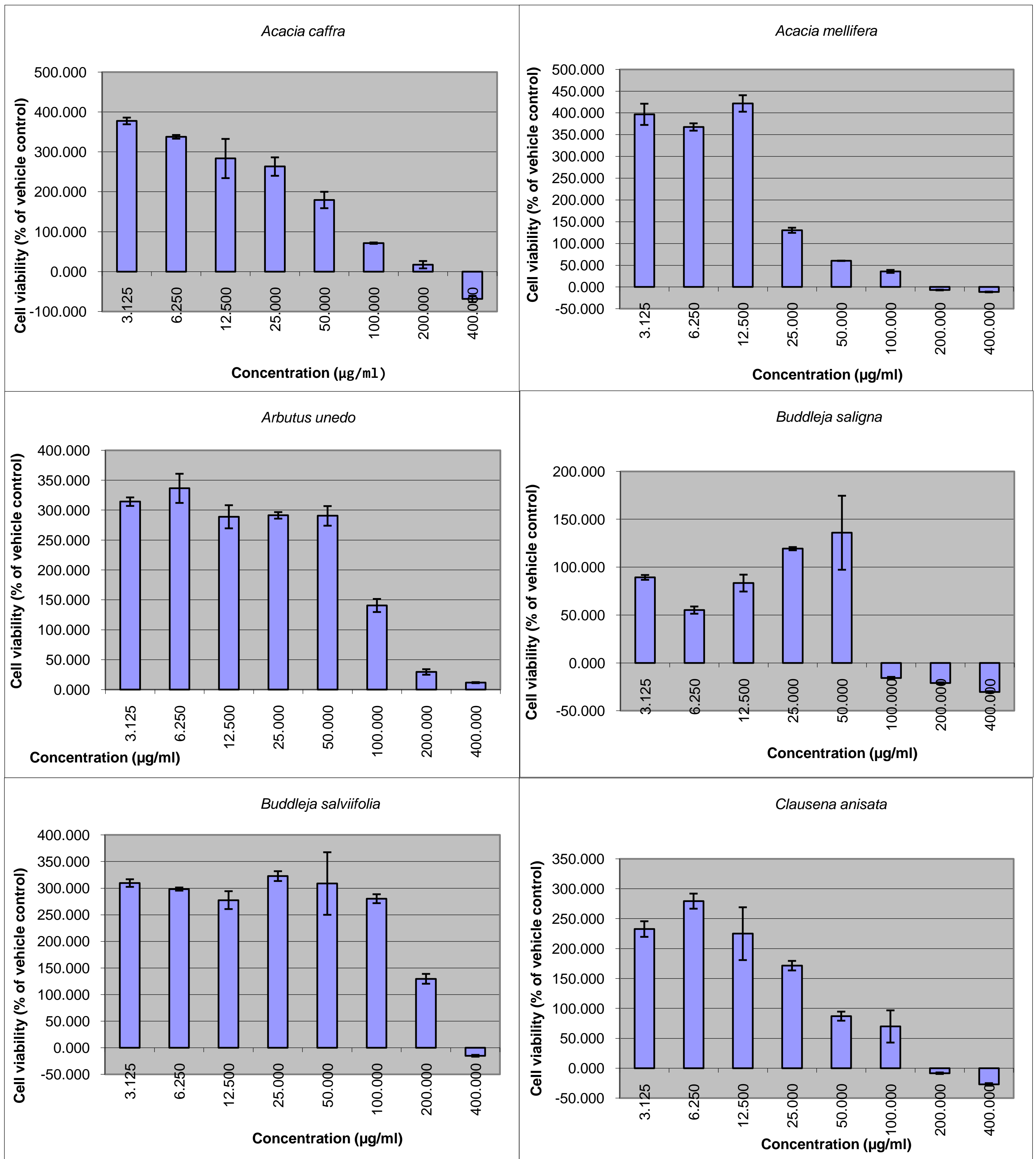

Fig 1: Dose-dependent curves of plant extracts on HeLa cells after 72h of exposure

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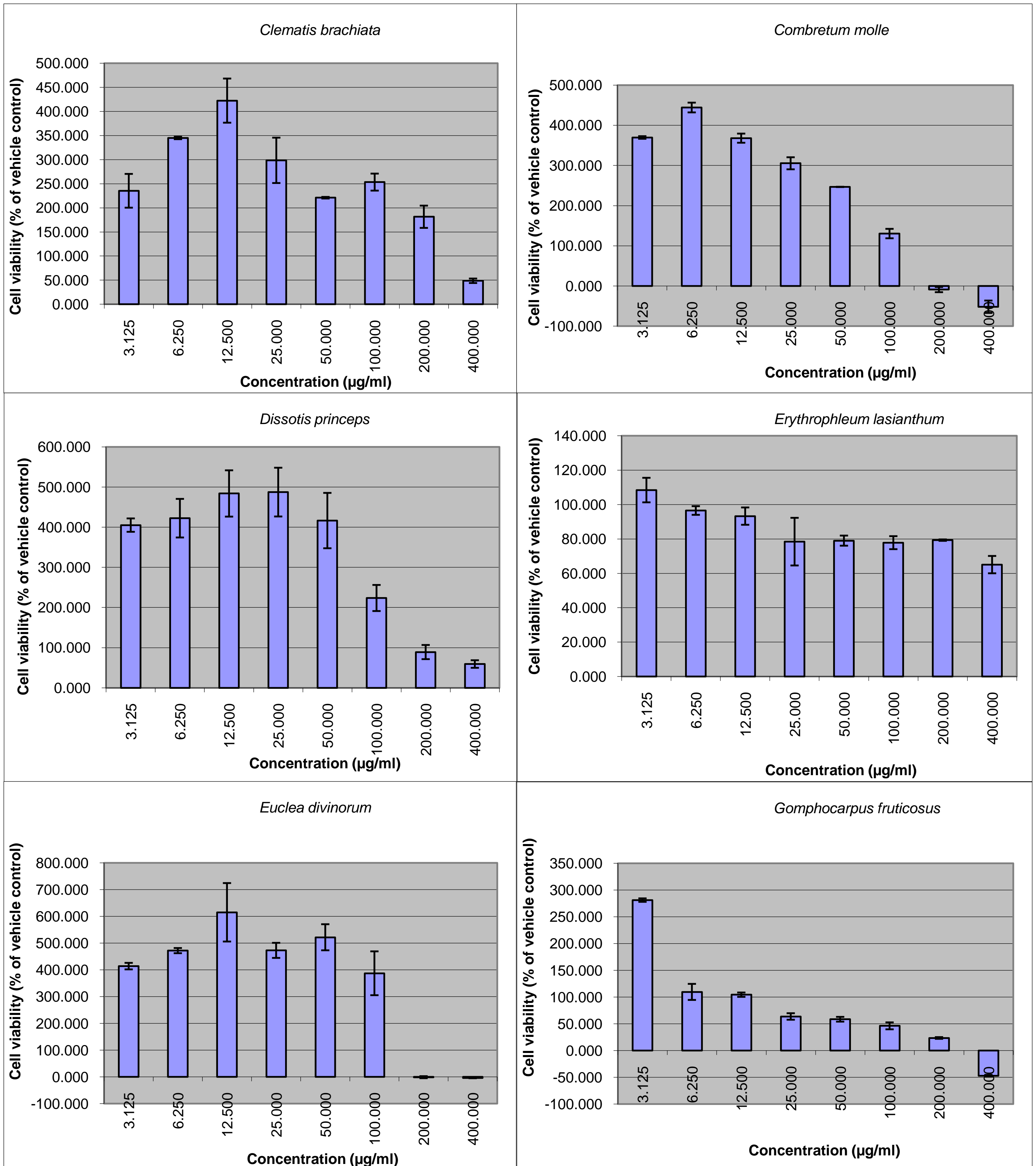

Fig 2: Dose-dependent curves of plant extracts on HeLa cells after 72h of exposure

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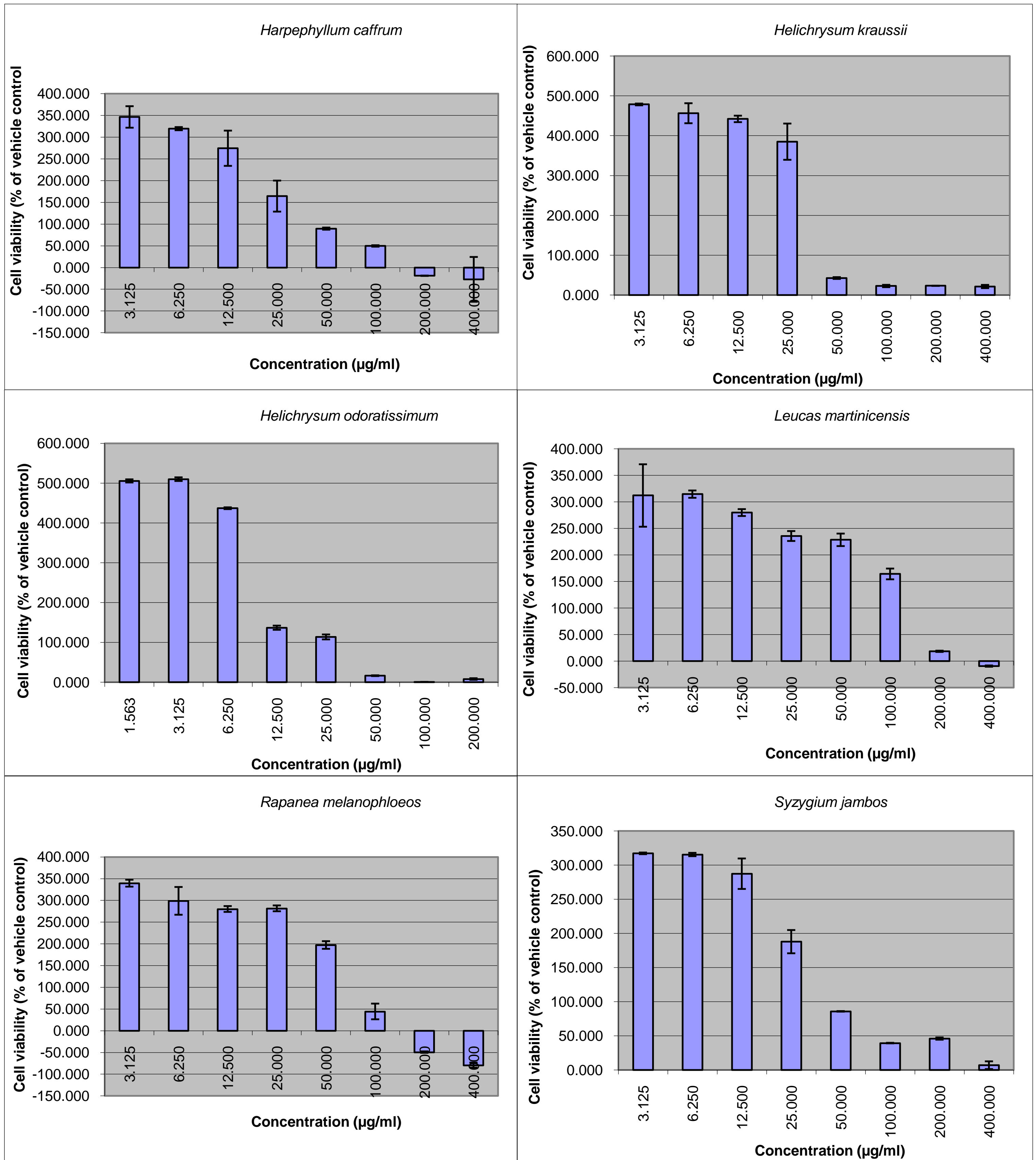

Fig 3: Dose-dependent curves of plant extracts on HeLa cells after 72h of exposure

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Tabernaemontana elegans
Cell viability (% of vehicle control)
700.000
600.000
500.000
400.000
300.000
200.000
100.000
0.000
3.125
6.250
12.500
25.000
50.000
100.000
200.000
400.000
Concentration (µg/ml)

Warburgia salutaris
Cell viability (% of vehicle control)
700.000
600.000
500.000
400.000
300.000
200.000
100.000
0.000
3.125
6.250
12.500
25.000
50.000
100.000
200.000
400.000
Concentration (µg/ml)

Actinomycin D
Cell viability (% of vehicle control)
90.000
80.000
70.000
60.000
50.000
40.000
30.000
20.000
10.000
0.000
0.00039
0.00078
0.00156
0.00313
0.00625
0.01250
0.02500
0.05000
Concentration (µg/ml)

Fig 4: Dose-dependent curves of plant extracts on HeLa cells after 72h of exposure

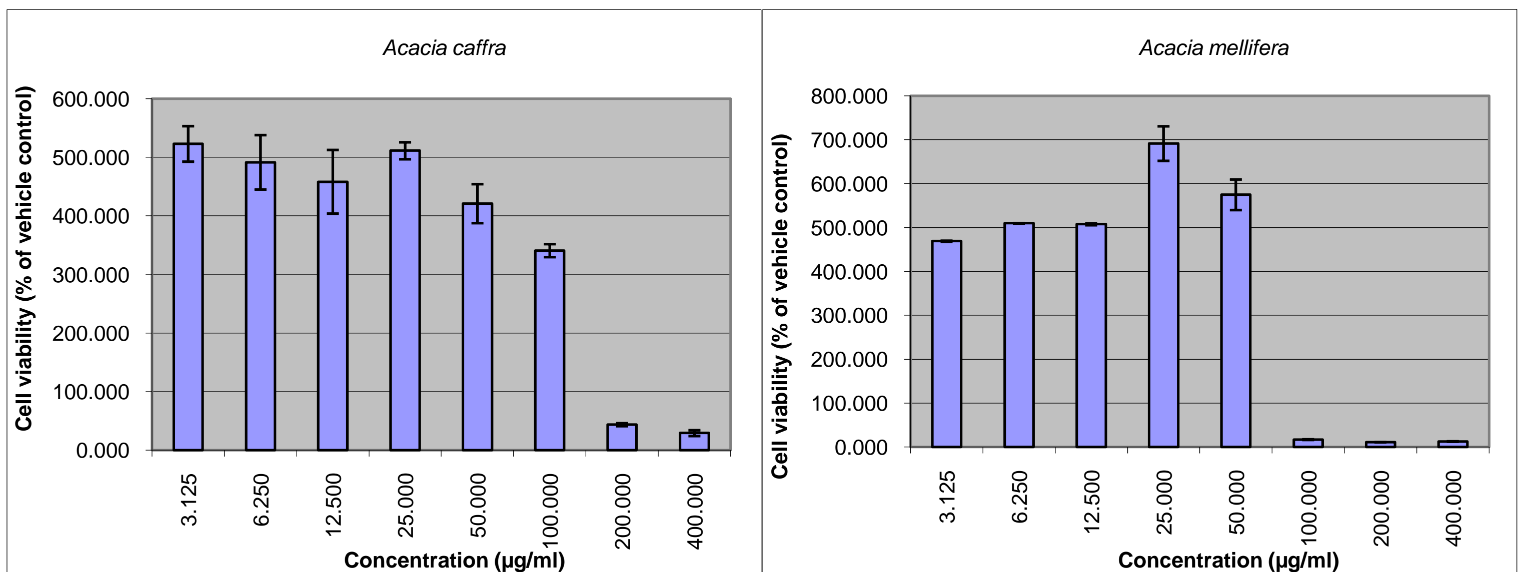

Fig 1: Dose-dependent curves of plant extracts on A431 cells after 72h of exposure

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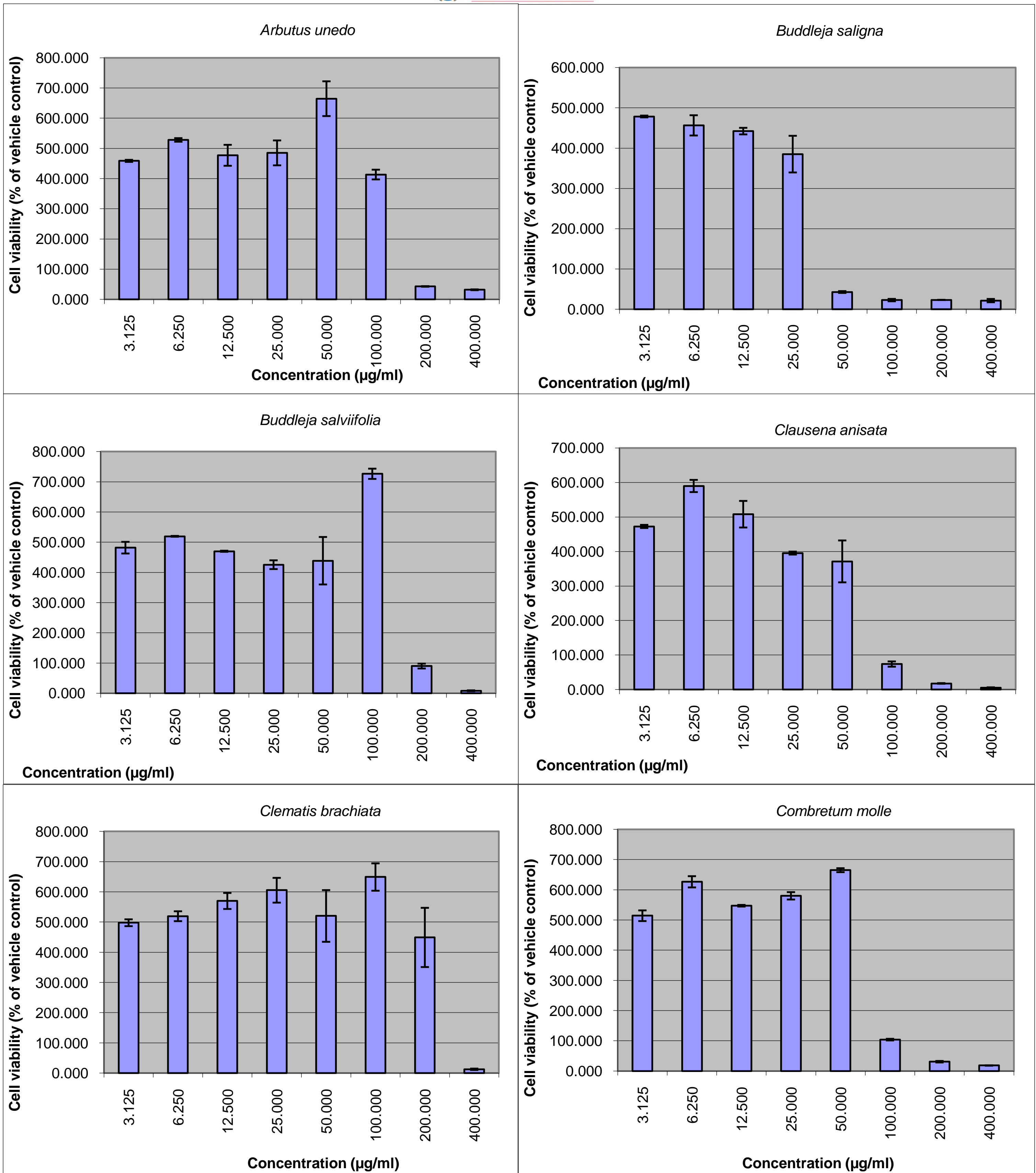

Fig 2: Dose-dependent curves of plant extracts on A431 cells after 72h of exposure

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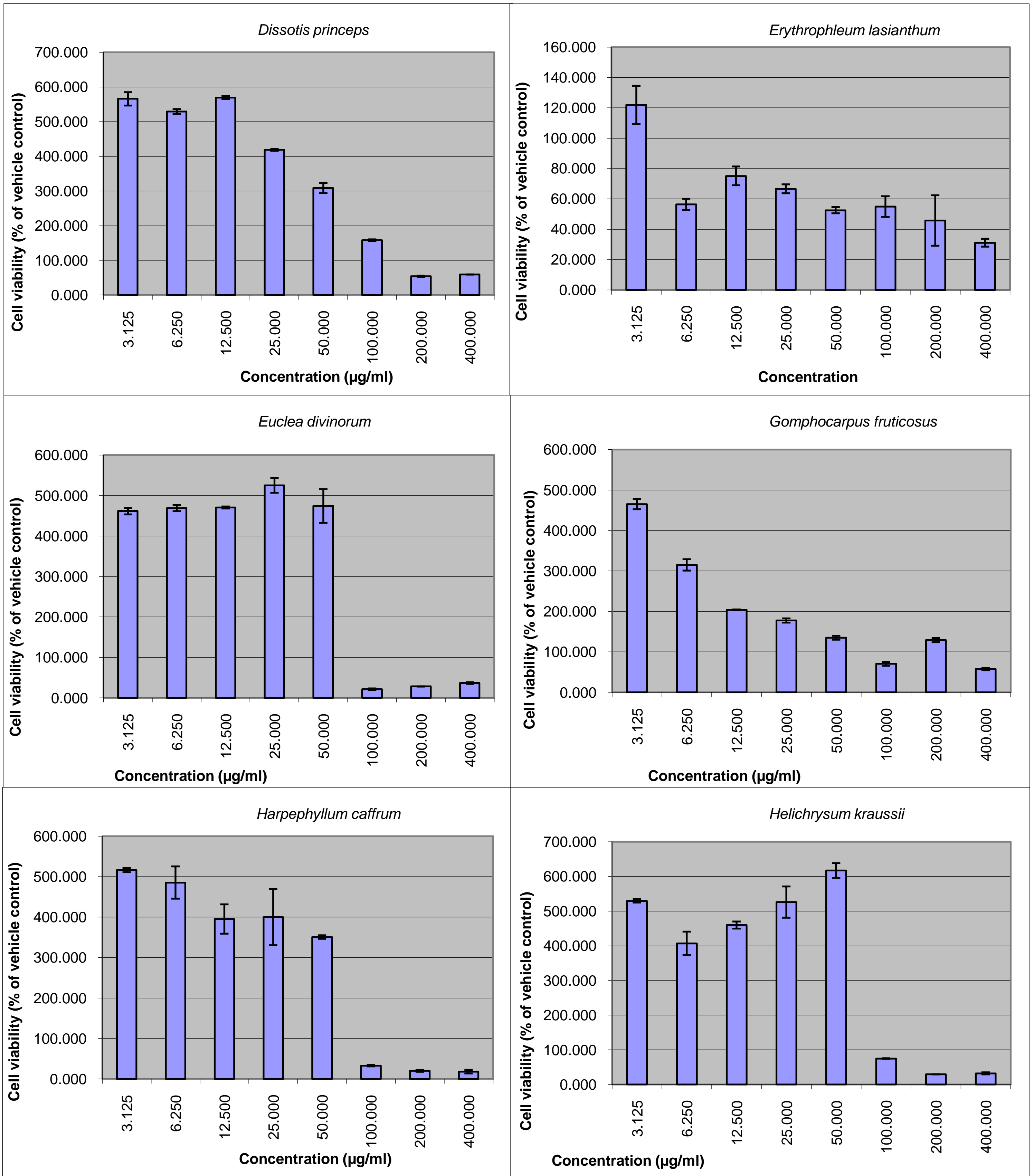

Fig 3: Dose-dependent curves of plant extracts on A431 cells after 72h of exposure

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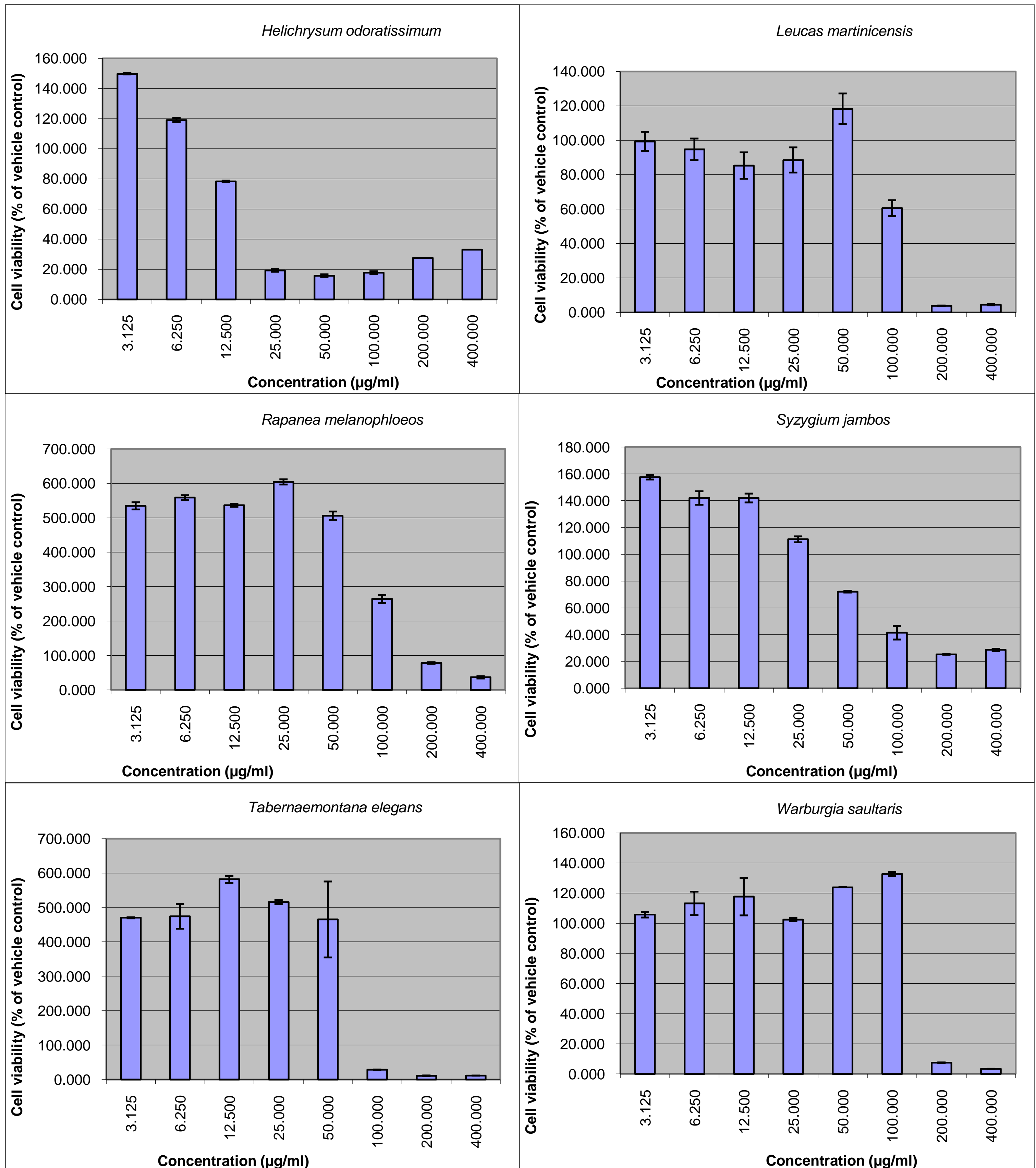

Fig 4: Dose-dependent curves of plant extracts on A431 cells after 72h of exposure

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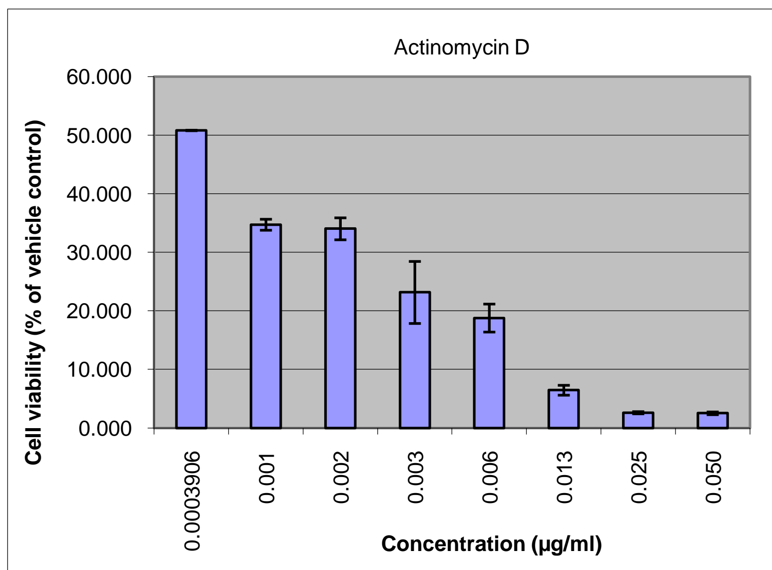

Fig 5: Dose-dependent curves of plant extracts on A431 cells after 72h of exposure

Acacia caffra

Cell viability (% of vehicle control)

350.000
300.000
250.000
200.000
150.000
100.000
50.000
0.000

3.125
6.250
12.500
25.000
50.000
100.000
200.000
400.000

Concentration (µg/ml)

Acacia mellifera

Cell viability (% of vehicle control)

450.000
400.000
350.000
300.000
250.000
200.000
150.000
100.000
50.000
0.000
-50.000

3.125
6.250
12.500
25.000
50.000
100.000
200.000
400.000

Concentration (µg/ml)

Arbutus unedo

Cell viability (% of vehicle control)

400.000
350.000
300.000
250.000
200.000
150.000
100.000
50.000
0.000

3.125
6.250
12.500
25.000
50.000
100.000
200.000
400.000

Concentration (µg/ml)

Buddleja saligna

Cell viability (% of vehicle control)

500.000
400.000
300.000
200.000
100.000
0.000
-100.000

3.125
6.250
12.500
25.000
50.000
100.000
200.000
400.000

Concentration (µg/ml)

Fig 1: Dose-dependent curves of plant extracts on A375 cell after 72h of exposure

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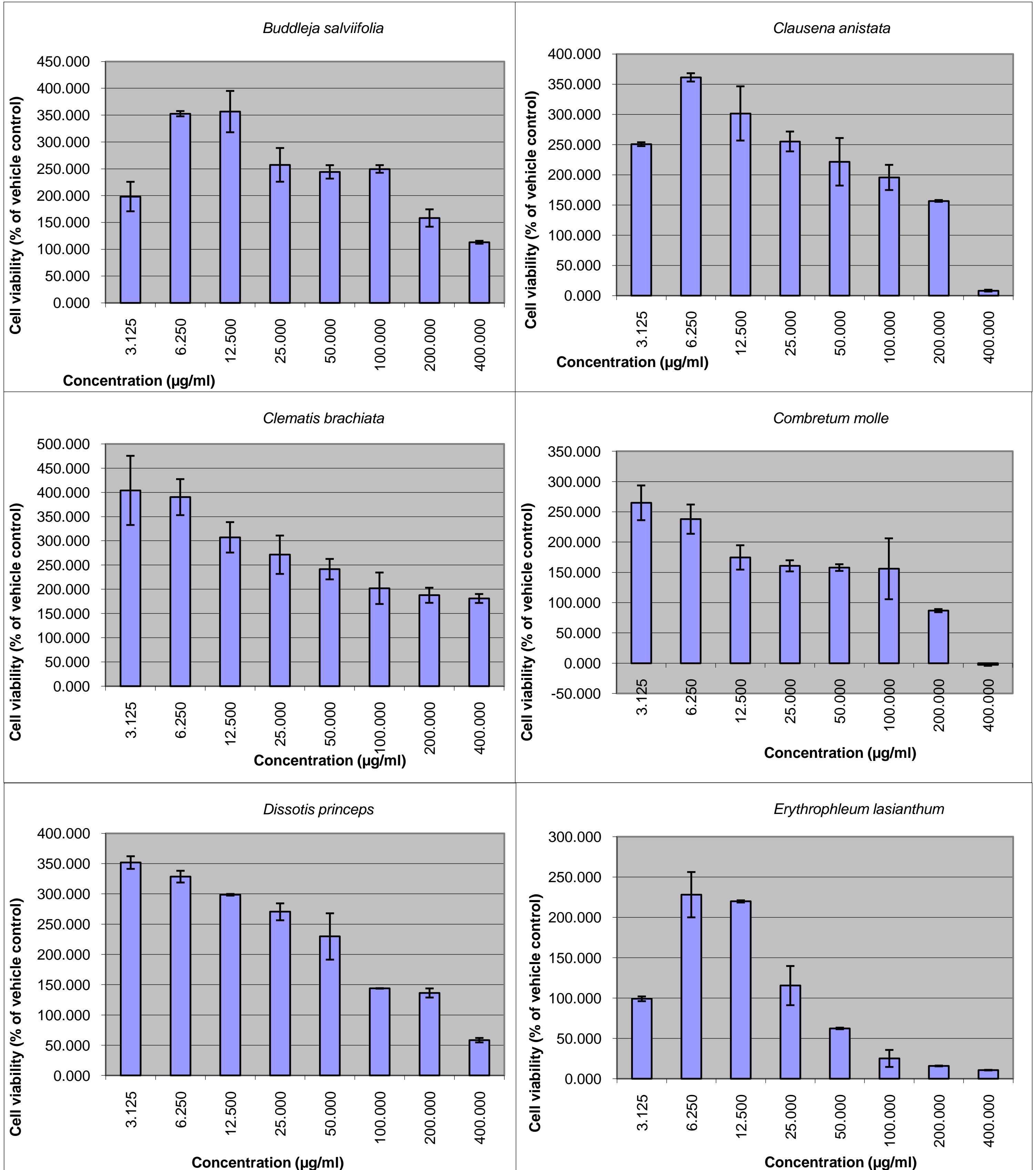

Fig 2: Dose-dependent curves of plant extracts on A375 cells after 72h of exposure

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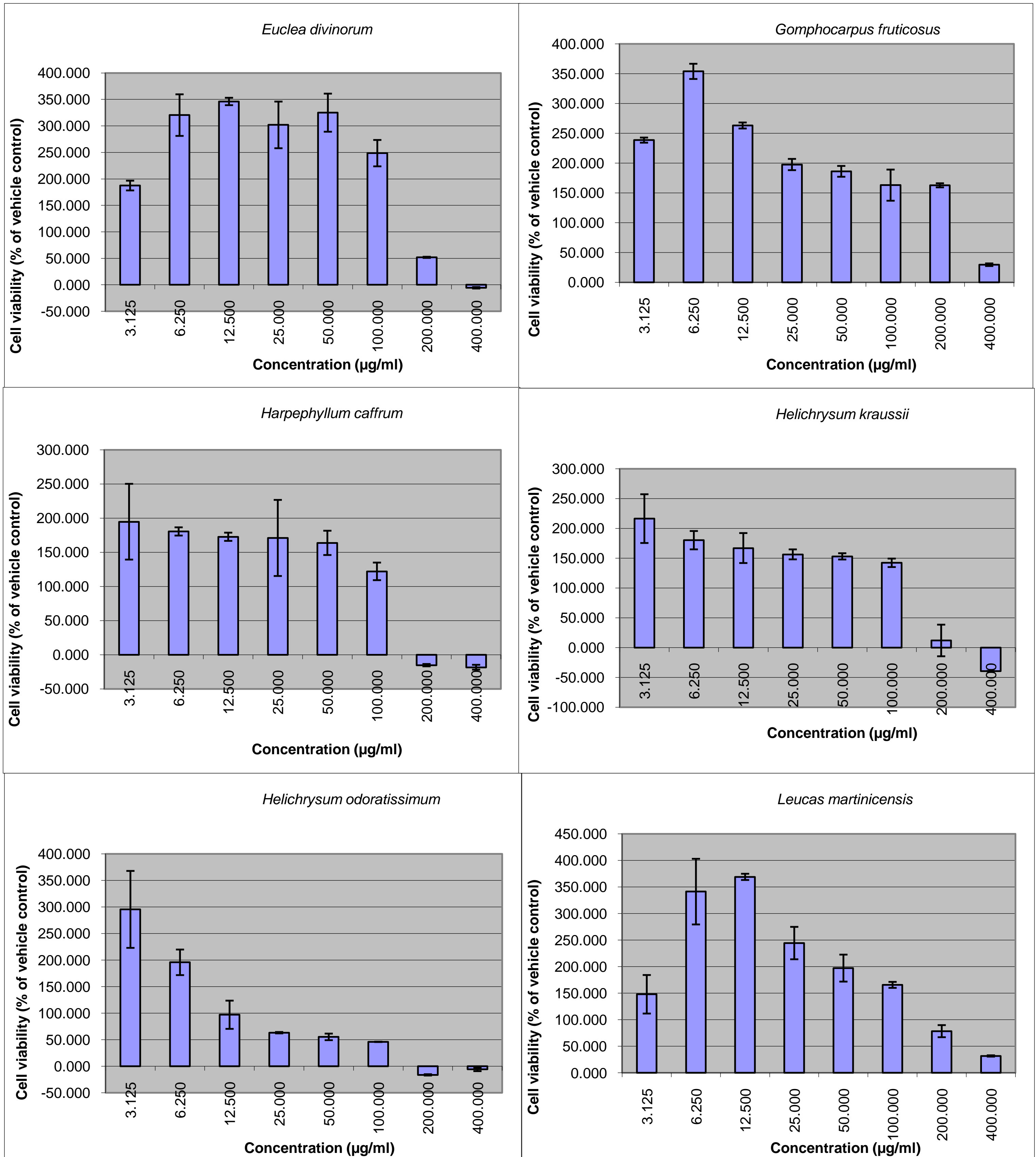

Fig 3: Dose-dependent curves of plant extracts on A375 cells after 72h of exposure

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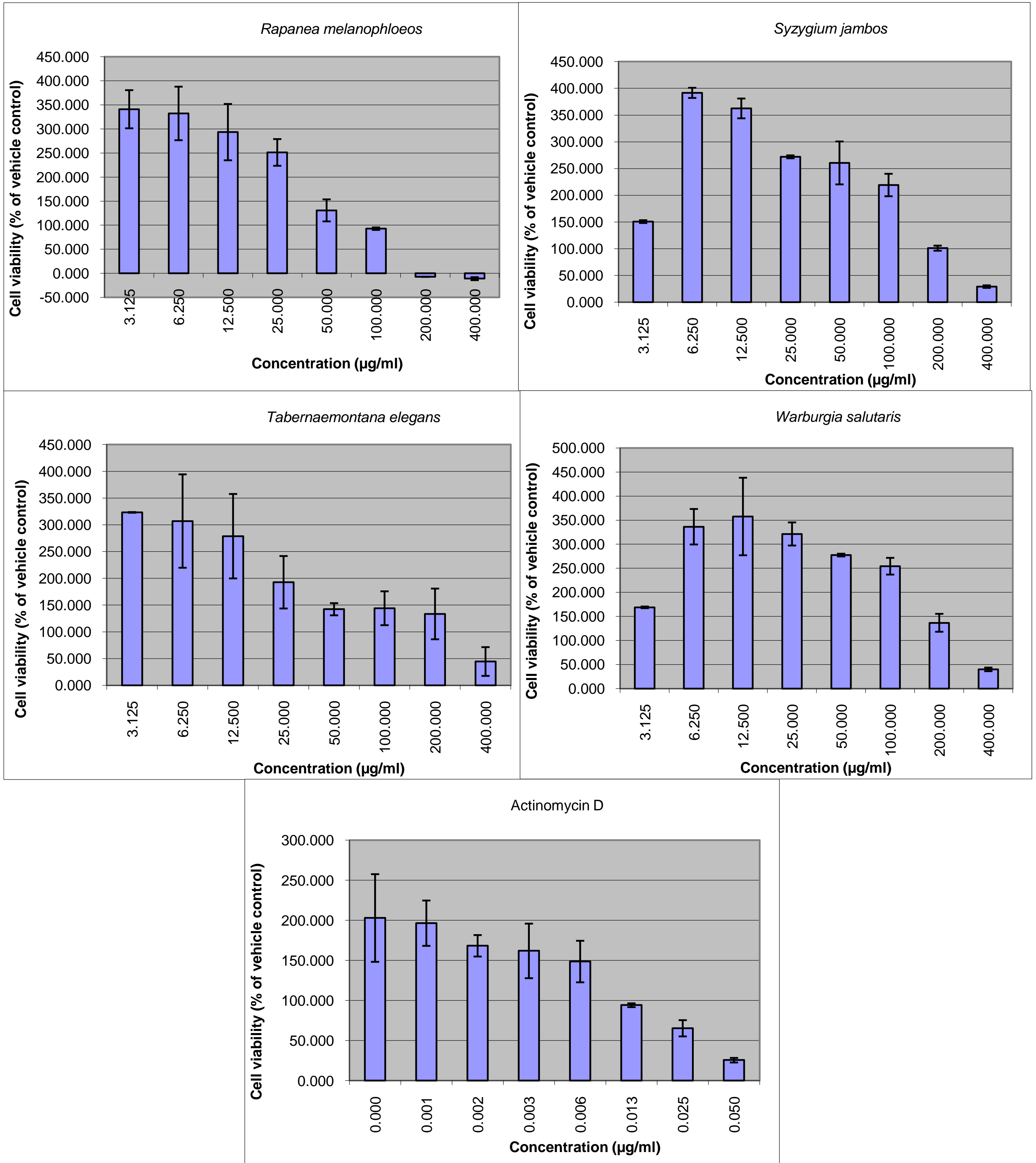

Fig 4: Dose-dependent curves of plant extracts on A375 cell after 72h of exposure

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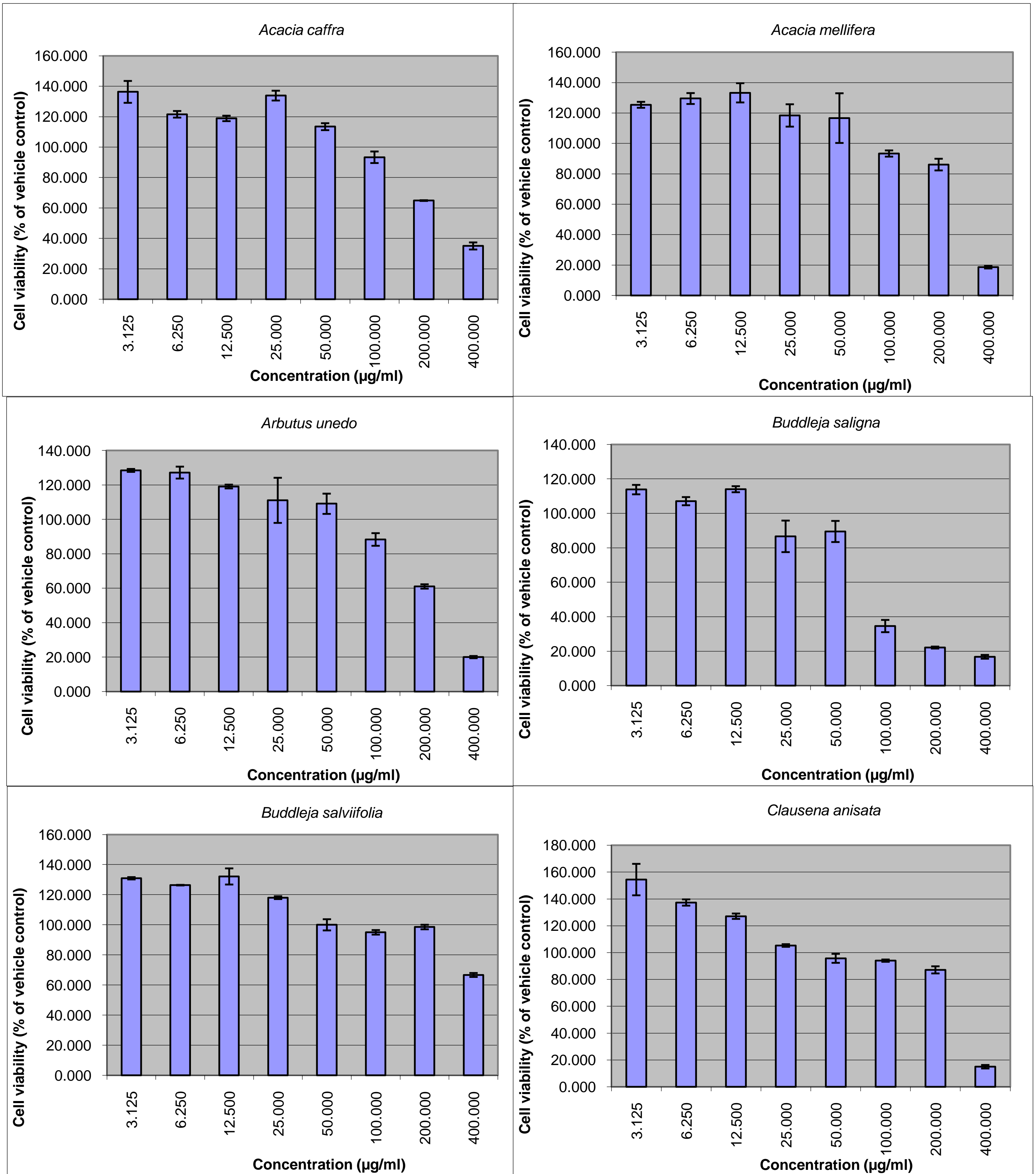

Fig 1: Dose-dependent curves of plant extracts on HEK-293 cells after 72h of exposure

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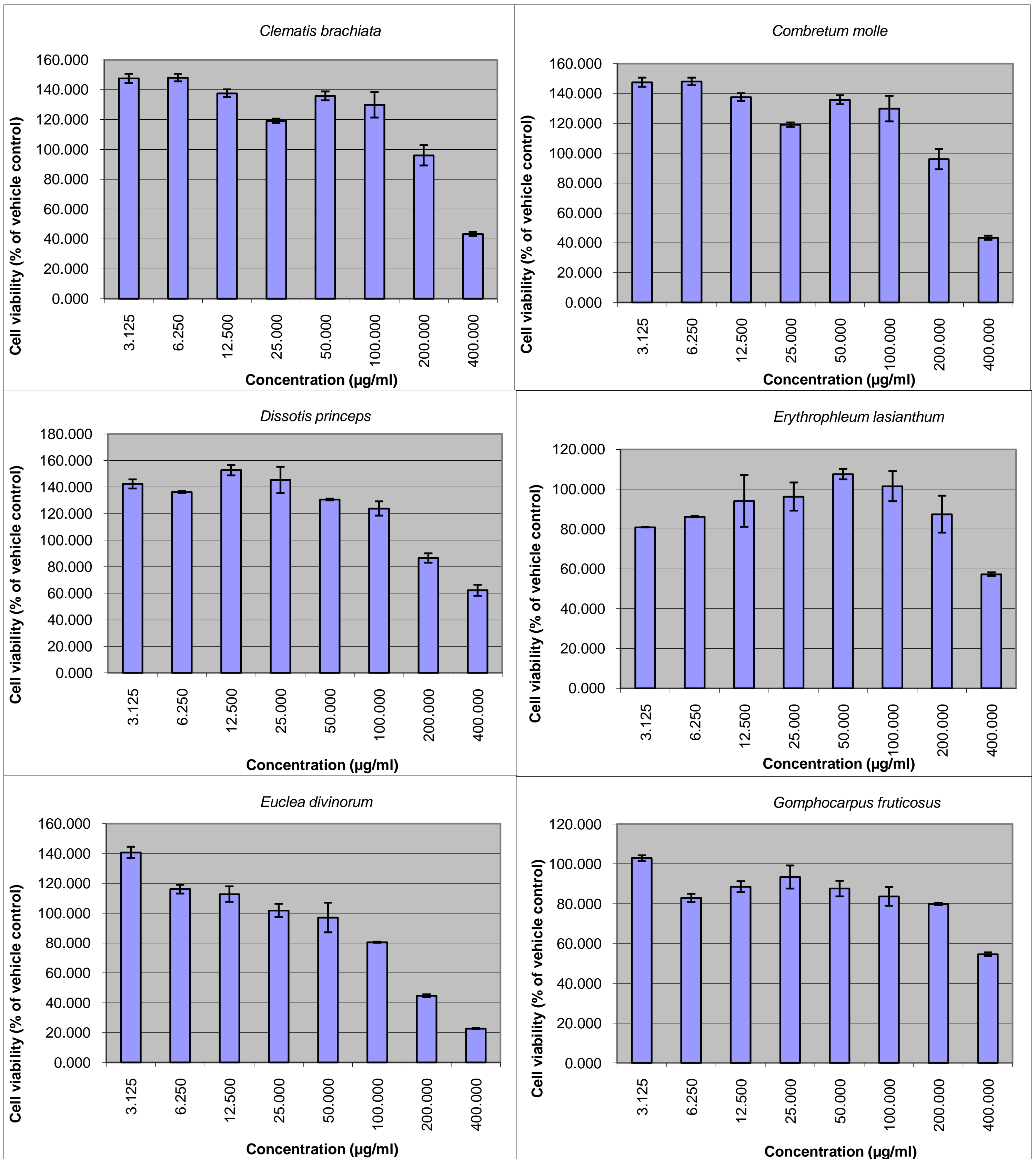

Fig 2: Dose-dependent curves of plant extracts on HEK-293 cells after 72h of exposure

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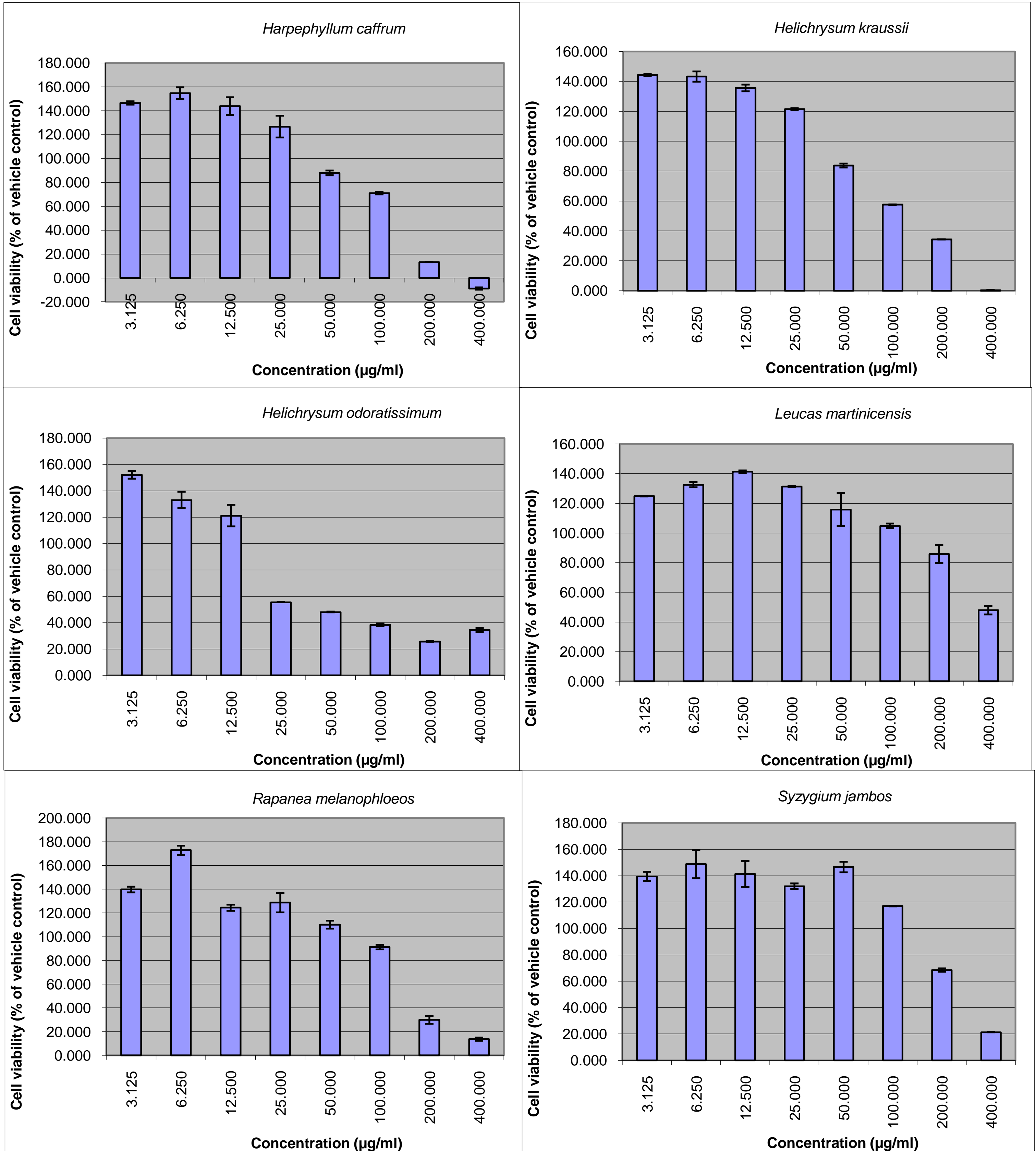

Fig 3: Dose-dependent curves of plant extracts on HEK-293 cells after 72h of exposure

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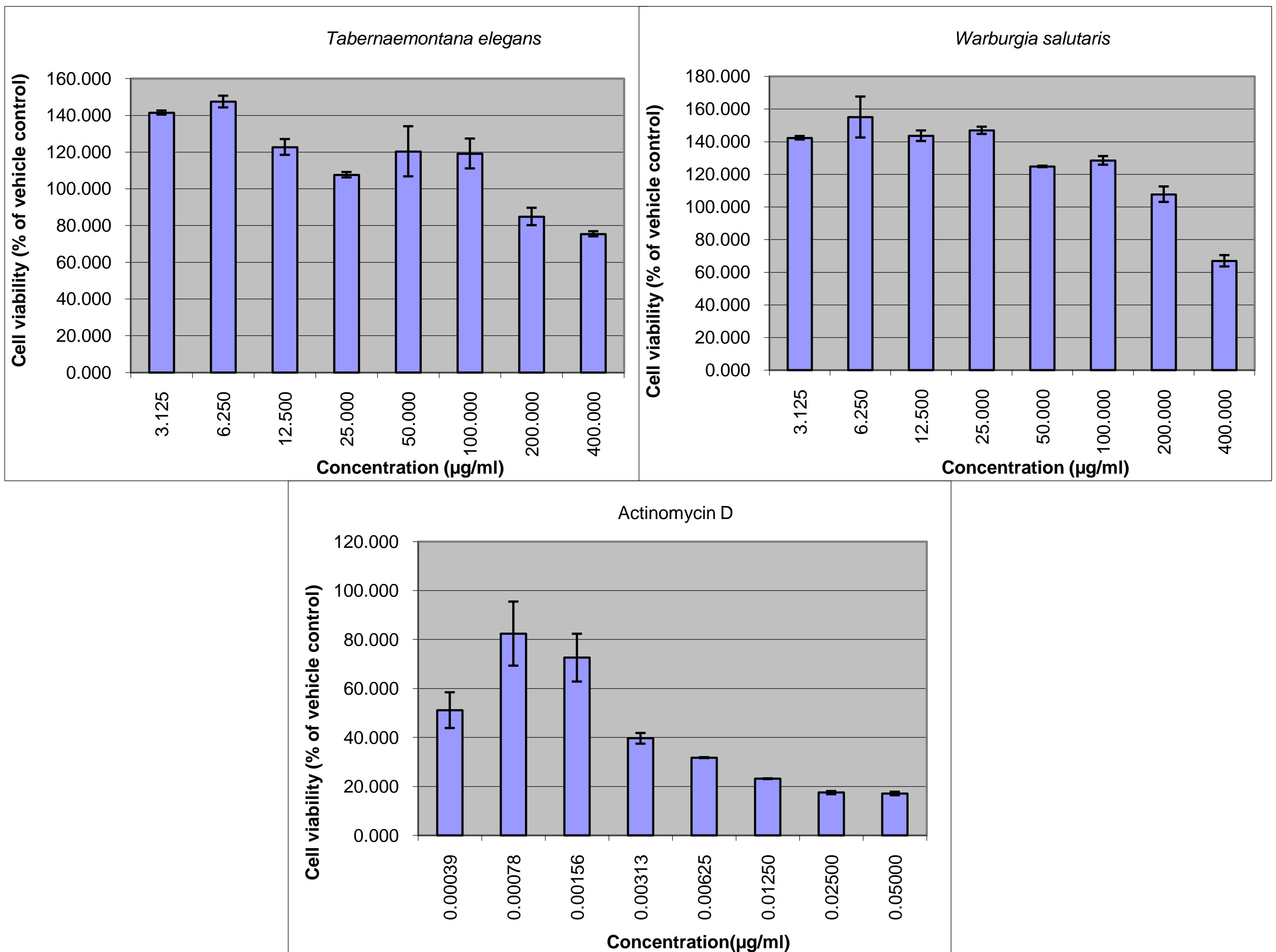

Fig 4: Dose-dependent curves of plant extracts on HEK-293 cells after 72h of exposure

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Appendix B

Cytokine evaluation

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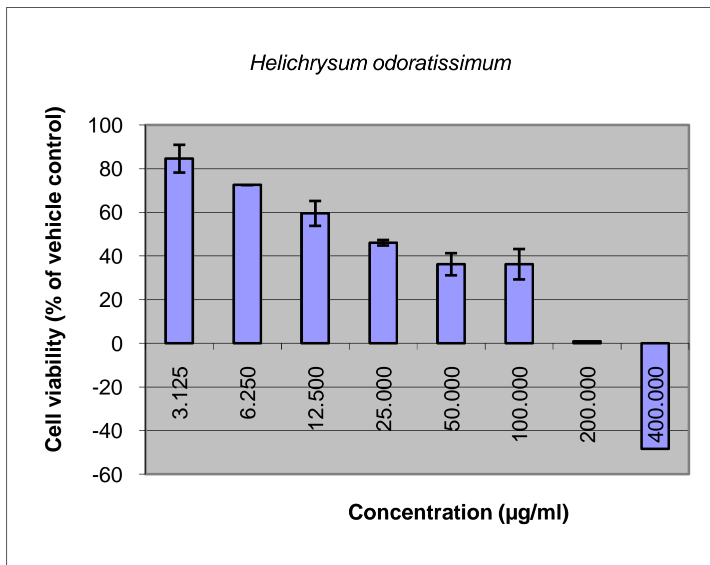

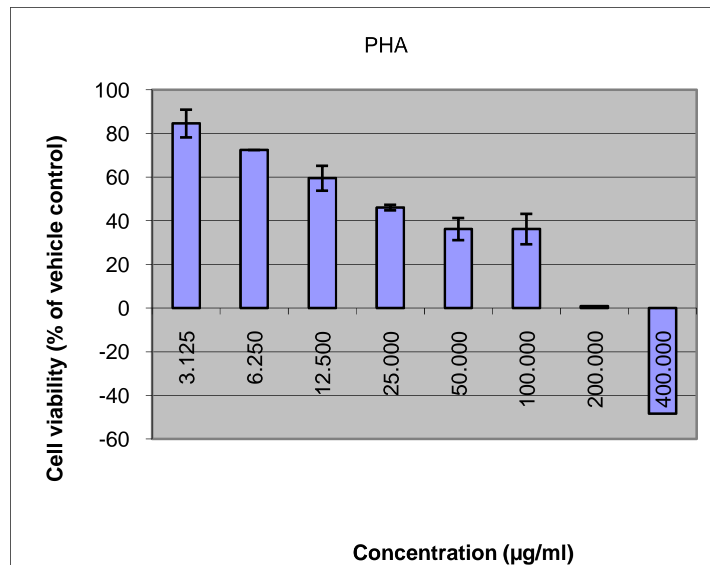

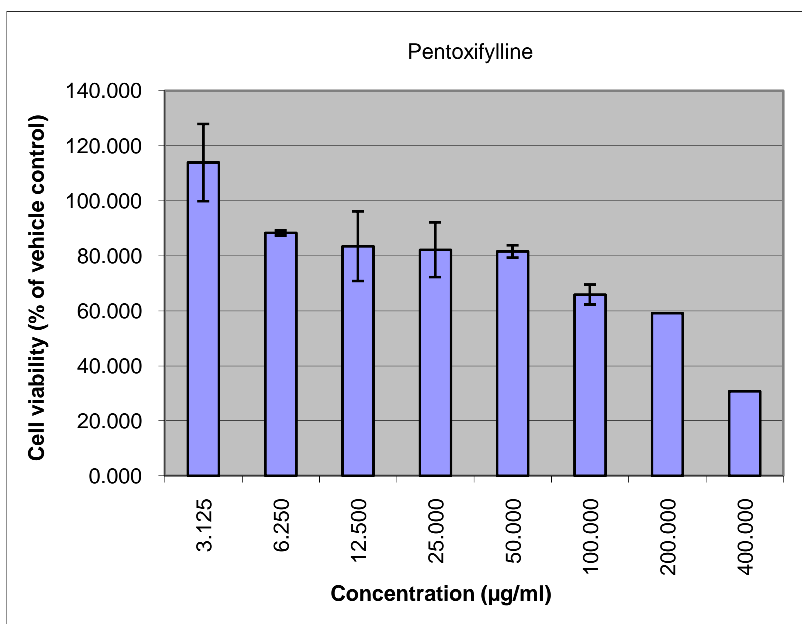

Fig 1: Dose-dependent curves of *H. odoratissimum* on U937 cells after 72hours exposure

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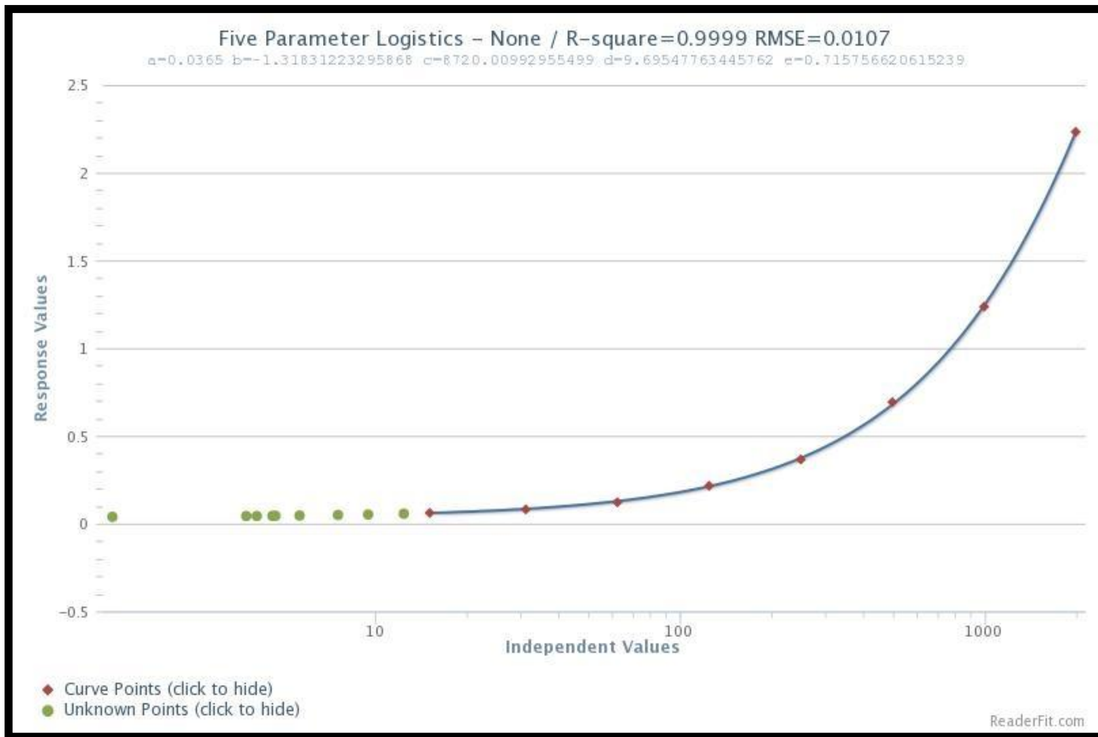

Fig 2: Standard curve of IL-12

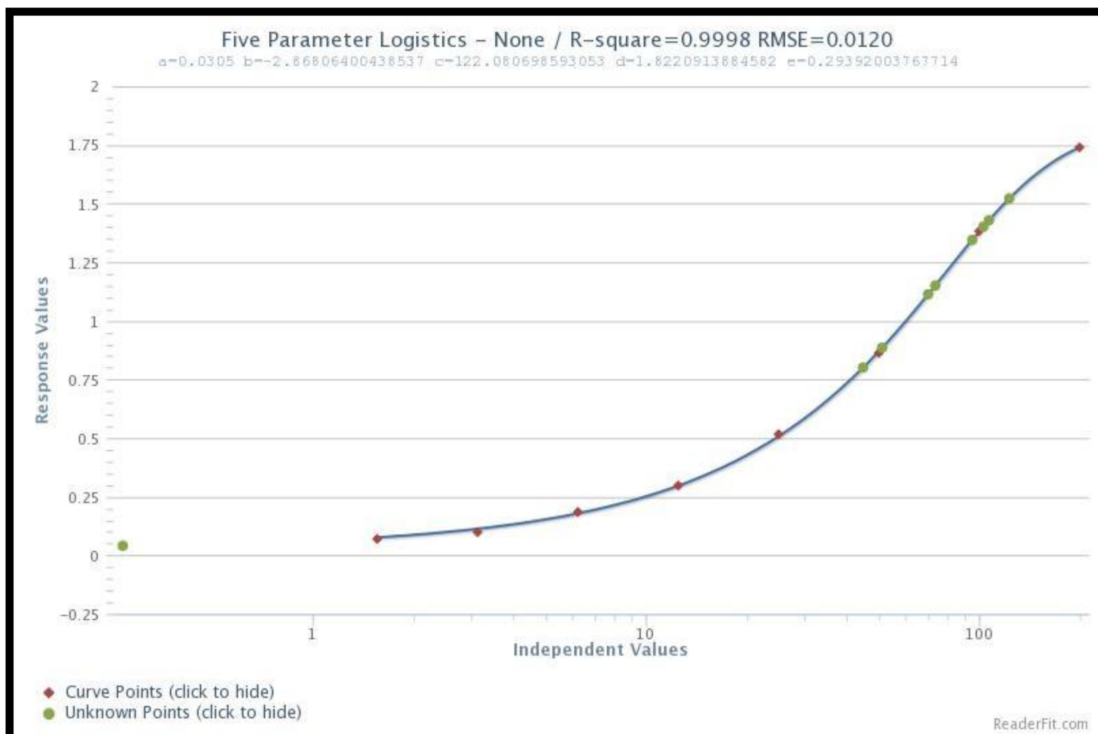

Fig 3: Standard curve of IL-8

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1. Manufacturers protocol for cytokine detection

1.1 Capture antibody, detection antibody and enzyme reagent preparation

The capture antibodies are specific for both IL-12 (Anti-human IL-12 (p40)) and IL-8 (Anti-human IL-8) and therefore, were prepared separately according to the manufacturer's instructions. Both capture antibodies were prepared at a recommended dilution of 1:250 in coating buffer. The detection antibodies are also specific for both IL-12 (Biotinylated Anti-human IL-12) and IL-8 (Biotinylated Anti-human IL-8) and were prepared at a dilution of 1:250 in assay diluent. Once the detection antibodies were prepared the enzyme reagent, which consisted of Streptavidin-horseradish peroxidase conjugate, was prepared at a dilution of 1:250 in diluted detection antibody.

1.2 Wash buffer preparation

The wash buffer was supplied at a concentration of 20X, which consisted of a detergent solution and a preservative known as ProClin-150. A working solution of wash buffer (1X) was prepared by adding 25ml 20X wash concentrate and 475ml distilled water to prepare a final volume of 500ml.

3.3 Standard preparation

Stock concentrations of 2000pg/ml and 400pg/ml were prepared for IL-12 and IL-8 respectively. The standards were added to the 96-well plate in duplicate. Briefly 200µl of a standard was added to the first two wells (1 and 2; Row A) and to the rest of the wells (1 and 2; Row B-H) 100µl of assay diluents was added (Fig 6.6). Serial dilutions were made by taking 100µl of standard solution and adding it to the next well and this was carried through to row H where the last 100µl was discarded. A zero standard was also prepared which consisted of assay diluent only and therefore concentrations ranged between 2000pg/ml-31.25pg/ml and 400pg/ml-3.125pg/ml for IL-12 and IL-8 respectively.

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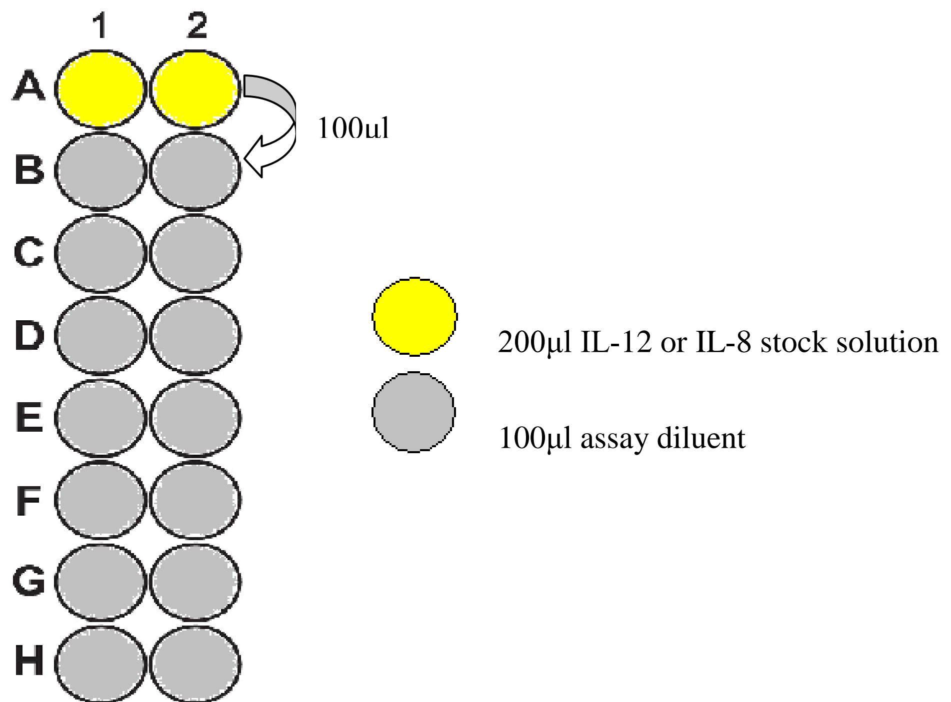

Fig 4: Preparation of IL-12 and IL-8 standards in a 96-well plate

3.4 Substrate preparation

A working concentration of (tetramethylbenzidine) TMB substrate solution was prepared 15 minutes before addition to the 96-well plates. To prepare the substrate solution an equal volume of substrate A (hydrogen peroxide in buffer solution) and substrate B (3, 3', 5, 5' TMB in organic solvent) was mixed together.

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Appendix C

Presentations, publications and patents

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1. The Society of Cosmetic Chemists Annual Conference (September 2013)

Title: Green sunscreen

Authors: Danielle Twilley and Namrita Lall

Type: Presentation

2. Extracts and Composition of *Helichrysum odoratissimum* for preventing and treating skin cancer.

Authors: Danielle Berrington and Namrita Lall

Type: Provisional South African patent

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