UNIVERSITY OF KWAZULU-NATAL ™

INYUVESI YAKWAZULU-NATALI

Effects of *Z*-venusol and other pure compounds from medicinal plants on prostate, cervical & breast cancer cells

by

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Submitted in fulfilment of the requirements for the degree of Doctor of Philosophy in the Discipline of Pharmaceutical Sciences, College of Health Sciences, University of KwaZulu-Natal

20th March 2017

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I, Revd. **Lehlohonolo John Mathibe (200500883)**, declare that,

i. The research reported in this thesis, except where otherwise indicated, is my own original work.

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Supervisors' Declaration

As the PhD candidate's supervisor I, **Dr Strinivasen Naidoo**, agree to the submission of this thesis.

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20 March 2017

As the PhD candidate's co-supervisor I, **Prof. Julia Hillary Botha**, agree to the submission of this thesis.

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20 March 2017

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Dedication

Completion of this PhD thesis is dedicated to the living and loving memory of Maseabata & David Mathibe (my late parents) as well as Lizzy & Simon Ditshego (my late parents-in-law), who were called to *higher service* in May 1998, August 2016, November 2006 & July 2015, respectively.

"As for you, go your way until the end… you will rest, and then at the end of the days you will rise again to receive the inheritance set aside for you" (Daniel 12 v 13, NLT Bible).

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List of Published Peer-Reviewed Papers

Mathibe L.J., 2015. The strength of *Gunnera perpensa*'s "evidence of traditional use". South African Family Practice 58 (Suppl. 1); S39 – S40. http://www.tandfonline.com/doi/pdf/10.1080/20786190.2014.976968

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Mathibe L.J., Botha J., Naidoo S., 2015. Apoptotic effects of *Z*-venusol, from *Gunnera perpensa*, on breast cancer cells *in vitro*. Pharmacology and Toxicology Congress, Wits University, Johannesburg, South Africa.

Mathibe L.J., Botha J., Naidoo S., Drewes S., 2012. *In vitro* effects of *Z*-venusol & *ent*-Beyer-15-en-19-ol on human cancer and normal cells. The International Congress of Pharmacology and Pharmaceutical Sciences, Pretoria, South Africa.

Mathibe L.J., Botha J.H., Naidoo S., Drewes S., 2010. Effects of *Z*-venusol (from *G. perpensa*) on Human Prostate and Cervical Carcinoma. Presented at the 16th World Pharmacology Congress, Copenhagen, Denmark.

Mathibe L.J., Botha J., Naidoo S., 2009. Effects of *Z*-venusol (from *G. perpensa*) on Human Cervical Carcinoma. The International Congress of Pharmacology and Pharmaceutical Sciences, Potchefstroom, South Africa.

List of Abbreviations

ABC	ATP-binding cassette
ACS	American Cancer Society
APAF	apoptotic protease activity factor
APC	adenomatous polyposis coli gene
AR	androgen receptor
ASCs	adult stem cells
ASR	age-standardised mortality rates
Atg	autophagy-related genes
ATP	adenosine triphosphate
Bcl-2	B-cell lymphoma
BP	British Pharmacopoeia
BPE	bovine pituitary extract
cAMP	cyclic adenosine monophosphate
CAPRISA	Centre for the AIDS Programme of Research in South Africa
Caspase	cysteine aspartate protease enzymes
CDKs	cyclin-dependent kinases
cGMP	cyclic guanosine monophosphate
CI	confidence interval
CO_2	carbon dioxide
COX	cyclo-oxygenase
CSC	cancer stem cells

Cyt-C	cytochrome C
DD1α	Death Domain 1α
DMEM	Dulbecco's modified eagle's medium
DMSO	dimethyl sulphoxide
dMVECs	dermal microvascular endothelial cells.
DNA	deoxyribonucleic acid
EBHC	evidenced-based healthcare
EBM	evidence-based medicine
EGF	epidermal growth factor
EIA	enzyme immune-assay
ELISA	enzyme-linked immune-sorbent assay
EMC	extra-cellular matrix
EMT	epithelial-mesenchymal transition
ER	estrogen receptor positive
ESCs	embryonic stem cells
FITC	fluorescein isothiocyanate
FBS	fetal bovine serum
GLOBOCAN	Global Burden of Cancer study
GLUT	glucose transporters
HCl	hydrochloric acid
hEGF	human epidermal growth factor recombinant

HEK293	human embryonic kidney cells
HeLa cells	Henrietta Lacks cells (cervical carcinoma)
HepG2	hepatocellular carcinoma cells
HER2	human epidermal growth factor 2
hFGF	human fibroblastic growth factor
HIF	hypoxia-inducible transcription factor
HMECs	human mammary epithelial cells
HPP	HIV/AIDS Pathogenesis Programme
HPV	human papilloma virus
HRP	horseradish peroxidase
IC_{50}	50% inhibitory concentration
Ifs	intermediate filaments
IgV	immunoglobulin V
IgG	immunoglobulin G
IL	interleukin
IL-6 sR	interleukin-6 soluble receptor
JAK	janus kinase
K-RITH	KwaZulu-Natal Research Institute for Tuberculosis & HIV
LDH	lactate dehydrogenase
LLQ	lower left quadrant
LPC	lysophosphatidylcholine

LRQ	lower right quadrant
MAPK	mitogen-activated protein kinase
MCC	Medicines Control Council
MCF-7	Michigan Cancer Foundation-7 cells
MEBM	mammary epithelial basal medium
MLKL	mixed lineage kinase domain-like protein
MOMP	mitochondrial outer membrane permeability
MMPs	matrix metalloproteinases
MTT	dimethylthiazol-diphenyltetrazolium bromide
MYC	myelocytomatosis
NAD	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide hydride
NCI	National Cancer Institute
NF-κβ	nuclear factor-kappaβ
NRF	National Research Fund
NT2/D1 cells	NTera-2/clone D1 cells
PCD	programmed cell death
PG	prostaglandin
PI	propidium iodide
PI3K	phosphoinositide 3-kinase (also called phosphatidylinositide)
PKA	protein kinase A
PPG	phenylpropanoid glycoside

PR	progesterone receptor
PS	phosphatidylserine
PSA	prostate-specific antigen
PSF	penicillin streptomycin fungizone
R^2	coefficient determination
R^3-IGF-1	R insulin-like growth factor 1
RCT	randomised controlled trial
RIP	receptor-interacting proteins
RIPK	receptor-interacting protein kinase
ROS/RNS	reactive oxygen/nitrogen species
SANCR	South African National Cancer Registry
SATMeRG	South African Traditional Medicines Research Group
SEM	standard error of the mean
SR	systematic reviews
SRB	sulforhodamine B
STAT3	signal transducer and activator of transcription
TAM	tumour-associated macrophages
TEA	tetraethylammonium
TGA	Therapeutic Goods Administration
TGF-beta	transforming growth factor-beta
TIM	T cell immunoglobulin and mucin domain
TM	traditional medicines
TMB	tetramethylbenzidine

TCM	traditional Chinese medicines
TNF-α	tumour necrosis factor alpha
TRAIL	tumour necrosis factor-related apoptosis-inducing ligand
Tris	trisaminomethane
TSG	tumour suppressor gene
UK	United Kingdom
UKZN	University of KwaZulu-Natal
ULQ	upper left quadrant
UP	University of Pretoria
URQ	upper right quadrant
USA	United States of America
UV	ultra-violet
VEGF	vascular endothelial growth factor
VTE	venous thromboembolism
WHO	World Health Organisation

Scientific Symbols and Units

%	percentage
α	alpha
β	beta
µ	micro
µg	microgram
µL	microlitre
µM	micromolar
cm	centimetre
g	gram
hr	hour
kg	kilogram
kPa	kilopascal
L	litre
M	molar
mg	milligram
min	minutes
mL	millilitre
mM	millimolar
mol	mole
p	pico
rpm	revolutions per minute
x g	times gravity

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Structure of this PhD Thesis

This PhD thesis is structured in accordance with the ***"GUIDELINES FOR PRESENTATION OF MASTERS AND PhD DISSERTATION/THESIS BY RESEARCH"***, as approved by the College of Health Sciences, University of KwaZulu-Natal in 2015. It is divided into the following five main parts;

a) Abstract

This part is an extended summary of the entire thesis. It provides key findings of this research as published in peer-reviewed papers as well as those that are not yet published.

b) Introduction & Literature Review

This chapter provides an overview of major issues central to this thesis. Most importantly, it is an honest attempt aimed at critically-appraising the latest and seminal, as well as classical literature on cancer, cell death and the use of medicinal plants. It also provides opinion on current relevant issues and identifies gaps in the literature which provided the rationale for the research conducted and subsequently published.

c) Materials & Methods

This chapter explains the study-design of this research and gives information about compounds which were chosen. It also outlines methods and assays which were used in this research. For the sake of independent reproducibility of findings, this chapter describes, in detail, what was done and exactly how essays were carried out to get the results.

d) Results

This chapter provides, in detail, what was discovered or the facts of this study in relation to the research questions. The findings are presented in figures and in written text.

e) Discussion, Recommendations and Conclusion

In this chapter, the main findings of this research are discussed and, where possible, the meaning of the results is explained. In addition, and where applicable, extrapolation of the results in a wider context is provided. A comparison of the key findings of this study with those from similar studies, which were carried out in both basic and clinical science settings, is done in this part. Furthermore, indications are provided in terms of which results were expected or unexpected, and an honest attempt in explaining the unexpected results is done. More importantly, this part contains brief points on the novelty and significance of this research.

The Harvard referencing style was used and full citations are presented in the references section in ascending alphabetical order.

All relevant additional documents are included in appendices. Notably, this section contains exact copies of published manuscripts which emanated directly or were written in a direct association with this research.

Drugs derived from natural products make up a significant part of the modern pharmacopeia, but so far few of them came from traditional Chinese Medicine (TCM)… Quality science is the only solution. But once science is in, is it still TCM (Normile, 2015)?

ABSTRACT

Introduction

According to recent World Health Organisation (WHO) estimates, cancer causes more deaths than coronary heart diseases globally (GLOBOCAN, 2012). While communicable diseases such as HIV/AIDS continue to burden African populations, cancer is increasingly recognised as a critical public and private health problem in Africa (Igene, 2008). It is estimated that by 2030, about 112 921 new cases of cancer will be diagnosed in South Africa (Singh *et al*., 2015). This would represent a 50% increase of new cancer cases as compared to 2012's estimates by the WHO.

Although there is little doubt about the incidences of cancer, there are, unfortunately, divergent theories in as far as tumourigenesis and the aetiology of cancer. Some researchers hold the view that cancer originates from malignant transformation of normal tissue progenitor and stem cells (Reya *et al*., 2001). Others believe that cancer is as a result of mature cells that have undergone de-differentiation (Sell, 2004). Notably, latest research has shown that there is a strong association between tissue-specific cancer risk and the lifetime cumulative number of cell divisions of tissue or organ-specific stem cells (Tomasetti & Vogelstein, 2015). Although there are still differing views on the origins of cancer, it is widely accepted that this devastating disease occurs as a result of abnormal cell development and is characterised by uncontrollable cell proliferation.

The majority of currently-available cancer treatments target cell proliferation. However, the effectiveness of many cytotoxic drugs, including those that were discovered from plants, is limited by their serious side-effects and cost (Abratt, 2016). Chemotherapeutic agents that were originally discovered from medicinal plants include vinblastine (isolated from *Catharanthus*

roseus), etoposide (isolated from *Podophyllum peltatum*), paclitaxel (isolated from *Taxus brevifolia*) and topotecan and camptothecin (isolated from *Camptotheca acumenata*). Thus, medicinal plants continue to play a critical role in the management of diseases in the world.

In Africa, decoctions, which contain extracts from various medicinal plants (Bruneton, 1995; Balunas & Kinghorn, 2005), are widely used for traditional management of many diseases including cancer. However, apart from subjective oral evidence regarding the effectiveness of extracts from various plants, the identity of ingredients, as well as the science and pharmacology of active compounds found in numerous popular concoctions and decoctions are not known.

Objectives

The main objectives of this study were:

- To assess anti-proliferative potential of three plant-derived-compounds, i.e. hypoxoside, *ent*-Beyer-15-en-19-ol and *Z*-venusol on human cancer cells, namely DU-145 (prostate), HeLa (cervical) and MCF-7 (breast) *in vitro*.
- To determine the type of cell death, i.e. whether a compound with potential causes apoptotic or necrotic cell death on both human cancer and normal cell lines (such as MCF-12, HMECs and dMVECs).
- To investigate how a potential compound exerts its cytotoxicity.

Materials and Methods

Initially dimethylthiazol-diphenyltetrazolium bromide (MTT) assays were conducted to find the concentrations which may inhibit proliferation in prostate (DU-145), cervical (HeLa) and breast (MCF-7) cancer cells. Normal human cell lines, which were used for control purposes, were the primary human mammary epithelial cells (HMECs), MCF-12 and the dermal microvascular endothelial cells (dMVECs). Initially, cells were exposed for 48 hr to hypoxoside, *ent*-Beyer-15-en-19-ol and *Z*-venusol, which were isolated from *Hypoxis hemerocallidea*, *Helichrysum tenax,* and *Gunnera perpensa,* respectively. The concentrations ranged from 2.34 µg/mL to 2400 µg/mL, dissolved in cell specific media. In subsequent experiments, the more sensitive sulforhodamine B (SRB) methodology was used, and cells were exposed to *Z*-venusol for 24 hr, 48 hr and 72 hr, to much lower concentrations, which ranged from 1.9 µg/mL to 240 µg/mL dissolved in dimethyl sulphoxide (DMSO).

To investigate possible pathways of observed cell death, two assays were conducted. These were the fluorescein isothiocyanate (FITC) Annexin V apoptosis detection assay (using the FACS Calibur "JO" E5637 flow cytometer for analysis), and the lactate dehydrogenase (LDH) assay. To explore possible mechanism(s) of action, the activities of interleukin-6 (IL-6) and cyclic adenosine monophosphate (cAMP) were assessed. To investigate the activity of IL-6, cells were exposed for 48 hr to various working concentrations of *Z*-venusol; that is, 37.5 µg/mL and 75 µg/mL. To investigate the activity of direct cAMP, cells were exposed for 48 hr to various working concentrations of *Z*-venusol; that is, 37.5 µg/mL, 75 µg/mL, and 150 µg/mL. Absorbance, which is inversely proportional to the concentration of cAMP in both the samples and the standards, was measured using a BioRad (Model 3550) microplate reader. Epinephrine

(10 µM) and propranolol (10 µM), were used separately and in combination, added to the highest concentration of *Z*-venusol for comparison.

Main Results & Discussion

Hypoxoside resulted in a statistically significant ($p < 0.001$) 38% and 77% increases in proliferation in MCF-7s at concentrations of hypoxoside 1200 µg/mL and 2400 µg/mL, respectively, after 48 hr exposure. In support of the current findings, Xulu (2013) also reported that hypoxoside, and its active derivative known as rooperol, significantly increases cell proliferation of both cancer and normal mammary cells *in vitro* (Xulu, 2013). This was considered an undesirable finding with regards to the aim of finding a cure for cancer. Therefore, no further test were carried out on this compound beyond the initial screening stages.

The highest concentration (i.e., 2400 µg/mL) of the second compound, that is *ent*-Beyer-15-en-19-ol, decreased proliferation in prostate cancer cells (DU-145) and in breast cancer cells (MCF-7) by 6% and 19%, respectively. Interestingly, much lower concentrations, i.e. 4.7 µg/mL and 9.4 µg/mL, of *ent*-beyer-15-en-19-ol significantly ($p < 0.05$) decreased cell proliferation in cervical cancer cells (HeLa) by 37% and 41%, respectively. The differences in expression of vimentin gene, which is over-expressed in HeLa cells and suppressed in MCF-7s and DU-145s may explain why this compound showed significant activity only in the cervical cancer cells (Oshima, 2002; Satelli & Li, 2011). More importantly, the ability of this compound to significantly inhibit cell proliferation in the HeLa cell line by almost 50% at lower concentrations offers an opportunity for further studies.

The findings with regards to the third compound, i.e. *Z*-venusol, were the most exciting. Hence investigations on it were developed beyond the screening stages. This compound demonstrated a statistically significant, concentration-dependent, apoptotic inhibitory effect on the proliferation of MCF-7 cells, with an IC_{50} of 53.7 µg/mL after 72 hr exposure, while the highest concentration (250 µg/mL) resulted in 69% inhibition. Both the FITC Annexin V and LDH results suggested that apoptosis contributed to most of the effects observed. Further, there was non-significant inhibition (20%) of HMEC proliferation observed when the concentration of *Z*-venusol was increased beyond 16.6 µg/mL. The highest concentration of *Z*-venusol used in this study resulted in a statistically significant ($p < 0.001$) 51% inhibition of IL-6 activity in the MCF-7 after 48 hr exposure. None of the *Z*-venusol concentrations, either alone or in combination with epinephrine, an agonist of the adrenergic receptors, showed any statistically significant effect on the levels of cAMP in the MCF-7s. Surprisingly, there was a significant ($p \leq 0.028$) 34% elevation of cAMP levels in cells which were exposed to a combination of *Z*-venusol and propranolol.

If *Z*-venusol was ever able to be used clinically, there might be a need to increase the dose high enough for the attainment of desired therapeutic effects with minimal cytotoxicity on normal cells, because its potency is much lower than that of cisplatin. Increasing *Z*-venusol to a therapeutically-effective concentration would be possible as there was no plateauing-off of inhibition of proliferation in MCF-7s. It was only in primary normal human mammary epithelial cells (HMECs) that formation of "plateaus" was observed. Favourably, this selective plateauing-effect might allow the 'gold-standard' attainment of the desired cytotoxic effect on cancer cells while preserving normal cells at higher concentrations.

There are no studies with which to directly compare the findings of this study. However, reports on effects of the extracts of *G. perpensa* on various other cancer cell lines provide an opportunity for comparison. For instance, the results of this research support the findings of Simelane and colleagues. They recently reported that *G. perpensa* extracts caused an inhibition of proliferation of hepatocellular carcinoma cells (HepG2) with an IC_{50} of 222.33 μg/mL and human embryonic kidney 293 (HEK293) cells, with an IC_{50} of 279.43 μg/mL both after 48 hr of treatment (Simelane *et al*., 2012).

Conclusion

Z-venusol, unlike other compounds studied, has a firm potential to play a role in the treatment of cancer in the future. Its mechanism of action involves IL-6 signaling, which may trigger other downstream mediators and may also involve cAMP "cross-talk".

Recommendations

- More basic science investigations using other hormone-dependent and highly invasive breast cancer cell lines such as the triple-negative MB-231 cells are needed.
- *In vivo* studies, such as using the nude mice model, are needed to confirm the *in vitro* results and to provide an insight into the benefits of *Z*-venusol in living systems.

CHAPTER 1: Introduction and Literature Review

1.1 Cancer

Cancer is understood to be a disease which occurs as a result of aberrant cell development and evolution (Johnson, 2013; Komarova, 2015). Mainly, in cancer there is excess proliferation of cells and lack of proper differentiation thus formation of primary tumour. A benign tumour is a localised mass of cells with no ability to spread to other parts of the body. The cells of malignant tumours, however, are invasive and spread to other parts of the body or metastasise forming secondary tumours which cause fatal systemic disease (Cheung *et al*., 2016).

1.1.1 Epidemiology

According to the World Health Organisation (WHO) International Agency for Research on Cancer report "The GLOBOCAN 2012", cancer is one of the leading causes of death globally with about 14 million new cancer cases and 8 million cancer deaths occurring in 2012 worldwide (GLOBOCAN, 2012). Recent data show that Africa, Asia and Central and South America regions account for 70% of the deaths due to cancer in the world (WHO, Fact Sheet No: 297, 2015). Breast cancer, in particular, is the most common cancer in women and the second most common cancer in the world, whereas lung cancer is the most common cancer among males, and it accounts for the most deaths due to any cancer-type in the world (Jemal *et al*., 2011; Ferlay *et al*., 2015). As at January 2016, in the USA, there were an estimated 3 560 570 and 3 306 760 females with breast cancer and males with prostate cancer, respectively (Miller *et al*., 2016).

In South Africa there is gross under-reporting of cancer incidences because of lack of adequate national surveillance systems (Singh *et al*., 2015). However, most recent estimated age-standardised mortality rates (ASR) due to lung cancer in 2008, was 15.0 and 7.1 per 100 000

for males and females, respectively. It is predicted that in 2030, about 112 921 new cases of cancer will be diagnosed in South Africa, which is about a 50% increase of new cancer cases as compared to 2012 (GLOBOCAN, 2012). The highest ASR, 23.4 per 100 000, was estimated among Asian males, in South Africa, because of high prevalence of smoking among them (Winkler *et al*., 2015). About 8000 women are diagnosed annually with cervical cancer, representing about 19% of cancers in women, and about 4000 women per year die due to cervical cancer in South Africa (Tathiah *et al*., 2014). The pathology-based National Cancer Registry in South Africa has recorded breast cancer as the most common cancer in females with age-standardised incidences of 25 per 100 000. Cervical cancer was recorded as the second most common cancers with age-standardised incidences of 20 per 100 000, respectively (SANCR, 2008). However, in the Eastern Cape Province, South Africa, reports extracted from population-based registries indicated that cervical cancer was the most common cancer reported in the region, accounting for about 34% of all cases (Somdyala *et al*., 2013). The study by Jemal and colleagues also places cervical cancer at the top of the most cancers in females in South Africa (Jemal *et al*., 2012). As seen internationally, prostate cancer was the most common cancer in males with age-standardised incidences of 24 per 100 000 in South Africa in 2008 (SANCR, 2008).

1.1.2 Classification and Types of Cancer

Cancer is a group of diverse diseases which can be classified into four main groups that share the hallmark characteristic of uncontrollable proliferation of the body's own mutant cells (NCI, 2015). Firstly, the carcinomas arise mainly from the epithelial cells which cover the surfaces of the internal and external body organs; secondly, sarcomas originate from cells found in the supporting tissues of the body; thirdly, lymphomas originate from lymph nodes as well as

tissues of the body's immune system; and finally leukaemia is a cancer of immature blood cells which accumulate in the cardiovascular system.

There are many different types of cancer which affect almost all parts of human body. For example, prostate cancer originates from the prostate gland in males and the prostate-specific antigen (PSA) plays a critical role in diagnosis of prostate cancer (Stephenson *et al*., 2006). More than 95% of prostate tumours are adeno-carcinomas that start in acinar wall and proximal ductal epithelium (Zelefsky *et al*., 2011); prostate cancer is more prevalent in men who are 60 years and older (GLOBOCAN, 2012).

Cervical cancer often occurs where the uterus meets the vagina. Infection with human papilloma virus (HPV), which occurs mainly during unprotected heterosexual intercourse, is the main cause of cervical cancer (Herbert & Coffin, 2008). The HPV initially infects the basal cells of mucosal or epidermal epithelium. Some HPVs can alter cell structures in the body, eventually becoming cancerous. There are more than 100 oncogenic strains of HPV, but the strains found in more than 50% of cases of cervical cancers are HPV 16 and 18 (WHO Fact Sheet No.: 380, 2015). HPV vaccines have been developed and are usually targeted at adolescents who are not yet sexually active (Saslow *et al*., 2007). Although sporadic cases of venous thromboembolism (VTE) have been reported (Liu *et al*., 2016), these vaccines offer protection against strains of HPV infections that cause more than 70% of cases of cervical cancer (ACS, 2011).

Breast cancer can occur both in males and females, although it is very rare in males. The mammary carcinoma, a mixture of both ductal and lobular breast cancers, is the most common type of breast cancer in human beings (Makki, 2015). Ductal carcinomas originate in the ducts

or tubes that carries milk to the nipples and they account for more than 80% of all mammary carcinomas while the lobular carcinomas originate from the milk-producing lobule tissues (ACS, 2011). Breast cancer can also begin in the stromal tissues, which include the fatty and fibrous connective tissues of the breast, but that accounts for less than 2% of invasive breast cancers (Makki, 2015). Clinically, breast cancer can be divided into three subgroups; oestrogen receptor positive (+ER), HER2 positive, and triple-negative which refers to the absence of ER, progesterone receptor (PR) and HER2 (Rimawi *et al*., 2015). HER2 protein increases proliferation of breast cancer cells and it is over-expressed in 20% of breast tumours (Mustacchi *et al*., 2015). Therefore, in therapeutics HER2 protein is often the main target of therapy when drugs such as trastuzumab and lapatinib are used in patients with breast cancer (Zanardi *et al*., 2015).

1.1.3 Causes of Cancer

There are many theories that attempted to explain tumorigenesis, especially at molecular level. These theories, however, can be divided into two main schools (Rivlin *et al*., 2011). The first group, or the old and widely-held school, maintains that cancer is as a result of normal, less or non-differentiated cells that have acquired additional traits which render them aggressive. The second school suggests that tumour is initiated by the stem cells with oncogenic properties (Reya *et al*., 2001; Max, 2003; Dean *et al*., 2005; Wu, 2008).

For many years, there were only two factors considered as the main causes of cancer; environmental factors (e.g., the use of tobacco, alcohol and unhealthy lifestyles, the so called "modifiable risk factors") and genetic predisposition (WHO, Fact Sheet No: 297, 2015). However, recently, Tomasetti & Vogelstein (2015), brought the theory of "cancer stem cells" (CSC) to the fore when they suggested an additional factor. The CSC theory proposes that, as

a result of accumulation of mutations, a small population of cells acquire limitless capability for self-renewal and the ability of these to cause indefinite proliferation play a vital role in tumorigenesis (Marx, 2003; Wang & Dick, 2005). CSCs possess self-renewal properties that the physiological embryonic stem cells (ESCs) and the adult stem cells (ASCs) exhibit (Alvarez *et al*., 2012). In addition, however, CSCs' are very small in size; they have slow rate of division; they have high expression of ATP-binding cassette (ABC) transporters, telomerase and key stem cell genes (e.g., SOX2); they have the ability to remain dormant for lengthy periods; and they are resistant to chemotherapy (Alvarez *et al*., 2012; Clevers, 2016). Therefore, Tomasetti & Vogelstein proposed that different regenerative capacities of tissues and the "stochastic effects" may play a critical role in aetiology of the majority of cancers. For example, in organ-specific cancers such as the colorectal and intestinal cancers, the genetic and environmental predispositions inadequately explain differences in risk in human beings. The regenerative capacities of tissues, which usually proliferate rapidly from the stem cells, are biologically more likely to have mutations. Therefore, Tomasetti & Vogelstein strongly maintain that cumulative number of DNA replications and the rates of stem cells division that happen in a life-time are the major causes of organ-specific cancer types in human beings (Tomasetti & Vogelstein, 2015).

The findings of Tomasetti & Volgelstein, especially their main conclusion that about 66% cases of cancer cases are as a result of "bad-luck" created debates in both lay media (Wright, 2015; Knapton, 2015; Boseley, 2015; Alexander, 2015), and in academic fields (O'Callaghan, 2015; Ashford *et al*., 2015; Gotay *et al*., 2015; Couzin-Frankel, 2015; Wild *et al*., 2015; Wodarz & Zauber, 2015). As a result, the WHO's International Agency for Research on Cancer issued a press release strongly disagreeing with Tomasetti & Volgelstein's conclusions (WHO Press Release No.: 231, 2015). Although the Lancet Editorial, 2015, recognised the complexity of

molecular events involved in cancer, they affirmed the public health agenda's role in promotion of healthy lifestyles and reduction of tobacco smoking efforts (Lancet Editorial, 2015; 201). O'Callaghan (2015) stated that it is possible that Tomasetti & Volgelstein missed a huge volume of data by not having used a systematic literature review or meta-analysis methodologies, because they did not include more aggressive and common cancers such as those of the breast and prostate.

However, many other researchers have embraced the Tomasetti & Volgelstein's proposal as a tissue micro-environment factor, which is valuable in the growth of transformed cells. Therefore, Crossan and colleagues suggest that the Tomasetti & Volgelstein's proposal deserve to be explored in order to understand how tumorigenesis occurs (Crossan *et al*., 2015). It appears that when genetic and environmental factors cannot fully explain why there are huge variations of cancer risk for different organs that are often exposed to the same potential mutagens or have the same inherited mutant genes (Crossan *et al*., 2015; Albini *et al*., 2015). Although CSCs have been implicated in the aetiology of prostate, cervical and breast cancers (Wang & Dick, 2011; Yao 2015), unfortunately, Tomasetti & Volgelstein's study did not include these common cancers in their analysis. However, regardless of the causes, the incidences of certain cancer (especially breast cancer in women) increase with age (Colditz *et al*., 2014).

A substantial number of cancers have also been associated with chronic inflammation. These include cervical, ovarian and oesophageal cancers, among many (Balkwill & Mantovani, 2001). Tumour-associated macrophages (TAM) and pro-inflammatory cytokines such the interleukins (IL) 1 & 6, tumour necrosis factor (TNF) and nuclear factor-kappaβ (NF-κβ) play a critical role in tumorigenesis (Colotta *et al*., 2009). More importantly, Lee and colleagues

have showed that TNF require STAT3, a gene which plays a critical role in cell proliferation, for carcinogenesis, especially in colon cancer (Lee *et al*., 2009).

1.1.4 Characteristics of Cancer Cells

The cells that make-up primary tumours can differ in terms of growth rate, drug sensitivity, metastatic potential and their prevalence (Hanahan & Weiberg, 2000; Huysentruyt & Seyfried, 2010). Generally cancer cells show at least five characteristics, which separate them from normal cells, namely:

1.1.4.1 They have uncontrollable proliferation

In cancer cells, the processes that usually control cell division and tissue growth do not adequately regulate cell proliferation. This can be due to abnormal production of, or responses to, growth factors or to altered feedback controls, such as increased cell activity. This increase may be caused by malfunctioning of cyclin-dependent kinases (CDKs) and/or Ras and/or other genes leading to genomic instability (Schwab, 2009; Gordeon *et al*., 2011). Mutations in the p53 gene, a member of the super tumour suppressor gene (TSG) family which are responsible for terminating cell division and proliferation once DNA is damaged, accounts for most cases of tumorigenesis (Rivlin *et al*., 2011).

Excess proliferation could also be the result of loss of Wnt proteins' signalling activity in the nearby aberrant or injured cells (Frede & Jones, 2013; Lim *et al*., 2013). Usually, in tumour cells, as well as in normal cells when Wnt signalling pathway is activated, there is a loss of Wnt genes (Komiya & Habas, 2008). Therefore, proteasomes remain inactive, resulting in accumulation of β-catenin proteins. In turn, high level of β-catenin proteins increases proliferation by activating oncogenes such as cyclin D1 and MYC (MacDonald *et al*., 2009).

Uncontrollable proliferation is also caused by decreased cell death due to diminished activity of apoptotic protease activity factor 1 (APAF-1), which normally binds to the "initiator" cysteine aspartate protease enzymes 9 (caspase-9) to activate the "executioner" caspase-3 (Sun & Peng, 2009). Decreased cell death can also be due to an increased activity of the B-cell Leukemia/Lymphoma gene (Bcl-2) and many other mediators which play a meaningful role in cell survival and apoptosis (Weinberg, 2007). It has also been recently reported that cancer cells, unlike normal cells, have the ability to proliferate indefinitely even after centrosome loss (Stearns, 2015; Wong *et al*., 2015), making the achievement of efficacy with medicines which inhibit proliferation medicines, such as vinblastine, arduous and complex.

1.1.4.2 They lose the ability to differentiate and function

In normal cells, cell multiplication involves division of the stem cell in a particular tissue to give rise to daughter cells. These daughter cells ultimately differentiate to become mature cells of the relevant tissue and carry out organised functions (Berthron, 1992). The transforming growth factor-beta (TGF-β) superfamily, and the Activin A gene, in particular, play an important role in differentiation processes in normal cells (Moses & Serra, 1996). Therefore, deregulation of TGF-β genes causes less cell differentiation and contribute to cancer progression and invasion (Loomans & Andl, 2014). Helczynska and colleagues have reported that, in breast cancer, high levels of hypoxia-inducing factors, such as the HIF-1, inhibit cell differentiation (Helczynska *et al*., 2003). In colon cancer, cells lose their capacity to differentiate due to defects in the adenomatous polyposis coli (APC) gene and therefore are unable to perform any function (Goss & Groden, 2000; Phelps *et al*., 2009; Fearon, 2011; Reed, 2011).

1.1.4.3 They expand and invade other tissues

Normal cells throughout differentiation and during the growth of tissues and organs, develop certain spatial relationships with respect to each other. This relationship is continually maintained even when the cells are involved in repair. However, cancer cells "acquire" the ability to invade other tissues (Weinberg, 2007). Mediators, such as the matrix metalloproteinases (MMPs), play a critical part (Corcoran *et al*., 1996; Parsons *et al*., 1997; Kähäri and Saarialho-Kere, 1999; Hua *et al*., 2011). Recently, Hammer & Diakonova have showed that serine-threonine kinase p21-activated kinase-1 (PAK1) increases motility and stimulates invasion of breast cancer cells in response to prolactin via transcription and secretion of MMP-1 and MMP-3 (Hammer & Diakonova, 2015).

1.1.4.4 They undergo metastasis

About 90% of deaths due to cancer are caused by metastasis (Gilkes *et al*., 2014). Metastases are secondary tumours formed by cancer cells that have been released from the initial tumour and travelled to another site in the body. They spread via the lymphatic system, through open cavities, such as the pleural cavity or abdominal cavity (peritoneal metastases), and through the blood stream (Schwab, 2009). For the metastasised cell to multiply at new sites, tumour angiogenesis takes place. During angiogenesis, vascular endothelial cells, which normally multiply very slowly, proliferate rapidly to form new capillaries which supply cancer cells with nutrients (and oxygen) and remove waste products (NCI, 2011). Vascular endothelial growth factor (VEGF) is one of the proteins which plays a critical role in tumorigenesis and in the spread of malignant cells in solid tumours (Taniguchi *et al*., 1998). At clinical level, other microenvironment factors that plays a critical role in metastasis is hypoxia and expression of the hypoxia-inducible transcription factors HIF-1 & HIF-2 (Rankin & Giaccia, 2016).

The contents of the extracellular matrix (EMC) also play an important part in metastasis (Gilkes *et al*., 2014). EMC is a complex meshwork which contains over 300 different proteins that serve many roles which include regulating intercellular communication (Naba *et al*., 2012). These proteins enable cancer cells to migrate to distant sites and to survive even when there is insufficient oxygen and they also stimulate VEGF, which ultimately causes angiogenesis. More importantly, Boyd and colleagues have showed that EMC stiffness increases the risk of breast cancer and resistance to treatment (Boyd *et al*., 2014).

It has been widely reported that, in many epithelial tumours, the epithelial-mesenchymal transition (EMT), a pathologic process which enhances the motility and migratory properties of epithelial cells, is crucial for metastasis and lethality of cancer (Micalizzi *et al*., 2010; Britton *et al*., 2011; Chao *et al*., 2012; Mallini *et al*., 2014). On the contrary, the latest studies show that although EMT causes cancer resistance to chemotherapy, it has no significant role in the many metastatic cancers (Zheng *et al*., 2015; Fischer *et al*., 2015). Therefore, more research is needed to clarify the role of EMT in the metastatic ability and the deadliness of epithelial breast cancer cells in particular.

1.1.4.5 They have altered metabolism – the "Warburg effect"

Normal mammalian cells produce energy in the form of adenosine triphosphate (ATP) by catalysing the conversion of glucose or fructose to pyruvate during glycolysis. In the presence of oxygen pyruvate molecules enter the mitochondria to produce 36 additional units of ATP. However, in cancer cells, even when there is sufficient oxygen, there is accumulation of lactate and only two additional molecules of ATP are produced from pyruvate; this is called "aerobic glycolysis" or the "Warburg effect" (Warburg, 1931). Although the exact cellular mechanisms upregulating aerobic glycolysis are not yet well understood, it is believed that altered

metabolism of glucose by various cancer cell-lines is more than an adaptation to hypoxia. For example, it has been reported that non-invasive MCF-7 breast cancer cells have much lower aerobic glucose consumption rates compared to the highly invasive MDA-MB-231 breast cancer cell line during normoxia and hypoxia (Gatenby & Gillies, 2004).

The metabolism of glucose to pyruvate without mitochondrial respiration requires the enzyme lactate dehydrogenase (LDH) to produce increased amounts of lactate and regenerate nicotinamide adenine dinucleotide (NAD) from nicotinamide adenine dinucleotide hydrogen (NADH) (van der Heiden, 2011). There is also increased glucose consumption and decreased oxidative phosphorylation, which are also features of oncogene activation (Warburg, 1931; Gatenby & Gillies, 2004). Shifting to aerobic glycolysis diminishes dependence of cancer cells on oxygen and it gives these cells an advantage over normal cells as they continue to proliferate (Gatenby & Gillies, 2004). This leads to "stabilization" of hypoxia-inducible transcription factor (HIF), which then up-regulates VEGF to promote angiogenesis (Hsu & Sabatini, 2008). Deranged metabolism in cancer cells increases biosynthesis of nucleotides, proteins, phospholipids, fatty acids and inhibits apoptosis, while glucose transporters (GLUT-1) are used to remove toxic metabolites and lactate (Larson *et al*, 2011).

1.1.5 Treatment of Cancer

Depending on the size and the location of the tumour, approaches for treatment include surgery and irradiation therapy, especially for localised lumps. Alternatively there is chemotherapy, whereby cytotoxic drugs are used before (i.e., primary or neoadjuvant) or after (i.e., secondary or adjuvant) tumour mass has been reduced with radiation and/or surgery (Lippman *et al*., 1986; Swain *et al*., 1987).

1.1.5.1 Origins of cytotoxic drugs and classification of chemotherapy

Systemic medicines that are used to treat cancer vary extensively in terms of whether they were initially derived from inorganic and metal-based compounds or from plants. They also vary in terms of their effectiveness and their ability to increase survival rates in humans. Both the inorganic agents, cisplatin (a platinum-based cytotoxic drug) and cyclophosphamide are alkylating agents. Cyclophosphamide, the most commonly used anticancer drug, is a pro-drug converted to an active metabolite by hepatic enzymes (Ludeman, 1999). Acrolein, cyclophosphamide's active metabolite, causes damage to urinary bladder (Kehrer & Biswal., 2000). Other side effects of cyclophosphamide include: Marked immune-suppression, leukopenia (sometimes called leukocytopenia; a reduced number of leukocytes) and cardiotoxicity (Hassan & Waller., 2015). Although cisplatin has a broad spectrum of action in cancer chemotherapy, it causes debilitating side effects such as nausea, vomiting and thrombocytopenia (Dasari & Tchounwou 2014; DeLozier *et al*., 2014; Gresham *et al*., 2014). Other systemic treatment includes hormonal therapy such as anastrazole, tamoxifen for breast cancer (Dalmau *et al*., 2014); androgen synthesis inhibitors such as abiraterone acetate for prostate cancer (Wolff *et al*., 2015; Zhang *et al*., 2015).

Recent advances in targeted cancer treatment include immunotherapy and the use of biological medicines. A number of potential immunotherapy agents have been reported to work through the immunoglobulin superfamily; the extracellular immunoglobulin V (IgV) domain, the T cell immunoglobulin and mucin domain (TIM) play a critical role in tumorigenesis (Baghdadi & Jinushi, 2014). Accumulation of TIM-3 gene within the tumour micro-environment, reduces anti-cancer immune-surveillance by producing inhibitory signals which causes apoptosis of T helper (Th-1) cells (Freeman *et al*., 2010). Therefore, evidence from pre-clinical studies indicate that TIM-3 inhibitors have potential in cancer therapy (Ngiow *et al*., 2011). Biological

and targeted therapies (which are generally classified into the monoclonal antibodies such bevacizumab and the protein kinase inhibitors such as imatinib) increase survival especially when used in combination with chemotherapy or cytotoxic drugs (Sikic, 1999; Liauw, 2013).

Since it was established that cAMP is upregulated in cancer cells, in both *in vitro* and *in vivo* studies (Lang *et al*., 2004), there have been many studies that have evaluated the effectiveness of β-adrenergic blockers in patients with cancer. For instance, several studies have reported that propranolol suppresses angiogenesis (Lamy *et al*., 2010), is safe and effective against various vascular tumours such as has haemangioma in infants (Bayart & Brandling-Bennett, 2015); it reduces triple-negative breast cancer brain metastases (Bai *et al*., 2010; Choy *et al*., 2016); it reduces breast cancer progression and mortality (Barron *et al*., 2011); it enhances the efficacy of other cytotoxic drugs (Pasquier *et al*., 2011); and it improves survival (Powe *et al*., 2011).

To improve the outcomes and to prevent drug-resistance, there are various chemotherapy regimens, or combinations of drugs, applied in the treatment of cancer. Firstly, there are typical or conventional regimens whereby maximum doses, over a controlled number of treatment cycles that can be tolerated, are administered to kill as many cancer cells as possible (Swain *et al*., 1987). Secondly, there are intermittent regimens whereby maximum doses are administered until the tumour diminishes whereupon therapy is stopped or missed and started once again should the cancer re-emerge (Enriquez-Navas *et al*., 2015). Thirdly, there are adaptive regimens whereby individualised treatment schedules are adapted from time to time depending on response and disease progression (Willyard, 2016; Enriquez-Navas *et al*., 2016).

1.1.5.2 Currently used plant-derived cancer drugs

Vincristine, vinblastine and vinorelbine, originally derived from *Catharanthus roseus*, act by binding to tubulin proteins during an M-phase and thereby prevent formation of spindles that are essential for the centriole duplication and ultimately resulting in the formation of daughter cells (Silverman & Deitcher, 2013; Wong *et al*., 2015). Other regularly-used anti-cancer drugs originally derived from plants include paclitaxel (isolated from *Taxus brevifolia*), topotecan (isolated from *Camptotheca acuminata*), etoposide (isolated from *Podophyllum peltatum*) and camptothecin (isolated from *Camptotheca acumenata*) (Wall *et al*., 1966). These agents, the "taxanes", act by inhibiting telomerase, a reverse transcriptase enzyme, which is upregulated during mitotic cell division in cancer cells (van Vuuren *et al*., 2015). Therefore, medicinal plant derivatives continue to play an important role in the current management of many diseases, including cancer, as it also happened in the past (Rates, 2001).

1.1.5.3 Plant-derived compounds with cytotoxic potential

Recently, many compounds, some extracted from various indigenous South African plants, have been reported to have anti-bacterial, anti-inflammatory and anti-tumour effects (Watt & Breyer-Brandwijk, 1926; Hutchings, 1989; Fouche *et al*., 2008). These indigenous plants and their relevant compounds include *Agapanthus africanus* (which contains saponins and sapogenins), *Bulbine natalensis* (chrysophanol and knipholone), *Gunnera perpensa* (which contains flavonoids), *Helichrysum species* (flavonoids), *Pallaea calomelanos* (triterpenoid saponins), *Heteropyxis natalensis* (limonene, ocimene and cineole), *Plumbago auriculata* (plumbagin), *Sanseviera hyacinthoides* (sapogenin and ruscogenin), *Scilla natalensis* (saponins and homoisoflavanoids), *Trichilia emetica* (limonoids), *Tulbagia violacea* (alliin), *Zanthoxylum capense* (sanguinarine), *Aster bakeranus* (ocimenediol), *Albiza adianthifolia*

(saponins and sapogenins) and *Gunnera perpensa* (Hutchings *et al*., 1996; van Wyk *et al.*, 1997).

Traditional uses of extracts from *Hypoxis hemerocallidea* (of the plant family Hypoxidaceae), commonly known the "African potato", include the management of conditions such as inflammation, diabetes and convulsion is common in South Africa (Laporta *et al*., 2007). There is evidence that suggests that pure compounds too, especially hypoxoside and rooperol (Figure 1.1), pentenyne derivatives, isolated from *H. hemerocallidea* have a role to play in the management of cardiovascular diseases (Coetzee *et al*., 1996), mycobacterial diseases (Muwanga, 1996), prostate cancer (Drewes *et al*., 2008) and skin cancer (Albrecht *et al*., 1995).

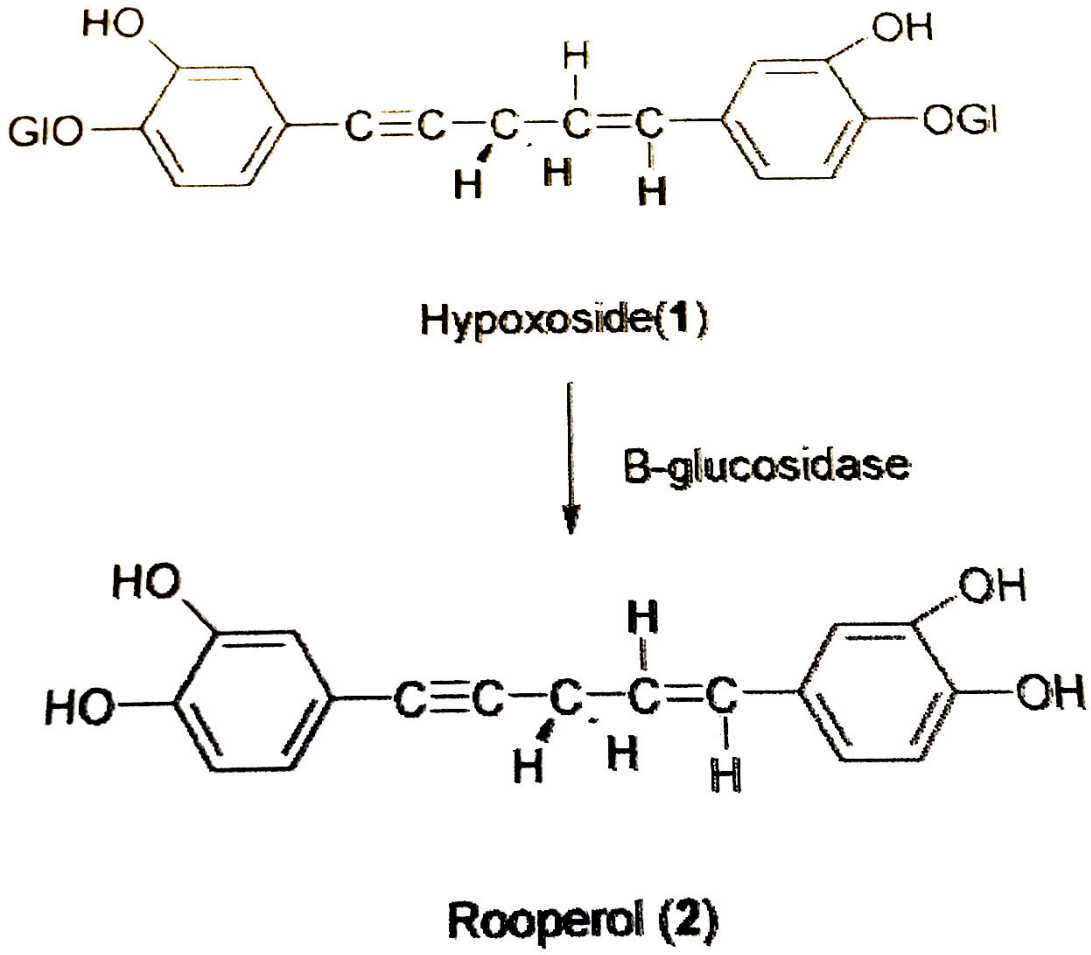


Figure 1.1: Structure of hypoxoside, which transforms into a more active metabolite (rooperol) by the β-glucosidase enzyme, isolated from the roots of *Hypoxis hemerocallidea* (Drewes *et al*., 2008).

Helichrysum petiolare, also known as *impepho* in isiZulu, is the most well-known member of the genus *Helichrysum* (of the plant family Asteraceae), and is widely distributed throughout South Africa. The dried plant is often burned because the resultant smoke is used as a ritual incense. Plant decoctions are used traditionally to treat cough, fever, infection and menstrual pain (Hutchings *et al.,* 1996; van Wyk *et al.,* 1997). The active ingredients of *H. petiolare*, have anti-inflammatory and antitumour (inhibiting cyclin B/cdc2 kinase enzyme) activities (Kobayachi *et al*., 2002). While traditional uses of crude extracts of *H. tenax* include treatment of sore eyes (Lourens *et al*., 2008), *ent*-beyer-15-en-19-ol (Figure 1.2), a diterpenes, isolated from the leaves of *H. tenax* was active against pathogens such as *Bacillus cereus* and *Staphylococcus epidermidis* (Drewes *et al*., 2006).

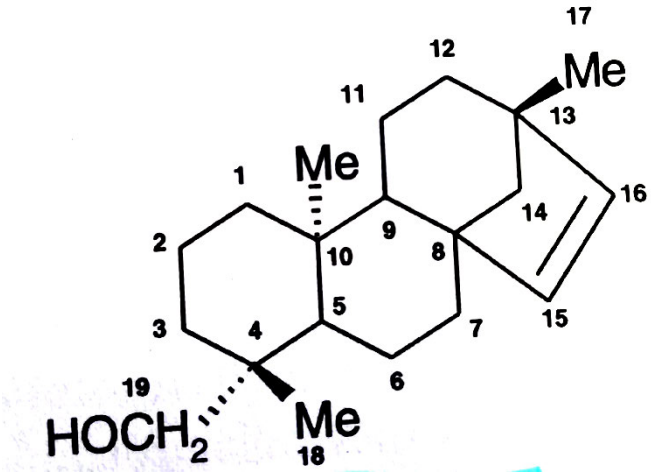


Figure 1.2: Structure of *ent*-beyer-15-en-19-ol isolated from the leaves of *Helichrysum tenax* (Drewes *et al*., 2006).

Gunnera perpensa (of the plant family Gunneraceae), also known as “river pumpkin”, *ughobo* or *uklenza* (isiZulu), *ipuzi lomLambo* (isiXhosa) and *qobo* (Sesotho), grows well along the river banks in many parts of South Africa (Khan *et al*., 2004). Its traditional uses (in decoctions; that are water-filtered from boiling the finely ground parts of the plants for a specified period of time) by African traditional healers vary widely and include; maternal/foetal ailments, other conditions such as: painful joints, “canker sore”, rheumatism and swelling (Iwalewa *et al*., 2007; Ndhlala et al., 2011), breast (Seleteng-Kose *et al*., 2015) and stomach cancer (Pujol, 1990), psoriasis (Hutchings *et al.,* 1996), wound dressing (Grierson & Afolayan, 1999), diarrhoea and for treatment and eradication of intestinal parasites (McGaw *et al*., 2000). Khan *et al*., (2004), isolated and identified *Z*-venusol (molecular formula – $C_{15}H_{16}O_8$, molecular weight – 324.28 $gmol^{-1}$), a phenylpropanoid glycoside (PPG), as the main active compound found in the roots of *G. perpensa* (Figure 1.3). Subsequently, Drewes *et al.* (2005) isolated a 1,4 benzoquinone compound from *G. perpensa*’s fresh stems and leaves (collected in spring), and reported that it inhibited the growth of various pathogens, such as *Staphylococcus epidermidis, Enterococcus faecalis, Staphylococcus aureas* and *Bacillus cereus*. In 1997 Kaido and colleagues reported that a *G. perpensa* (dried roots boiled in distilled water for 4 hr) extract potentiated an oxytocin-like response to rat uterine contractions (Kaido *et al*., 1997). Thus, it is encouraging that recent studies are not only reporting on the claims or on traditional uses associated with *G. perpensa*, but are scientifically verifying some of these claims (McGaw *et al*., 2000; Khan *et al*., 2004; McGaw *et al*., 2005; Basson, 2005 & Drewes *et al*., 2005).

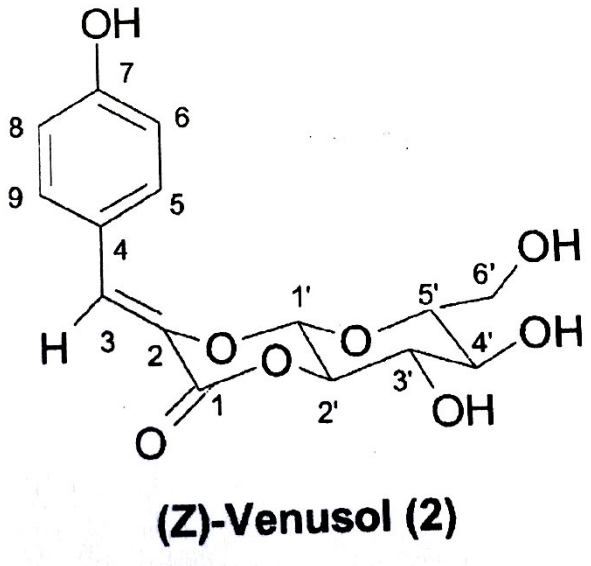


Figure 1.3: Structure of *Z*-venusol isolated from the roots of *G. perpensa* (Khan *et al*., 2004).

Although there is evidence that support the claims attributed to the effectiveness of many plant extracts against various diseases, including cancer, the strength of such evidence needs to be investigated (Mathibe, 2015). Recently, the Medicines Control Council (MCC) in South Africa published "draft guidelines", which propose that evidence be provided on the effectiveness of products' active ingredients and their excipients before registration and sale is allowed (MCC, 2015) Unfortunately, while awaiting the promulgation of the law which will regulate the use of traditional medicines, many unregistered herbal products and concoctions are commonly-used in South African (van Wyk, 2008). Fortunately, huge national effort is now being put into establishing a scientific basis for understanding how compounds derived from plants stop proliferation of cancer cells and how they cause cell death in cancer and normal cells.

1.2 Cell Death

For many years, cell death was divided into necrosis and apoptosis (Kerr *et al*., 1972; Karantza & White, 2011). Necrosis is characterised by cell membrane rupture and the release of organelles which are non-functional, while during apoptosis the membrane remains intact and the DNA fragments into small pieces (Nanji, *et al*., 1997). Initially, necrosis was considered as a bad or "passive" and uncontrolled process of cell death, as compared to apoptosis, an epitome of "programmed cell death" (PCD). However, recent studies have demonstrated that PCD can occur in at least three distinct types, namely; apoptosis, autophagy and necroptosis (Clarke, 1990; Golstein and Kroemer ,2007; Galluzzi *et al*., 2008; Kroemer *et al*., 2009; Sun *et al*., 2009).

1.2.1 Type I Programmed Cell Death – Apoptosis

Apoptotic cell death, a process which was first reported by Kerr and colleagues in 1972, involves activation of a serial cascade of different caspases which act "upstream" or as initiators (caspases 8, 9 & 10) and those that act at "downstream" or as executioners (caspases - 3, 6 & 7) of cell death (Kerr *et al*, 1972; Thornberry & Lazebnik, 1998). Cell death could happen through either the intrinsic or the extrinsic pathway. The intrinsic pathway involves mitochondria while the extrinsic pathways happens via activation of the cell surface receptors (Kerr *et al*., 1972; Kroemer *et al*., 2007).

Briefly, a complex intrinsic pathway is activated when the mitochondrion receives apoptotic signals when DNA is damaged. These signals activate the oligomerisation proteins which include Bcl-2 associated proteins, which in turn increases mitochondrial outer membrane permeability (MOMP). The MOMP activates the release of cytochrome c (Cyt-C) which in turn activates Apaf1. Induction of Apaf1 protein activates the release of caspases 3 and 7, which ultimately causes cell death (D'Amelio *et al*., 2010; Mirzayans *et al*., 2016).

The extrinsic pathway is activated by the tumour necrosis factor super-family, which comprise tumour necrosis factor alpha (TNF-α), the tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) and the Fas ligand (FasL), among others (Kroemer *et al*., 2007). Signals from these protein trigger the membrane-bound death-inducing signalling complex (DISC) to induce caspases 3 and 8 directly (Nicholson & Thornberry, 1997; D'Amelio *et al*., 2010). *In vitro* studies have shown that DNA fragmentation associated with apoptosis occurs through activation of caspase 7 in cells that are caspase 3 deficient such as the MCF-7s (Kagawa *et al*., 2001; Mc Gee *et al*., 2002).

1.2.2 Type II Programmed Cell Death – Autophagic Cell Death

In autophagic cell death, autophagosomes bind to lysosomes to form a double membrane around the cell organelles and subsequently the sequestered cell is digested (Tsujimoto & Shimizu, 2005; Kuo, 2006). Autophagy can be stimulated by hypoxic conditions and high temperature. It is often used by the cells as a temporary survival strategy (Karanza & White, 2011), but an up-regulation of the autophagy-related genes (Atg5 & Atg6), as was demonstrated by Tsujimoto & colleague (Figure 1.4), leads to cell death (Tsujimoto & Shimizu, 2005). Although many studies have focused on the autophagic processes in the cytoplasm, recently Dou and colleagues (2015) reported that nucleic lamin B1 degradation during "oncogenic insults" induces autophagic cell death to protect cells from tumorigenesis (Dou *et al*., 2015).

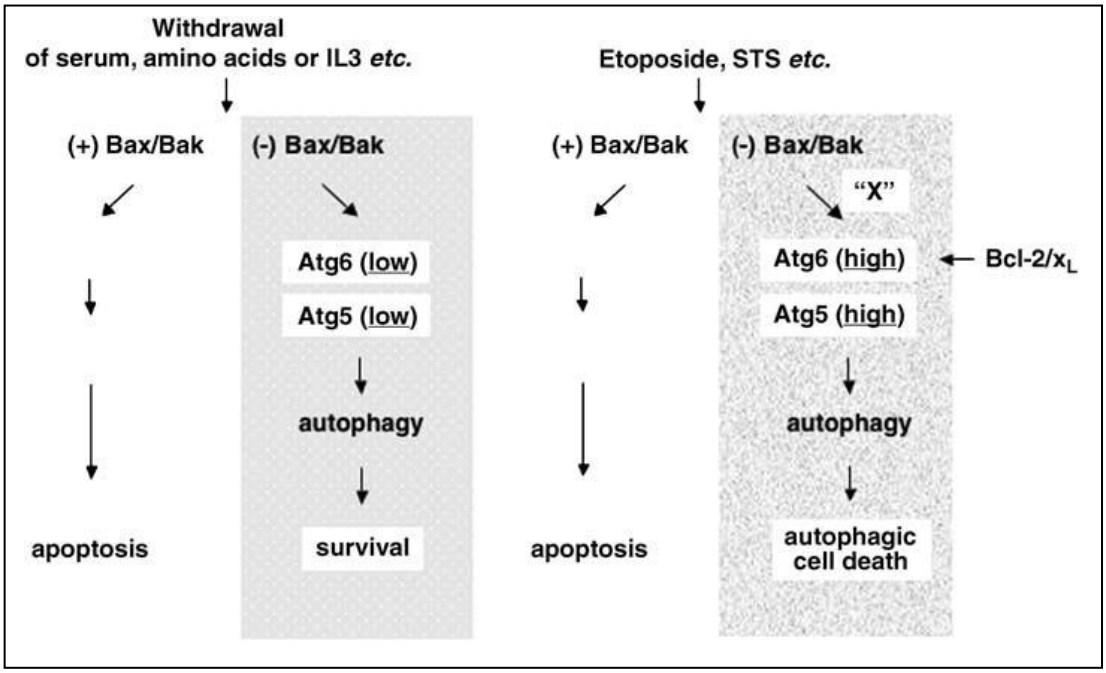


Figure 1.4: A cascade showing survival of cell and autophagic cell death (by Tsujimoto & Shimizu, 2005) as a result of different concentrations of autophagy-related genes (Atg5 & Atg6). When there is a low concentration of level of Atg5 & Atg6 cell survive, but high levels induces autophagic cell death.

1.2.3 Type III Programmed Cell Death – Necroptosis or Pyroptosis

Necroptosis, or programmed-necrosis is initiated by the death domain proteins such as the TNF family, the interleukin (IL-6) gene and the receptor-interacting proteins (RIP-1 and RIP-3) (Wallach *et al*., 2016), and it can take place independent of caspases (Sperandio *et al*., 2000; Broker *et al*., 2005; Kroemer *et al*., 2009). Its execution involves the active disintegration of mitochondrial, lysosomal and plasma membranes (Vandenabeele *et al*., 2010). Fink & Cookson (2005) proposed the use of the term "pyroptosis" in reference to cell death which involves both caspases and inflammatory cytokines such as IL-1 and IL-8; further, these authors define pyroptosis as a pro-inflammatory pathway resulting from caspase-1 activity leading to membrane breakdown and pro-inflammatory cytokine processing (Fink & Cookson, 2005). Another cytokine, which plays a crucial role in pyroptosis and facilitates deadly changes in the cell, is the mixed lineage kinase domain-like protein (MLKL). This is thought to be a pro-inflammatory cellular component (Wallach *et al*., 2016).

In human beings and in *in vivo* studies, apoptosis is not complete until phagocytes remove or dispose of dead cells (deCathelineau & Henson, 2003). Therefore, the final stage of apoptosis is characterised by the release of the lysophosphatidylcholine (LPC) and phosphatidylserine, the "find-me" and "eat-me" signals, respectively (Hochreiter-Hufford & Ravichandran, 2013). Thus, these signals appear on the surfaces of the dead cells for the phagocytes to remove dead cells and to prevent inflammation (Grimsley & Ravichandran, 2003; Lauber *et al*., 2004). Inflammation happens during necroptosis as a result of activation of damage-associated molecular patterns (DAMPs) and the NLRP3 inflammasome gene (Kang *et al*., 2014; Lawlor et al., 2015). Recently, Yoon and colleagues have reported that the p53 gene, in addition to controlling proliferation and inducing apoptosis, also plays a significant role in the removal of dead cells (Yoon *et al*., 2015). They reported that the p53 gene activates the "Death Domain

1α" (DD1α), a member of the immunoglobulin family, to appear on the surfaces of dying cells, thereby avoiding inflammation by enabling removal of dying and dead cells (Yoon *et al*., 2015; Zitvogel & Kroemer, 2015).

Recently, Newton and colleagues have demonstrated that both apoptosis and programmed necrosis involve the intersection of receptor-interacting protein kinase 3 (RIPK3), and they further illustrated how unsettling one pathway can incite death by the other (Newton *et al*., 2014; Zhang & Chan, 2014). Therefore, it is clear that various pathways complement each other for both the demise of cells and clearance of "dead cells" once death has occurred.

1.3 Assessing Cell Proliferation, Turnover and Cell Death

There are various colourimetric assays used to assess cytotoxicity of various plant extracts and pure compounds on cells *in vitro*. These include the tetrazolium salt, 3-4,5 dimethylthiazol-2,5 diphenyl tetrazolium bromide (MTT) assay (Mossman, 1983; Twentyman & Luscombe, 1987) and the sulforhodamine B (SRB) assay (Skehan *et al*., 1990), lactate dehydrogenase and Annexin V assay (del Bino *et al*., 1999). Studying mechanisms of action of drugs is complicated by the fact that observed effects are as a result of complex interactions of compounds with various proteins and metabolites (NRC, 2007; 109). Thus, there many assays used to explore mechanism of action of compounds with potential health benefits. These include the caspase, cAMP and IL-6 assays (Madden *et al*., 2011).

1.3.1 Colourimetric Assays

In the MTT assay, the soluble yellow tetrazolium salt is reduced in metabolically active/viable cells to form insoluble blue formazan crystals which are quantified colourimetrically. In the

SRB assay, the bright-pink aminoxanthene SRB dye adheres to basic-amino acid residues under mildly-acidic conditions and dissociates under alkaline conditions. The SRB dye from stained cells is directly proportional to cell density (Vichai & Kirtikara, 2006). Recently, Sodde and colleagues have demonstrated that the sensitivity of the SRB assay is better than that of the MTT assay in studies assessing proliferation of MCF-7 breast cancer cells *in vitro* (Sodde *et al*., 2015). Other ways of assessing cell death *in vitro*, are to use other chromagen-based assays such as the lactate dehydrogenase (LDH) assay (King, 1965), the cysteinyl-aspartic acid proteases (caspase) assay (Debatin, 2004) and the Annexin V assay.

The main function of LDH is to convert pyruvic acid into lactic acid (Guyton, 1991: 751). In the presence of diaphorase, LDH converts lactic acid back into pyruvic acid. LDH is mainly found inside the cell and high concentrations in the extracellular fluid are indicative of cell membrane damage and cell death.

Caspase activity assay involves a series of steps which involve cleaving a tetrapeptide which gives measurable colourimetric signal. Briefly, as caspases become activated, they split specific substrates either activating or inactivating them. Ruptured substrates bind to a preferred tetrapeptide cleavage sequence at the aspartate residue. Ultimately, a cleaved substrate produces a colourimetric signal, which is an indication of caspase activity (Roche, 2003; Schulze-Osthoff, 2008).

With a flow cytometer, the uptake of Annexin V and propidium iodide (PI) dyes, at the beginning of cell death, has been used to differentiate between cells undergoing apoptotic and necrotic cell death (del Bino *et al*., 1999; Yue *et al*., 2010). As the process of death starts, cells will absorb both dyes to varying degrees and the predominant uptake of one dye indicates the

particular mechanism of death. Cells undergoing early necrosis absorb only the PI dye and are therefore dispersed in the upper left quadrant, while cells undergoing early apoptosis are in the lower right quadrant as the Annexin V dye attaches to the phosphatidylserine (PS) protein exposed to the outer surface of the cell membrane. Cells in late apoptosis and/or necrosis, are dispersed into the right upper quadrant because they absorb both the PI and the Annexin V dyes.

1.3.2 Assays used to explore mechanism of action of compounds

Cyclic nucleotides such as cAMP and cyclic guanosine monophosphate (cGMP), have both been reported to have effects on breast cancer cell proliferation and apoptosis. Elevation of cGMP activity inhibits proliferation and induces apoptosis of breast cancer cells (Follin-Arbelet *et al*., 2011; Dong *et al*., 2015). However, the effects of cAMP have been more contentious, with cAMP reported to either stimulate or inhibit proliferation depending on the type of the cancer cells studied (Kung *et al*., 1983; Fentiman *et al*., 1984). The assay uses polyclonal antibody to bind to free cAMP in the sample and the intensity of the yellow colour generated is inversely proportional to the cAMP concentration.

Interleukin 6 (IL-6) is a pleiotropic cytokine produced by many different types of cells (Adams *et al*., 1991; Saglam *et al*., 2015). IL-6 has the ability to upregulate VEGF and its high concentration is associated with shorter survival in various patients with prostate and breast cancer (*Bachelot et al*., 2003). The IL-6 activity immunoassay utilises tetramethylbenzidine (TMB) substrate to indicate the concentration of IL-6 in the supernatants, plasma and other body fluids (Singh *et al*., 1995; Jiang *et al*., 2011).

1.4 Cell Lines

Various *in vitro* models are available and an appropriate cell line can be chosen when studying different cancers (Holliday & Spires, 2011). The DU-145, HeLa, and MCF-7 cell lines are well characterised and have been used regularly to study prostate, cervical and breast cancer respectively in human beings. Along with cancer cell lines normal cell lines such as human dermal micro-vascular endothelial cells (dMVECs) and mammary epithelial cells (HMEC and MCF-12A), are available to use as controls.

The prostate cancer DU-145 cells were originally derived from metastatic human prostate adenocarcinoma (Stone *at el*., 1978). Androgen receptor (AR) protein expression plays a critical role in proliferation of DU-145 cells and it is also used to determine prognosis in patients with prostate cancer (Yeap *et al*., 2004). However, various studies have reported conflicting results with regards to expression of androgen receptor (AR) protein in DU-145 cells (Scaccianoce *et al*., 2003; Alimirah *et al*., 2006). When growing optimally *in vitro*, DU-145 cells have a doubling time of about 48 hour or less (Carey *et al*., 2009).

The epithelial cervical cancer cells, HeLa, were originally derived from patient Mrs Henrietta Lacks (Scherer *et al*., 1953). Encouragingly, the entire genome of the HeLa cell line has been recently sequenced (Callway, 2013; Landry *et al*., 2013). However, contaminations of the HeLa cell line limit the use and the usefulness of these cells (Nelson-Rees *et al*., 1981; Lacroix, 2008). Other than contaminations, another problem identified with HeLa cells is the varying telomerase activity in different cultures of these cells, which may explain the inability of some cells to proliferate (Ivanković *et al*., 2007). HeLa cell line has a very low expression of p53 and a doubling time of about 24 hr (Sigma-Aldrich, 2006).

The epithelial breast cancer cell line, MCF-7, which was established in 1973 by the Michigan Cancer Foundation, is used widely in *in vitro* models (Holliday & Spires, 2011). Unlike a more aggressive and triple negative (i.e., oestrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) negative) MD Anderson's (MDA-MB231) breast cancer cell line, MCF-7s are less invasive and they form tumours in the presence of oestrogen (Cailleau *et al*., 1978; Clarke *et al*., 1990; Zhang *et al*., 1991). Other studies have reported that MCF-7s lack caspase 3 proteins which play a critical role in apoptosis cascade (Kagawa *et al*., 2001; Mc Gee *et al*., 2002). Madden and colleagues have reported that MCF-7 breast cancer cell line has a very low cAMP density as compared to the MDA-MB231s (Madden *et al*., 2011). Xie and colleagues, in addition, have reported that MCF-7 cell line have a low Cyr61 protein; a gene that is associated with the extracellular matrix and angiogenesis (Xie *et al*., 2001). MCF-7s have a doubling time of about 38 hr when growing optimally (Sutherland *et al*., 1983; ATCC, 2012).

Normal breast cancer cell lines, in contrast, have a longer doubling time and finite propagation when cultured *in vitro*, unless immortalised. For instance, HMEC cell line has a doubling time of about 46 hr and can be passaged for 32 generations without showing signs of senescence (Berthon *et al*., 1992). Ades and colleagues have, however, reported that HMEC cell line can immortalised with PBR-322-based plasmid cloning vector to produce HMEC-1 cell line, which can be passaged for over 95 generations without showing any signs of senescence (Ades *et al*., 1992).

1.5 Rationale and Aim

Although there are many currently available anti-cancer therapies, they remain almost universally very toxic. For example, cisplatin (Dasari & Tchounwou, 2014) inhibits proliferation of both normal and cancer cells, and cause serious nausea and vomiting as well as numerous endocrine side effects (Graeppi-Dulac *et al*., 2014). Therefore, there is a need for effective anti-tumour medicines with fewer adverse effects. Since the treatment of tumours places a huge financial burden on patients (Ubel *et al*., 2013; Meisenberg, 2015), research and development into more affordable and efficacious anti-cancer drugs are needed.

Potential exists among as-yet-to-be-explored plants and their extracts and therefore the aim of the present study was to investigate this. Since extracts and decoctions from *Hypoxis hemerocallidea* and *Helichrysum tenax* and *Gunnera perpensa* are used regularly in many parts of South Africa, pure compounds isolated from these plants were investigated. In KwaZulu-Natal Province (KZN) in particular, extracts from these indigenous plants are commonly used as part of traditional remedies for a variety of inflammatory conditions without concrete scientific evidence with regards to their effectiveness.

1.6 Study Objectives

1.6.1 Screening

To assess anti-proliferative potential of pure compounds isolated from *Hypoxis hemerocallidea*, *Helichrysum tenax* and *Gunnera perpensa* on human cancer cells, namely DU-145 (prostate), HeLa (cervical) and MCF-7 (breast), *in vitro*.

1.6.2 Further assessment of potentially useful compounds

If a potentially promising candidate was found, the next objective would be to determine the type of cell death, i.e. whether it causes apoptotic or necrotic cell death of both human cancer and normal cell lines.

1.6.3 Determination of possible mechanism of action

To investigate how a potential compound exerts its cytotoxicity by assessing the activity of cAMP, LDH, IL-6 and caspases.

CHAPTER 2: Materials and Methods

2.1 Ethics Approval

The study protocol was approved by the UKZN Biomedical Research Ethics Committee (Ref.: BE061/09), please refer to Appendix B.

2.2 Study Design and Screening of Products Using MTT Assay

Since no similar studies had been done using the various compounds investigated in this study, MTT assays were initially conducted to find the concentrations which may inhibit proliferation. MTT assays were carried out because, at the time, the more sensitive SRB methodology was not yet available in this laboratory. Experiments were done on various cancer cell lines, i.e. MCF-7 (breast), HeLa (cervical) and DU-145 (prostate). The effectiveness of currently available cancer drugs is limited by their toxicity on normal cells (Florea & Büsselberg, 2011). Therefore, we also, at this stage, investigated the cytotoxic effects of various compounds on normal dermal microvascular endothelial cells (dMVECs).

Various cell lines were exposed for 48 hr to hypoxoside, *ent*-Beyer-15-en-19-ol and *Z*-venusol isolated from *Hypoxis hemerocallidea*, *Helichrysum tenax*, and *Gunnera perpensa* respectively. The concentrations ranged from 2.34 µg/mL to 2400 µg/mL, dissolved in cell specific media. The times were chosen based on the doubling time of each cell line (Sutherland *et al*., 1983; Carey *et al*., 2009; Sigma-Aldrich, 2006). The concentrations range chosen was based on the work of Drewes and colleagues who reported that 750 µg/mL to 8000 µg/mL *G. perpensa* aqueous extracts, in particular, were active against various human pathogens *in vitro* (Drewes *et al*., 2005).

Microscopic examinations were carried out using 100X magnification and 20X objective. Images were captured using the Leica Microsystems' LAS AF Version 2.0.2 (Mannheim, Germany).

2.3 Assessment of Proliferation using SRB Assay

Following screening studies, more experiments were carried-out using *Z*-venusol on breast cancer cells (MCF-7s) and on more appropriate and resemblant normal human mammary cells (MCF-12s and HMECs). Further tests were not done with other compounds because of their limited effectiveness. The MTT assay was replaced with the SRB assay to strengthen reproducibility of findings and because of the latter's superior sensitivity (Skehan *et al*., 1990).

Various cell lines were exposed for 24 hr, 48 hr and 72 hr to a concentrations range (1.9 µg/mL to 240 µg/mL) of *Z*-venusol. Cisplatin, a known chemotherapy drug against breast cancer, was used as a positive control. The concentration range of cisplatin was informed by the findings of Smith (1999). The stock solutions of *Z*-venusol powder and cisplatin were prepared by dissolving them in DMSO, ensuring that the final concentration of DMSO that would be in contact with cells was equal or less than 0.5% (van Tonder *et al*., 2014). Cancer and normal cell lines were also exposed in the same way to Re-Jooneva™ mixture. This is a commercially available product which claimed to contain extracts of *G. perpensa.* Re-Jooneva™ concentration range used was similar to that of *Z*-venusol and was calculated using information on the product label.

2.4 Investigation of Cell Death Pathways

Human cells can die by apoptosis (the so called "good" death), necrosis (the so called "bad" or "messy" death), or by a combination of both processes (Newton *et al*., 2014). Therefore, after identifying compounds which caused tumour cytotoxicity, possible pathways of cell death were explored for only those which demonstrated potential.

A flow cytometer was used to assess the extent of exposure of phosphatidylserine (PS) protein to the outer surface of the cell membrane. To assess this, fluorescein isothiocyanate (FITC) Annexin V apoptosis detection assay was done. After 24 hr incubation, cells were exposed to 150 μg/mL *Z*-venusol (for 24 hr and 48 hr). For purposes of comparing findings with a known apoptotic drug, cells were also exposed for 4hr to an established apoptotic concentration (0.15 μM) of camptothecin (Del Bino *et al*., 1999). The FACS Calibur "JO" E5637 flow cytometer was used for analysis. Verifying a possible cytotoxicity pathway due to a potential compound was necessary to inform which path to be taken as mechanism of action was to be explored.

Still focusing on what happens at the cell membrane, the direct LDH activity assay (Clontech Laboratories, Inc., USA), which gives an unspecific and broad indication of the leakage of the cell membrane as the death process begins, was used. After 24 hr incubation, cells were exposed to 150 μg/ml *Z*-venusol for 24 hr and 48 hr. At the end of exposure periods, LDH activity was colourimetrically measured in the supernatants. Camptothecin was used as a positive control at a necrosis-inducing concentration (4 μM) for 4 hr as per manufacturer's instructions.

2.5 Possible Mechanism of Action

To explore possible mechanism of action, activity of IL-6, cAMP and caspases were assessed. The activity of IL-6 and cAMP were explored because of regular traditional uses of *G. perpensa* extracts for management of inflammatory conditions and to cause contraction of the pregnant uterus (Gumede, 1978). The activity of caspases were investigated because of established apoptotic effects of potential compound on human breast cancer cells in this study.

To investigate the activity of IL-6, cells were exposed for 48 hr to various working concentrations *Z*-venusol; i.e. 37.5 µg/mL and 75 µg/mL. Absorbance, which is directly proportional to IL-6 activity, was read in the supernatants using a BioRad (Model 3550) microplate reader. The highest final working concentration of the drug used as a positive control, pitovastatin, was 1.5 µM (Wang & Kitajima, 2007).

To investigate the activity of direct cAMP, cells were exposed for 48 hr to various working concentrations *Z*-venusol; that is, 37.5 µg/mL, 75 µg/mL, and 150 µg/mL. Absorbance, which is inversely proportional to the concentration of cAMP in both the samples and the standards, was read in the supernatants using a BioRad (Model 3550) microplate reader. Epinephrine (10 µM) and propranolol (10 µM), were used separately, in combination and added to the highest concentration of *Z*-venusol for comparison (Kelly *et al*., 2009; Madden *et al*., 2011).

To investigate the activity of various caspases, cells were exposed for 48 hr to various working concentrations *Z*-venusol; i.e. 600 µg/mL, 1200 µg/mL and 2400 µg/mL. Absorbance, which is directly proportional to the concentration of caspases in both the samples and the standards, was read in the supernatants using a BioRad (Model 3550) microplate reader.

2.6 Compounds and Drugs Used in this Study

2.6.1 Study Compounds Derived from Medicinal Plants

Isolation, purification and characterisation of hypoxoside, *ent*-Beyer-15-en-19-ol and *Z*-venusol was carried out in an assay-guided isolation as previously reported (Potgieter *et al*., 1998; Khan *et al*., 2004; Drewes *et al*., 2006). This was done in order to justify that these pure compounds were indeed the main active chemical constituents found in the dried roots of the *Hypoxis hemerocallidea,* fresh air-dried leaves of *Helichrysum tenax*, and dried roots of the *Gunnera perpensa*. Pure compounds, in dry powder form, were supplied by Professor Siegfried Drewes, Department of Chemistry, University of KwaZulu-Natal.

2.6.2 Drugs Used as Positive Controls

All agents used as positive controls viz., cisplatin, camptothecin, epinephrine, propranolol and pitovastatin, were purchased from Sigma-Aldrich. Initial concentrations of all positive controls were based on published data in related studies (Wang & Kitajima, 2007; Kelly *et al*., 2009; Madden *et al*., 2011)

2.7 Aseptic Technique

This study used an *in vitro* cell culture model to investigate the objectives of this study. To maintain sterility, separate clean coats were kept in the tissue culture laboratory and latex-free gloves were worn all the time when experiments were conducted (Freshney & Liss, 1987; Freshney, 2006). Bench-tops and surfaces of the incubator (Function Line, Heraeus, Germany), the centrifuge (Megafuge 1.0R, Heraeus, Germany) and the water-bath (Scientific Group, SA), were also swabbed thoroughly with a 5% anti-bacterial solution (Virkon, Antec International, U.K). Before starting cell culture work, item therein such as the pipettes, tips and

1mL tubes were exposed to UV light for at least a 30 min before use. The microscope along with the contents of the biological cabinets was also sterilised with 70% alcohol. At the end of daily sterilisation process, the room was allowed to settle for 20 min. All pipette tips and tubes used for cell culture were autoclaved at 121ºC at 100 kPa (Speedy Autoclave, SA) for 20 min, and thereafter exposed to UV light for 1 hr before use to ensure sterility. To assess mycoplasma contamination in the cell cultures, the Myco®Alert Mycosplama Detection Kit (Biotech, USA), was used regularly. After every four weeks of continuous use the floor, celling, interior wall and windows were cleaned with commercially available disinfectants.

2.8 Cell Lines, Media and Maintenance of Cell Culture

2.8.1 Cell Lines and Media

The epithelial prostate cancer cells (DU-145) and the epithelial cervical cancer cells (HeLa) were grown in (Dulbecco's Modified Eagle's Medium (DMEM), purchased from Biowhittaker, USA. DMEM was supplemented with 10% Fetal Bovine Serum (FBS), L-Glutamine and Penicillin Streptomycin Fungizone® (PSF), all purchased from Sigma, St. Louis, USA.

The epithelial human breast cancer cells, the Michigan Cancer Foundation-7 (MCF-7s), were obtained from Highveld Biological, National Repository of the Cancer Association of South Africa. These cells were grown in DMEM (Biowhittaker, USA) supplemented with 10% FBS, L-Glutamine and PSF and 0.1% Sodium Pyruvate, all purchased from Sigma, St. Louis, USA.

The dermal microvascular endothelial cells (dMVECs) were sub-cultured using EGM®-2 MV Bulletkit media (basal medium MCDB 131; Cambrex Bio Science, USA). The basal media

was supplemented with single aliquots of human epithelial growth factor (hEGF), 10% FBS, VEGF, human FGF-ß, heparin, long R insulin-like growth factor 1 (R^3-IGF-1), ascorbic acid, hydrocortisone and gentamycin sulphate amphotericin ß (GA-1000), all of which were supplied together with the basal media. The period needed for sub-culturing was given as 5 – 9 days when plated at a density of 5000 cells/cm^2.

The human mammary epithelial cells (HMECs) were grown in MEBM (Biowhittaker, USA) supplemented with 0.2% Bovine Pituitary Extract (BPE), 0.05% hEGF, 0.05% human insulin recombinant, 0.05% hydrocortisone, 0.05% GA-1000 purchased from BioWhittaker, USA. To determine the yield of healthy cells, we utilised the trypan blue exclusion dye technique. The period needed for sub-culturing was given as 4 – 7 days when plated at a density of 5000 cells/cm^2.

The MCF-12 grown in a mixture of DMEM and Ham's F12 Kaighn's modification. The medium is supplemented with 1% PSF, hydrocortisone, cholera toxin, hEGF and insulin from bovine pancreas, purchased from Sigma-Aldrich. The supplements were prepared as follows; hydrocortisone 100 mg was dissolved in 10 mL water. Then the solution was divided into 50 µL aliquots and were stored at -84°C until utilised. One 50 µL aliquot was used for every one litre of medium. Cholera toxin 1mg was dissolved in 1mL water. Then the solution was divided into 100 µL aliquots and were stored at -84°C until utilised. One 100 µL aliquot was used for every one litre of medium. Insulin 100 mg was dissolved in 5 mL of sterile water. Then the solution was divided into 500 µL aliquots and were stored at -84°C until utilised. One 500 µL aliquot was used for every one litre of medium.

A solution of the EGF were made by mixing 5.7 µL acetic acid with 10 mL of sterile water. Thereafter, bovine serum albumin 0.01 g was added to the acetic acid solution. Then, 2 mL of EGF solution, which was made by dissolving 0.2 mg EGF in 2 mL of sterile water was added into acetic acid solution. The final solution was divided into 200 µL aliquots and stored at -84°C until utilised. One 200 µL aliquot was used for every one litre of medium. Then to make a DMEM and Ham's F12 Kaighn's mixture, 10 mL DMEM was used to dissolve all supplements. Thereafter, additional 490 mL DMEM was added and finally 500 mL Ham's F12 was added. Prior to use, PSF and 10% FBS were added.

2.8.2 Plating and Maintenance of the Cell Lines

The primary cultures of all cell lines had been purchased and stored at -85°C (NuAir ultra-freezer, Model Nu-9334E, Japan) prior to the commencement of the project. Cells were supplied as cryo-frozen cells in 1 mL aliquots. Cell culture flasks (200 mL flasks with surface areas of 75 cm^2, CellStar, USA) containing 8 mL of the appropriate cell-specific medium were pre-warmed in a humidified incubator (Function Line) at 37°C/5% CO_2 for 10 min prior to the plating of the cells.

The cryo-frozen vials containing each cell line were removed from storage one by one. They were first thawed in a 37°C water-bath (Scientific Group) for 1 – 3 min (or until the ice-crystals had melted). To avoid the possibility of introducing water-borne contamination, care was taken so as not to immerse the seals of the vials into the water. The contents of the vials were thereafter plated into the cell culture flasks, labelled with the cell type, date, passage number and number of cells, under the sterile environment of the biological cabinet (BioFlow-II, Labotec). To ensure no possible cross contamination occured between the different cell lines, the biological cabinet was then swabbed with 70% alcohol and a half hour time period was

allowed before the plating of the next cell line. The same procedure was then followed for the next cell line as above. The culture plates were incubated overnight in the humidified incubator (Function Line) at 37°C/5% CO_2 to allow the cells to adhere.

On the following day, the cells were examined using an inverted microscope (Leica) to ensure that the cells had adhered. The media was removed using glass Pasteur pipettes (Lasec, SA) attached to a hand-held electronic pipette (Finnpipette, Thermo-electron Corporation, USA). Cell lines were handled one at a time under the biological cabinet. Appropriate fresh, pre-warmed medium (8 mL) was then added gently to the flask. The same protocol was followed for the other cell lines once the biological cabinet was swabbed clean with 70% alcohol and a half hour had lapsed. The plates were then incubated again to allow the cells to proliferate. The cells were fed and examined microscopically each day to ensure they were healthy, growing and multiplying.

2.8.3 Trypsinisation

The trypsinisation procedure was the same for all cell lines upon reaching ± 80% confluency. First, the media was aspirated from the culture flasks under sterile conditions and discarded. Thereafter, the cells were gently rinsed with 4 mL of pre-warmed Hanks balanced saline solution (HBSS; Clonetics, USA) which was then also aspirated and discarded. This was followed by the addition of 4 mL pre-warmed Trypsin-versene solution (Clonetics). The flasks were then incubated for 2 – 3 min to allow the cells to dislodge from the bottom of the plate or flask. Throughout this period, the cells were closely monitored microscopically and the trypsinisation process considered successful once most of the cells had retracted their plasma membranes towards their cell bodies, the cells appeared shiny and began sloughing off the surface. The trypsin solution was then inactivated with appropriate pre-warmed media at a

volume twice that of the trypsin. Then cell-solution was aspirated and released back into the flask (no more than 5 times) so as to completely dislodge the cells from the surface. This cell suspension was then added to 15 mL centrifuge tubes (Greiner, Germany) and centrifuged (Megafuge 1.0R, Heraeus) at 1800 rpm (± 332 x g) for 5 min at room temperature to produce a cell pellet. After the centrifugation step, the supernatant was aspirated and the cells gently re-suspended in an appropriate volume of medium (depending entirely on the size of the pellet). Then, the numbers of viable cells were established using the trypan-blue exclusion method and the cell numbers quantified to determine plating numbers for the propagation of the cell lines.

2.8.4 Cell viability and Cell Counting

For manual counting of the cells, a haemocytometer was used to determine both the viability and the density of cells harvested. Briefly, 25 µL of the cell suspension was added to a 1 mL tube (Greiner, Germany) and to this 25 µL of the trypan blue solution (Sigma, USA) was added. This was mixed gently with a Gilson pipette, and allowed to stand for no more than 5 min to allow any dead cells to take up the dye. During this time the haemocytometer (Neubauer, Assistant, Germany) and coverslip was cleaned thoroughly with 70% ethanol and wiped dry. This was to ensure proper loading of the chambers by capillary action. Next, the trypan blue-cell suspension solution was gently re-suspended and 25 µL loaded into each chamber of the haemocytometer slide using a Gilson pipette. The cells were allowed to settle briefly and the numbers of viable cells (not staining blue) in the 4 squares in each grid were counted under the light microscope. The cell concentration was calculated as follows:

$$\frac{\text{Total cell count (excluding blue-stained cells)}}{\text{dilution factor of 2}} \times 10^4 = \text{Cells/mL}$$

Depending on the cell numbers, the cells were then used for experiments, passaged further, and/or cryo-preserved in order to build up the cell bank. For sub-culturing, culture dishes were pre-warmed in the incubator with their respective cell-specific media. Optimal cell densities used for the 60 mm culture dishes that allowed the cells to grow to approximately 60% confluency within 3 – 4 days.

2.8.5 Cryo-preservation of Cells

The cells that were not used for either experimentation or passaging were cryo-preserved to build up a cell bank. Depending on the volume of the cell suspension left behind, DMSO (Sigma) was added to the cell-suspension to give a 10% ($^{v}/_{v}$) final DMSO concentration. The DMSO was added slowly and the cell suspension left aside for approximately 15 min to allow the cryo-preservative to equilibrate within the cells. The cell suspension was then transferred into properly labelled cryo-vials (Corning Costar, USA) and placed into a polystyrene container (to slowly reduce the temperature at an approximate rate of 1^0C/minute) and placed in the ultra-freezer overnight. They were then transferred to freezer boxes in the ultra-freezer and stored at -85^0C until such time that more cells were required from storage.

2.9 Details of Cell Proliferation Assays

2.9.1 MTT Assay

All tumour (DU-145, HeLa and MCF-7) and normal human cells (dMVECs) were plated on sterile Corning (Corning Glass Works, USA) 96-well plates at a density of 4000 cells/cm^2. Plates were incubated at 37^0C/5% CO_2 and 100% relative humidity for 48 hr prior to addition of various concentrations of *Z*-venusol, *ent*-Beyer-15-en-19-ol and hypoxoside. A conventional MTT assay was then used to measure cell proliferation, where soluble, yellow tetrazolium salt,

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide was reduced to insoluble blue formazan crystals by metabolically active cells. Absorbance was measured spectrophotometrically and a linear relationship between cell number and absorbance established for each cell line. Any change in absorbance was an indication of change in proliferation and the percentage mean differences are calculated as follows:

$$\frac{(\text{mean absorbance}_{(\text{test})} - \text{mean absorbance}_{(\text{non-treated control})})}{\text{Mean absorbance}_{(\text{non-treated control})}} \times 100 = \text{Percentage mean difference}$$

The absorbance$_{595nm}$ of non-treated cells (control) represented 100% cell proliferation for that particular cell line, and was used as a reference. Cells were exposed to the test compounds at increasing concentration, up to 4800 µg/mL for both 24 hr and 48 hr treatment periods in order to determine the changes in cytotoxicity. The concentration of each compound which would inhibit cell growth by at least 50% (IC_{50}) was also established. Dilutions were performed in quadruplicate and experiments were repeated five times on all tumour cell lines and three times on dMVECs.

2.9.2 SRB Assay

A conventional SRB assay (Vichai & Kirtikara, 2006) was used to assess the effects of test compounds on proliferation of the human normal and tumour cells. Briefly, cells were harvested and 80 µL of the cell suspension were seeded into 96-well microplates at a density of 2000 cells/well. Cells were then incubated for 24 hr at 37°C/5% CO_2 and 100% relative humidity to allow for attachment and acclimatisation. Thereafter, appropriate wells were exposed to various concentrations of *Z*-venusol, Re-Joovena™, DMSO (less than 0.5%) and 4 µM cisplatin for 24 hr, 48 hr and 72 hr. Control cells were incubated with media only. At the end of each treatment period, 80 µL of trichloroacetic acid (30%) was added to each well to fix

the cells at the bottom of the 96 well plates which were left in the fridge (at 4°C) for three days. Thereafter, the plates were washed three times with distilled water, and left to dry in an oven (at 40 C) for about 2 hr. Thereafter, 100 µL of SRB dye (0.057% in 1% acetic acid) was added to each well and plates were left at room temperature (protected from light) for 30 min. Then plates were washed three times with 400 µL of acetic acid 1% and left in the oven (at 40°C) for about 2 hr. Finally, 200 µL Tris buffer (10 mM, pH 10.5) was added and plates were placed on the shaker to dissolve the contents of the wells. Absorbance was read using the BioRad microplate reader (Model 3550), at 595 nm. Three independent experiments were carried out in quadruplicate and no outliers were removed.

2.10 Details of Assays Used to Investigate Cell Death Pathways

2.10.1 Fluorescein Isothiocyanate (FITC) Annexin V Apoptosis Detection Assay

The FITC Annexin V apoptosis detection assays (BD Biosciences) were conducted as per manufacturer's protocol. Briefly, MCF-7s were harvested and 80 µL of the cell suspension was seeded at a density of 5 X 10^4 cells per well in 12-well culture plates containing 2 mL of medium/well. After 24 hr incubation, test wells were exposed to 150 µg/mL *Z*-venusol (for 24 hr and 48 hr). Cells were also exposed for 4 hr to an apoptotic concentration (0.15 µM) of camptothecin (Del Bino, *et al*., 1999), which thus served as a positive control. Afterwards, treated and untreated cells were detached using 2 mL of Accutase™ Cell Detachment solution, washed with 2 mL of HBSS and then suspended in 1X Binding Buffer solution. Subsequently, 100 µL of cell suspension (with density of not less than 5 x 10^4 cells) was transferred into 5 mL culture tubes. Thereafter, 5 µL of FITC Annexin V and 5 µL of propidium iodide (PI) dyes were added. These suspensions were analysed using a flow cytometer (the FACS Calibur "JO" E5637), which were verified by an application specialist from the BD Biosciences (Please refer

to Appendix C for the FACS Calibur's instrument settings). Two independent experiments in duplicate were carried out. Scatter plots were interpreted as follows: Cells with intact cell membrane (viable cells) are scattered in the left lower quadrant of the scatter plot. As the process of death begins, cells take up both dyes to varying extents and the predominant uptake of one dye indicates the particular mechanism of demise; that is, cells undergoing early necrosis absorb only the PI dye and are therefore scattered in the upper left quadrant, while cells undergoing early apoptosis are in the lower right quadrant as the FITC Annexin V dye attaches to the phosphatidylserine (PS) protein exposed to the outer surface of the cell membrane. Dead cells, i.e. cells in late apoptosis and/or necrosis, are scattered into the right upper quadrant because they absorb both the PI and the FITC Annexin V dyes. Apoptotic (lower) concentration (0.15 µM) of camptothecin, which was originally isolated from the tree *Campotheca acuminate* by Wall and colleagues (Wall *et al*., 1966), were used as a positive control.

2.10.2 Lactate Dehydrogenase (LDH) Assay

The LDH cytotoxicity experiment was done as per manufacturer's (Clontech Laboratories, Inc.) instructions. The LDH enzyme is released to the extra-cellular environment when the cell membrane breaks. Therefore, the higher the LDH activity (compared to the untreated control cells), the more the necrotic cell death. Briefly, cells were harvested and 80 µL were seeded into 96-well plates at a density of 2000 cells/well. After 24 hr incubation, appropriate wells were exposed to 150 µg/mL *Z*-venusol and necrosis-inducing camptothecin concentration (4 µM) for 4 hr as per manufacturer's instructions. After centrifuging the plates at 1800 rpm (± 332 x g) for 10 min, 100 µL of supernatant were transferred to corresponding optically-clear, flat-bottom 96-well plates. Thereafter, 100 µL of a freshly-prepared enzymatic reaction mixture, containing diaphorase enzyme and tetrazolium salt, was added to each well. After 30 min incubation (protected from light) at room temperature, absorbance was measured at 495

nm using the Biorad Model 3550 microplate reader. Three independent experiments were carried out in quadruplicate.

2.11 Details of Assays Used to Explore Mechanism of Action

2.11.1 Human Interleukin-6 (IL-6) Activity Assay

The human IL-6 sR ELISA kits, purchased from Sigma, were used as per manufacturer's protocol. Briefly, all reagents were brought to room temperature. Thereafter, 100 µL of each standard and sample were added into appropriate wells coated with human IL-6 sR antibodies, covered and incubated at 37°C/5% CO_2 and 100% relative humidity. On the following day, solutions were discarded and each well was washed 4 times with 300 µL of 1 X Wash Solution. Then, 100 µL of prepared biotinylated detection antibody solution was added to each well and incubated on the gentle shaker at room temperature for 1 hr. Thereafter, the solution was discarded and each well was washed with 1 X Wash Solution as described above. Then, 100 µL of prepared HRP-Streptavidin solution was added to each well, and incubated with gentle shake at room temperature for 45 min. Thereafter, the solution was discarded and each well was washed with 1 X Wash Solution as described above. Then, 100 µL of ELISA colourimetric 3, 3́,5,5́-Tetramethylbenzidine (TMB) reagent was added to each well, and incubated in the dark with gentle shake at room temperature for 30 min. Finally, 50 µL Stop Solution was added to each well and absorbance was read using the BioRad (Model 3550) microplate reader, at 595 nm wavelengths. The highest final working concentration for a drug used as a positive control, pitovastatin, was 1.5 µM (Wang & Kitajima, 2007). Three independent experiments were carried out in quadruplicate and outliers were not removed.

2.11.2 Direct Acetylated Cyclic Adenosine Monophosphate (cAMP) Assay

The direct cAMP enzyme immunoassay kits, purchased from Sigma were used as per manufacturer's protocol. Briefly, all reagents were thawed to room temperature. The acetylation reagent (or solution) was prepared by adding 0.5 mL of acetic anhydride to 1 mL of triethylamine. Subsequently, 10 µL acetylation solution was added to every 200 µL of standard and sample solutions to enhance the detection of cAMP activity, because there is a low expression of β-adrenergic receptors in MCF-7s (Madden *et al*., 2010). Various concentrations (0.078, 0.312, 1.25, 5 and 20 pmol/mL) of standard solution were prepared by serial dilution of cAMP solution with 0.1 M HCl. Then, 50 µL of neutralising reagent was added into each well of the 96-well-plate coated with goat anti-rabbit IgG antibodies. Thereafter, 100 µL of standard solutions and 100 µL of sample solutions added to appropriate wells. This was followed by addition of 50 µL of the blue cAMP-Alkaline Phosphatase Conjugate into appropriated wells and inoculation of 50 µL of the yellow cAMP EIA antibody solution. Then, the plate was incubated on a gentle shaker at room temperature for 2 hr. Thereafter, the contents were emptied and the wells were washed three times with a freshly prepared 10% wash buffer solution. Then, 200 µL of the p-nitrophenyl phosphatase conjugate substrate solution was added to each well and thereafter the plate was incubated at room temperature, protected from light, for 1 hr. Finally, 50 µL Stop Solution was added to each well and absorbance was read using the BioRad (Model 3550) microplate reader, at 415 nm wavelengths. The measured optical intensity is inversely proportional to the concentration of cAMP the samples. Epinephrine (10 µM) and propranolol (10 µM), both purchased from Sigma, were used separately, in combination and added to the highest concentration of *Z*-venusol for comparison (Kelly *et al*., 2009; Madden *et al*., 2011). Four independent experiments were carried out in duplicates.

2.11.3 Caspase Profiling Assay

The caspase profiling assay was done as per manufacturer's (Clontech Laboratories, Inc.) instructions. Briefly, cells were harvested and 80 µL were seeded into 96-well plates at a density of 2000 cells/well. After 24 hr incubation, appropriate wells with different cell lines were exposed 0, 600, 1200 and 2400 µg/mL *Z*-venusol for 48 hr, 250 000 cells from each plate were re-suspended in a chilled 200 µL of appropriate media mixed with 50 µL of Cell Lysis buffer and incubated on ice for 10 min. Cell lysates were centrifuged at 1800 rpm (± 332 x g) for 10 min at 4^0C. A 100 µL of supernatant was transferred to 96 well plates containing 5 µL of 1 mM caspase substrate (DEVD-AFC). 50 µL of 2 X Reaction Buffer/DTT mixtures was added to each well and the plate incubated at 37^0C for 3 hr in a water bath. Fluorescence was read at 355 nm excitation filter and 520 nm emission filter. All experiments were performed in quadruplicates and the experiments were repeated three times.

2.12 Statistical Analyses

GraphPad Prism v5.0 with the freeware package R v2.13.1 was used for statistical manipulations and analyses. To determine the IC_{50} of test compounds, concentrations were converted into $\log_{10}$ values to produce non-linear regression concentration–response curves. Standard curves of absorbance data of known concentrations were used to extrapolate unknown concentrations indicative of activity of protein/messenger of interest. Where applicable, co-efficient determination (R^2) was used to compare relationship co-relation between cell densities and concentrations of test compounds. To determine whether any observable differences between the treatment group means (with the ± SEM) were statistically significant, either Student's T-tests (for normally distributed data) or Mann–Whitney tests (for non-normal distributed data) were applied. Findings were considered statistically significant if *p*-value was ≤ 0.05. Relevant blanks and vehicle-treated controls (DMSO 0.5% or less) were included. Where appropriate, possible background noise (the mean) was deducted from experimental values.

CHAPTER 3: Results

3.1 Cell Viability

Throughout the study period the cell cultures showed no contamination by mycoplasma and the yield of healthy cells ranged from 98.5% to 99.0%.

3.2 Screening of Products

3.2.1 Effects on Proliferation Using MTT Assay

As can be seen in Figure 3.1, hypoxoside resulted in a concentration-dependent increase in proliferation in MCF-7 cells. There were a statistically significant ($p < 0.001$) 38% and 77% increases in proliferation at concentrations of hypoxoside 1200 µg/mL and 2400 µg/mL respectively after 48 hr exposure. *Ent*-Beyer-15-en-19-ol, however, had no effect on proliferation in MCF-7s at the concentrations studied. Although not statistically significant, *Z*-venusol showed an inhibitory trend and the highest concentration (2400 µg/mL) resulted in 27% inhibition of proliferation.

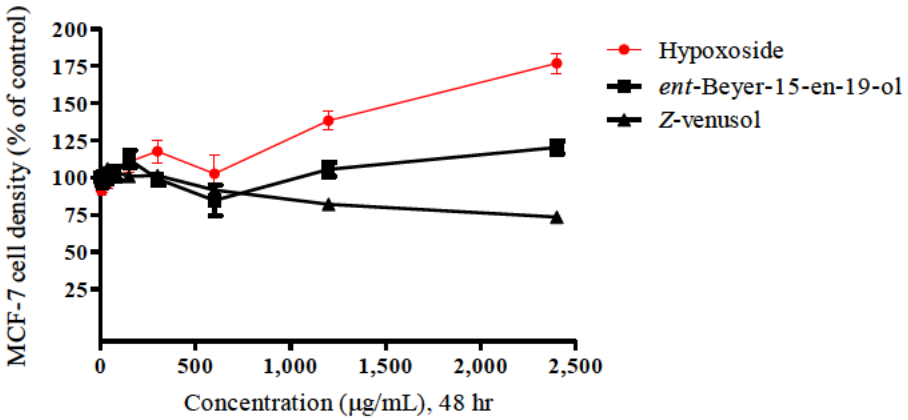


Figure 3.1: Cell density of breast cancer cells (MCF-7) after 48 hr exposure to hypoxoside, *ent*-Beyer-15-en-19-ol and *Z*-venusol

In cervical cancer cells (HeLa), low concentrations (75 µg/mL) of hypoxoside caused a significant 27% decrease ($p = 0.05$) in proliferation (Figure 3.2). Higher concentrations (600 µg/mL to 2400 µg/mL) caused an increase ($p > 0.05$) in proliferation. Similarly, at lower concentrations (4.7 µg/mL and 9.4 µg/mL), *ent*-beyer-15-en-19-ol resulted in a significant 37% and 41% ($p < 0.05$) decreases in proliferation in HeLa cells. As concentrations of *ent*-beyer-15-en-19-ol increased, they returned proliferation to normal. However, with *Z*-venusol there was a consistent concentration dependent decrease in proliferation in HeLa cells. The highest concentration of *Z*-venusol studied (2400 µg/mL) resulted in a 21% decrease in proliferation in cervical cancer cells ($p = 0.05$).

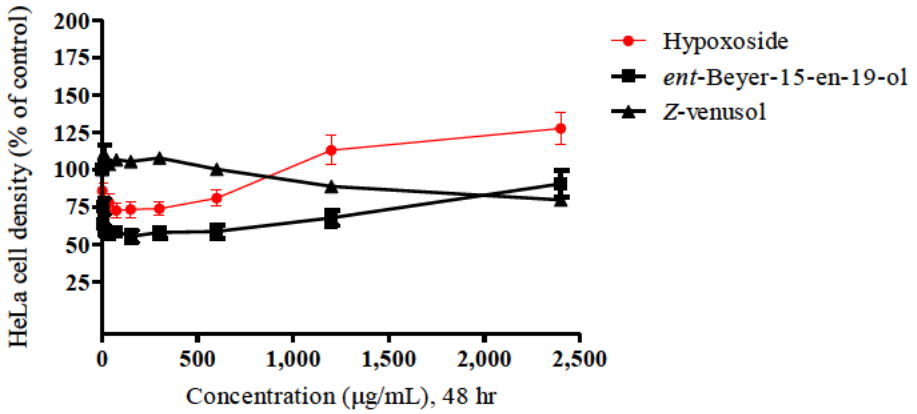


Figure 3.2: Cell density of cervical cancer cells (HeLa) after 48 hr exposure to hypoxoside, *ent*-Beyer-15-en-19-ol and *Z*-venusol using MTT assay

As can be seen from Figure 3.3, only the highest concentration (2400 µg/mL) of *ent*-Beyer-15-en-19-ol decreased proliferation in prostate cancer cells (DU-145) by only 6%. Similarly, the highest concentration of *Z*-venusol decreased proliferation by only 19%. In dMVECs after 48 hr exposure, neither *ent*-Beyer-15-en-19-ol nor *Z*-venusol, any of concentrations studied, showed any significant effects in proliferation (Figure 3.4).

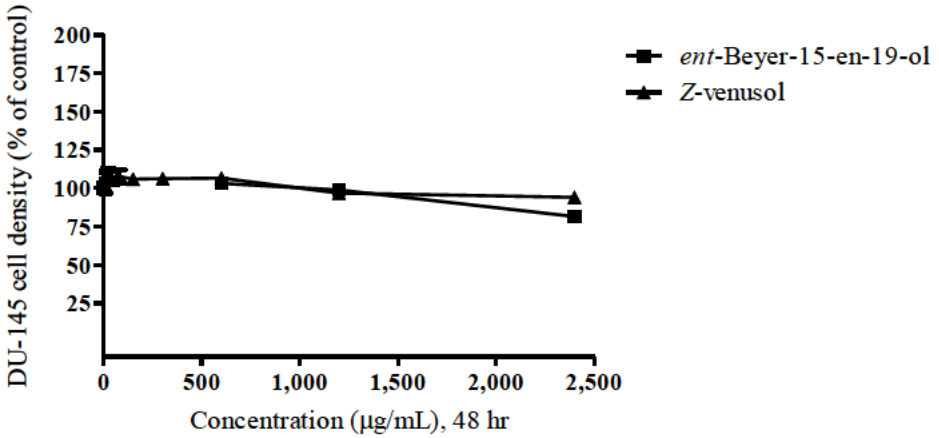


Figure 3.3: Cell density of DU-145 prostate cancer cells after 48 hr exposure to *ent*-Beyer-15-en-19-ol and *Z*-venusol using MTT assay

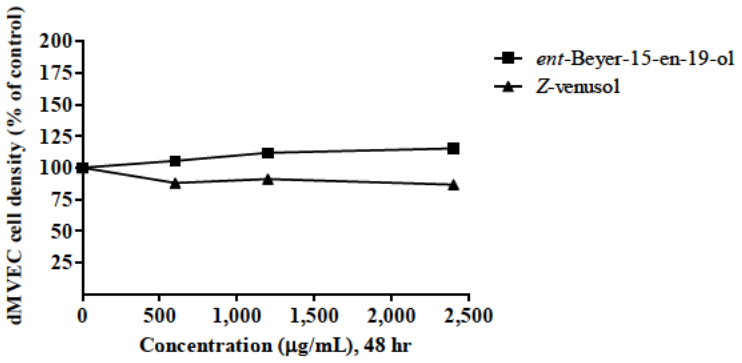
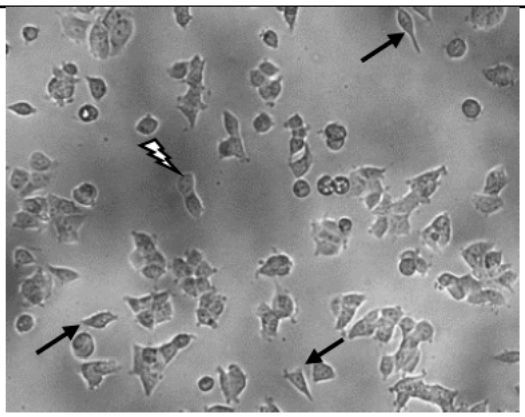


Figure 3.4: Cell density of dMVECs after 48 hr exposure to *ent*-eyer-15-en-19-ol and *Z*-venusol using MTT assay

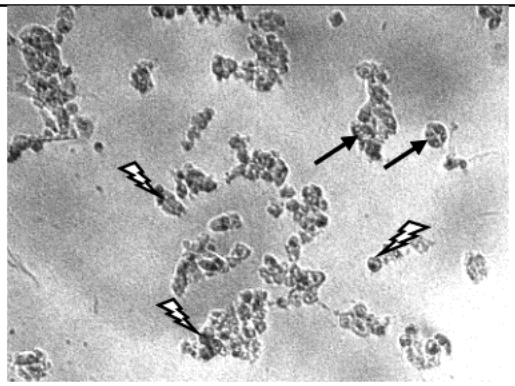
3.2.2 Qualitative Microscopic Assessment

As shown in the (a) panels of Figures 3.5 to 3.8, untreated normal and cancer cells were viable, with irregular and spindle shapes (as indicated by dark arrows). For cancer cells, there were multitudes of colonies dividing next to each other. However, as expected, in normal cells, there were spaces between dividing cells.

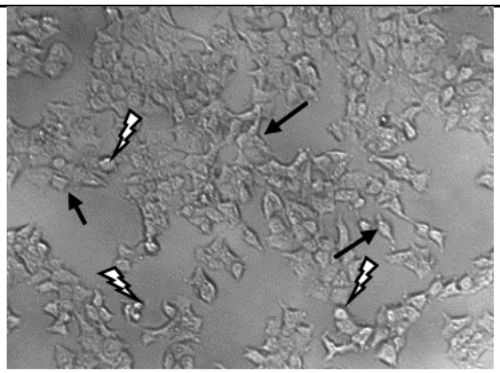
In this qualitative assessment, there were more MCF-7 cells showing signs of apoptosis when exposed to *Z*-venusol 2400 µg/mL for 48 hr (Figure 3.5 b) than HeLa (Figure 3.6 b) and DU-145 (Figure 3.7 b). Apoptotic cells, as indicated by lightening arrows, were "shiny" and the majority showed chromatin condensation surrounded by a clear halo. All cancer cell lines exposed to *ent*-Beyer-15-en-19-ol 2400 µg/mL for 48 hr (Figures 3.5c, 3.6c and 3.7c) had very few cells with the signs of apoptosis. Samples of dMVECs, exposed to either *Z*-venusol 2400 µg/mL or *ent*-Beyer-15-en-19-ol 2400 µg/mL for 48 hr (Figures 3.8b and 3.8c), had very few cells showing apoptosis.



(a) Untreated MCF-7s

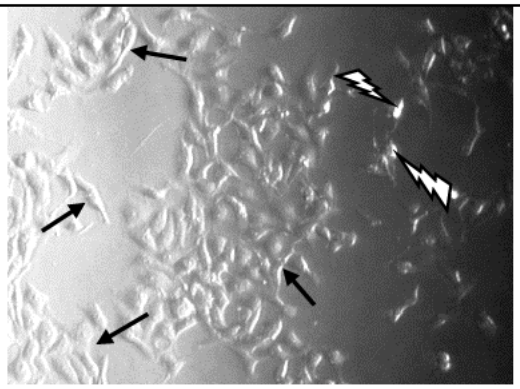


(b) MCF-7s treated with *Z*-venusol

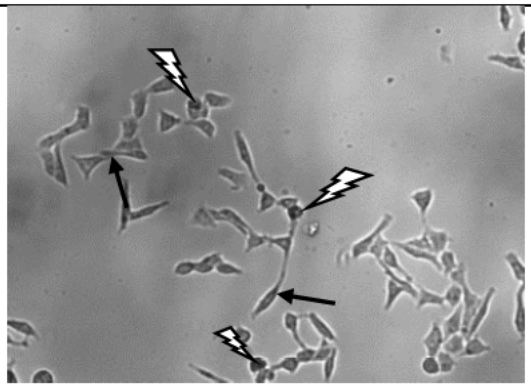


(c) MCF-7s treated with *ent*-Beyer-15-en-19-ol

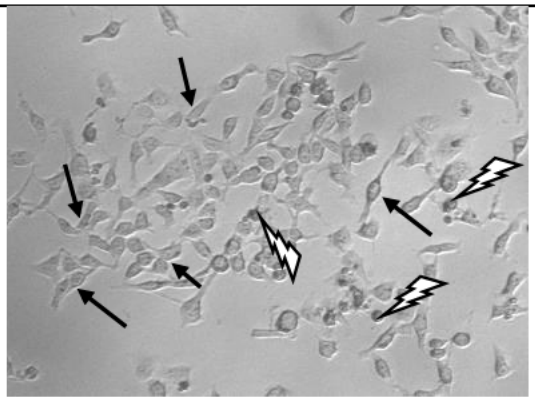
Figure 3.5: Microscopic images (100X magnification and 20X objective, using Leica Microsystems App Suite, Version 3.3.0) showing morphological changes in breast cancer cells (MCF-7) after 48 hr exposure to *ent*-Beyer-15-en-19-ol 2400 µg/mL and *Z*-venusol 2400 µg/mL. ↯ - pointing at some on the cells which are probably undergoing apoptosis. ↖ - pointing at viable cells undergoing proliferation



(a) Untreated HeLa cells

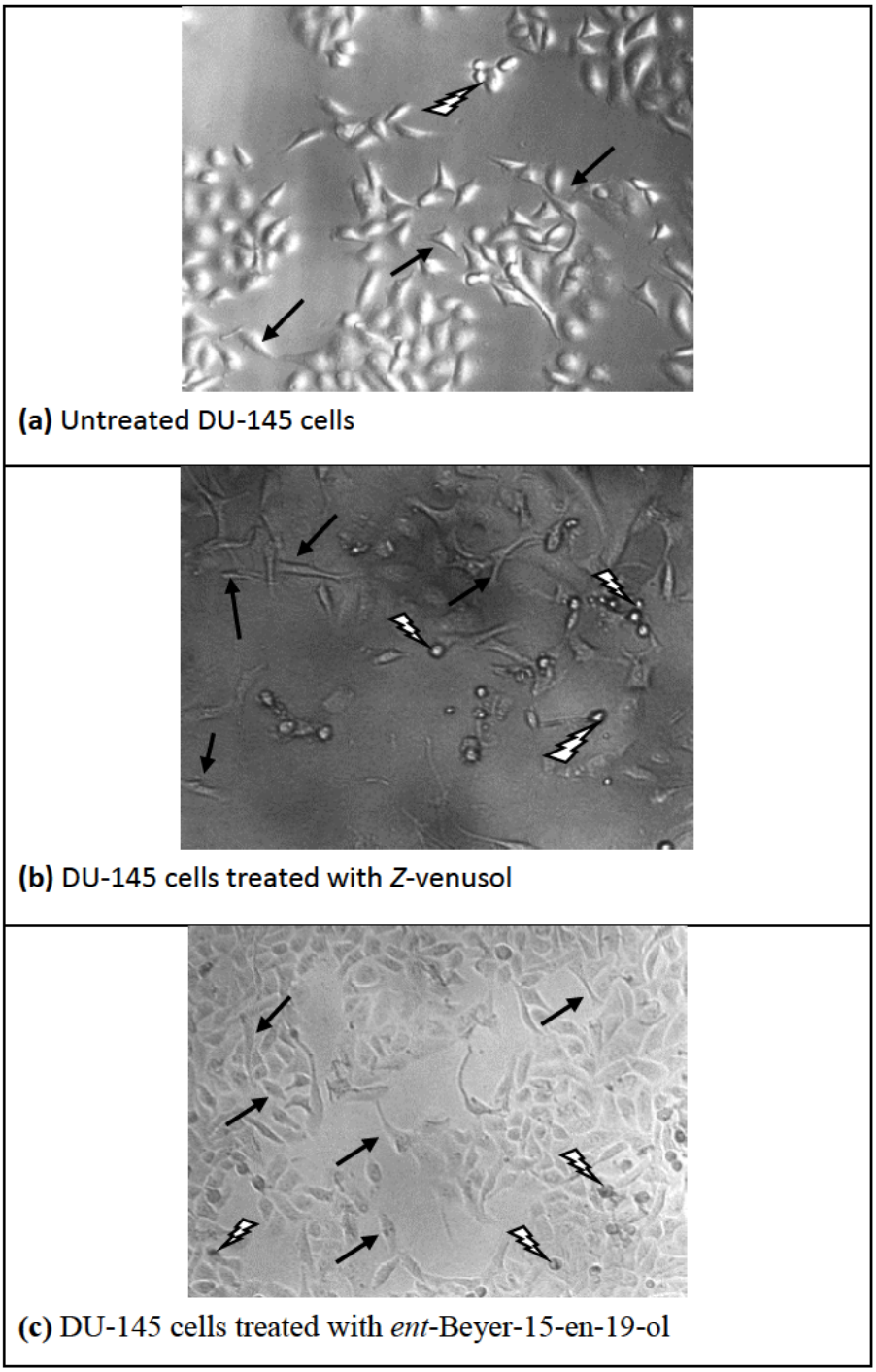


(b) HeLa treated with *Z*-venusol



(c) HeLa treated with *ent*-Beyer-15-en-19-ol

Figure 3.6: Microscopic images (100X magnification and 20X objective, using Leica Microsystems App Suite, Version 3.3.0) showing morphological changes in cervical cancer cells (HeLa) after 48 hr exposure to *ent*-Beyer-15-en-19-ol 2400 µg/mL and *Z*-venusol 2400 µg/mL. ↯ - pointing at some of the cells which are probably undergoing apoptosis. ↖ - pointing at viable cells undergoing proliferation

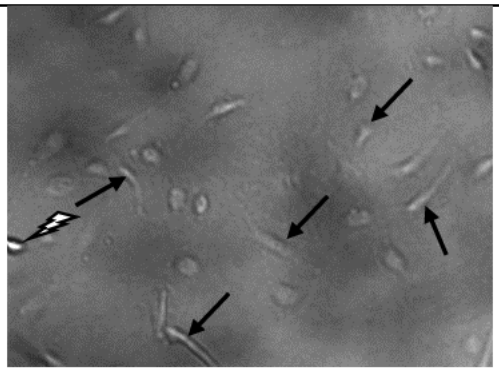


(a) Untreated DU-145 cells

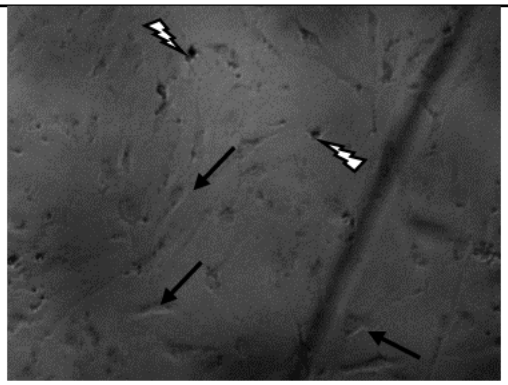
(b) DU-145 cells treated with *Z*-venusol

(c) DU-145 cells treated with *ent*-Beyer-15-en-19-ol

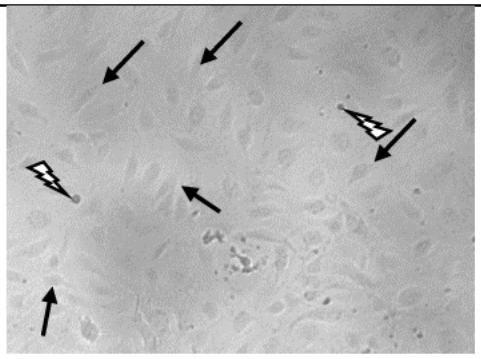
Figure 3.7: Microscopic images (100X magnification and 20X objective, using Leica Microsystems App Suite, Version 3.3.0) showing morphological changes in prostate cancer cells (DU-145) after 48 hr exposure to *ent*-Beyer-15-en-19-ol 2400 µg/mL and *Z*-venusol 2400 µg/mL. ↯ - pointing at some of the cells which are probably undergoing apoptosis. ↖ - pointing at viable cells undergoing proliferation



(a) Untreated dMVECs



(b) dMVECs treated with *Z*-venusol



(c) dMVECs treated with *ent*-Beyer-15-en-19-ol

Figure 3.8: Microscopic images (100X magnification and 20X objective, using Leica Microsystems App Suite, Version 3.3.0) showing morphological changes in dermal microvascular cells (dMVECs) after 48 hr exposure to *ent*-Beyer-15-en-19-ol 2400 µg/mL and *Z*-venusol epithelial 2400 µg/mL. ↯ - pointing at some of the cells which are probably undergoing apoptosis. ↖ - pointing at viable cells undergoing proliferation

3.3 Effects of Various Compounds on Cell Proliferation Using SRB Assay

3.3.1 *Z*-venusol

As depicted in Figure 3.9, after 24 hr exposure, *Z*-venusol was without any significant effect on proliferation of either MCF-7s, or the normal human breast cell lines MCF-12s and HMECs.

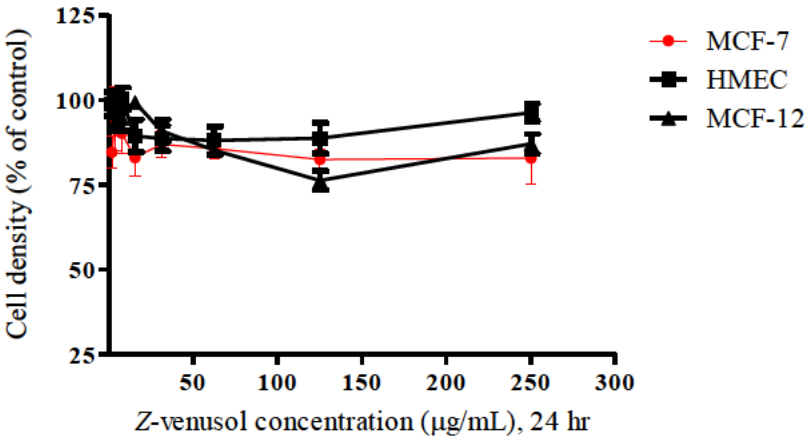


Figure 3.9: Concentration-response curves depicting density (as percentage of control) of MCF-7, MCF-12 and HMEC cells following 24 hr exposure to *Z*-venusol

However, after 48hr exposure (Figure 3.10), *Z*-venusol caused significant ($p < 0.05$) inhibition in MCF-12 cells, with an IC_{50} of 51 µg/mL, but it had minimal effect in proliferation of the HMECs. At a concentration of 125 µg/mL, *Z*-venusol showed more cytotoxicity in MCF-7s than in HMECs, with 28% and 14% inhibition in proliferation, respectively.

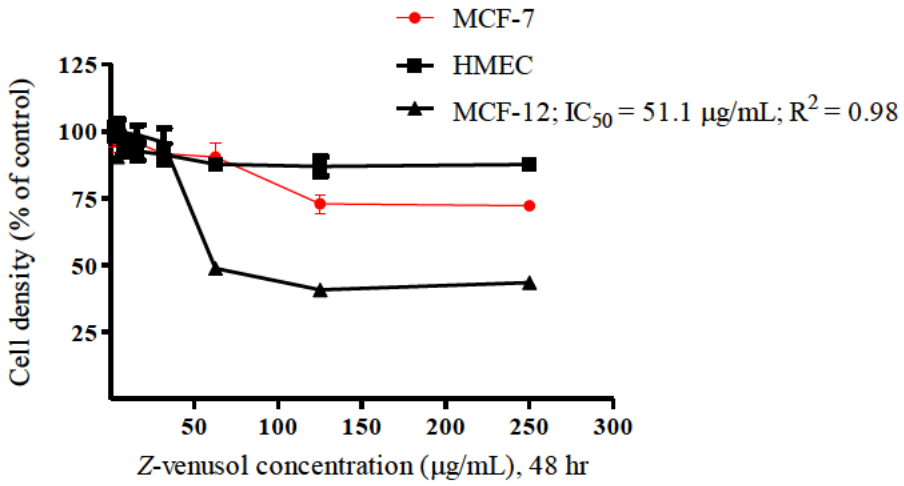


Figure 3.10: Concentration-response curves depicting density (as percentage of control) of MCF-7, MCF-12 and HMEC cells following 48 hr exposure to *Z*-venusol

After 72 hr exposure to *Z*-venusol there was a significant ($p < 0.05$) concentration-dependent inhibition in proliferation of MCF-7s with an IC_{50} of 53.7 µg/mL (95% CI 29.7 - 96.9 µg/mL), (Figure 3.11). The highest concentration of *Z*-venusol (250 µg/mL), used in this study, caused a statistically significant ($p < 0.001$) inhibition of about 69% in MCF-7s. The pattern of these results suggests that more inhibition could be achieved when the concentration is increased beyond that which was used.

Similarly, there was statistically significant ($p < 0.05$) inhibition in proliferation of the normal breast cell line, the MCF-12s, with IC_{50} of 35.6 µg/mL (Figure 3.11). The highest concentration of *Z*-venusol used in this study caused a statistically significant ($p < 0.001$) inhibition of about 84% in this normal breast cell line. However, *Z*-venusol did not have any significant effects on proliferation in other normal breast cell line, the HMECs, used in this study. The highest concentration (250 µg/mL) yielded only 28% inhibition in proliferation in these cells Interestingly, the plateau nature of the concentration-response curve, suggests that the most cytotoxicity to normal mammary cells occurs with 72 hr exposure to *Z*-venusol 31.1 µg/mL. This means that further increases *Z*-venusol concentration would yield no additional inhibition in cell proliferation in the HMEC cell line.

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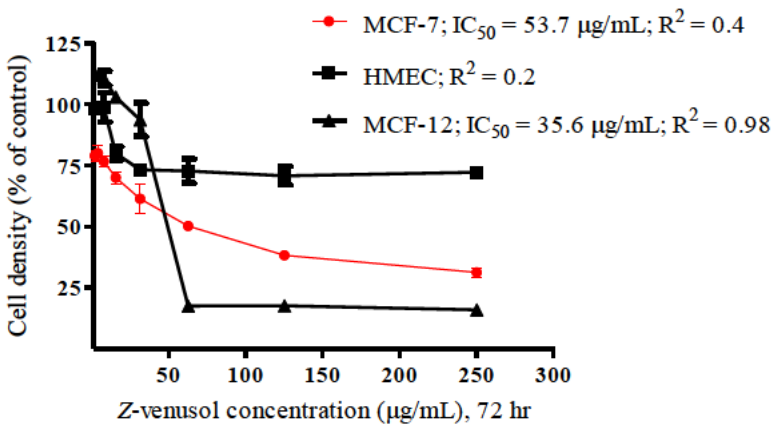


Figure 3.11: Concentration-response curves depicting density (as percentage of control) of MCF-7, MCF-12 and HMEC cells following 72 hr exposure to *Z*-venusol. R^2 = co-efficient of determination.

3.3.2 Re-Joovena™

The highest concentration (250 µg/mL) of *G. perpensa* extract purported to be in Re-Joovena™ caused a 67% ($p < 0.001$) and 31% ($p < 0.05$) induction in proliferation in the normal mammary cells after 24 hr and 48 hr exposure, respectively (as shown in Figures 3.12 and 3.13). However, there were no effects in proliferation of HMEC cells after 72 hr exposure to all the concentrations the Re-Joovena™ solution studied, as shown in Figure 3.14. Similarly, none of the Re-Joovena™ concentrations showed any effects in proliferation in the MCF-7 cells after 24 hr, 48 hr or 72 hr exposure.

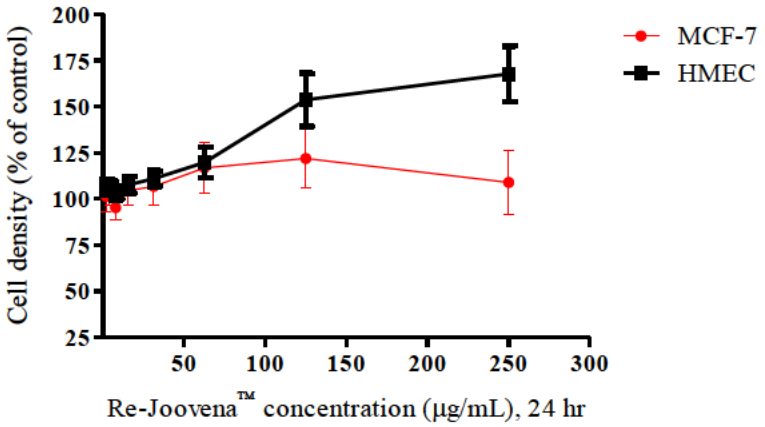


Figure 3.12: Concentration-response curves depicting density (as percentage of control) of MCF-7 and HMEC cells following 24 hr exposure to Re-Joovena™

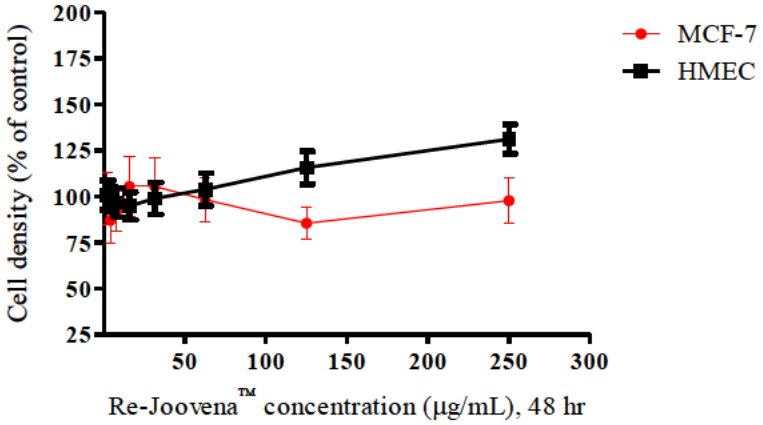


Figure 3.13: Concentration-response curves depicting density (as percentage of control) of MCF-7 and HMEC cells following 48 hr exposure to Re-Joovena™

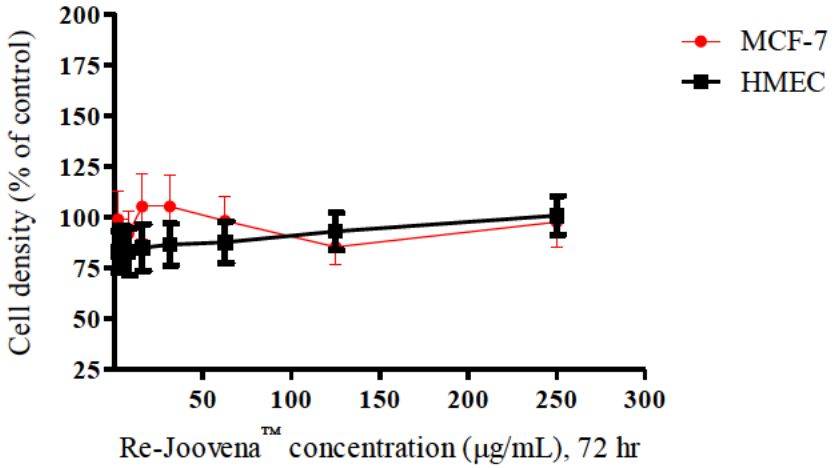


Figure 3.14: Concentration-response curves depicting density (as percentage of control) of MCF-7 and HMEC cells following 72 hr exposure to Re-Joovena™

3.3.3 Cisplatin

As was expected, cisplatin caused a statistically significant ($p < 0.01$) concentration-depended inhibition in proliferation in MCF-12 cells, with IC_{50} of 7.9 µM, 7.0 µM and 1.7 µM for cells which were exposed to 24 hr, 48 hr and 72 hr, respectively (Figure 3.15). Significant ($p < 0.05$) concentration-dependent inhibition in proliferation in MCF-7s was seen in cells exposed to cisplatin for 72 hr only, as can be seen in Figure 3.16.

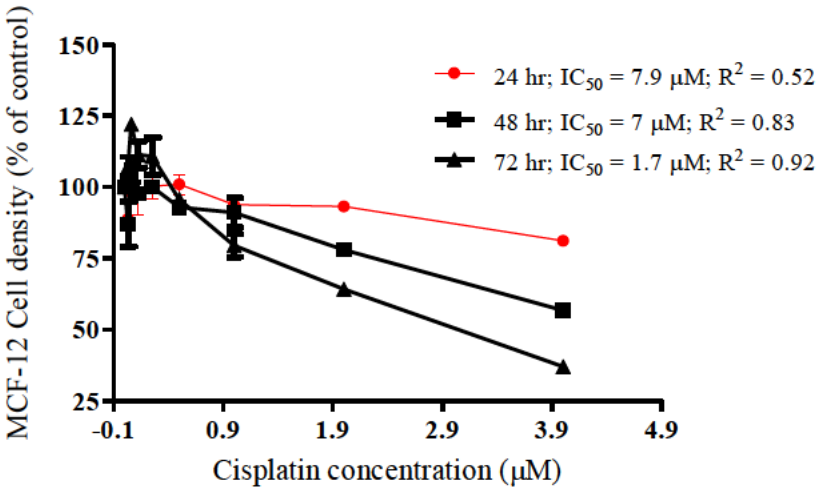


Figure 3.15: Concentration-response curves depicting density (as percentage of control) of MCF-12 following 24 hr, 48 hr and 72 hr exposure to Cisplatin

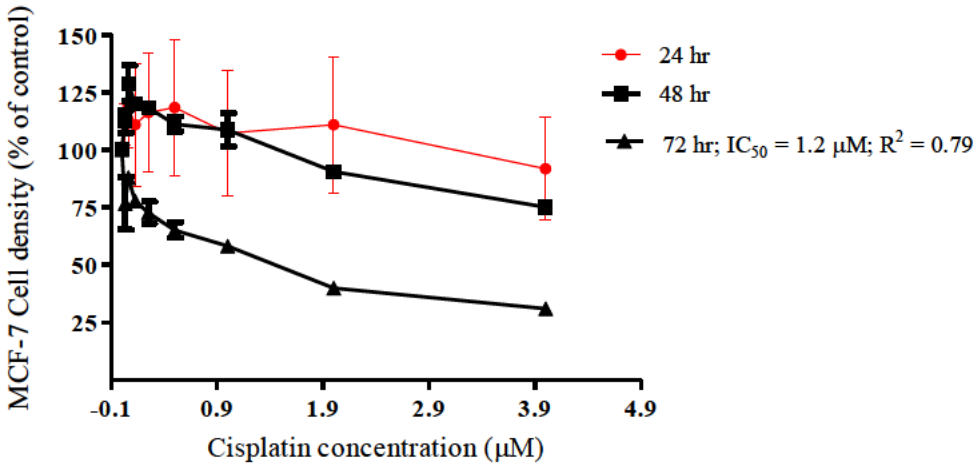


Figure 3.16: Concentration-response curves depicting density (as percentage of control) of MCF-7s following 24 hr, 48 hr and 72 hr exposure to Cisplatin

3.4 Cell Death Pathways

3.4.1 Confirmation of Apoptotic Pathway Using FITC Annexin V Assay

As shown in Figure 3.17a (LLQ), after 24 hr, 99% of untreated MCF-7 cells were still viable. However, 45% of cells which were exposed to 0.15 µM camptothecin showed early apoptosis (Figure 3.17b RLQ) after 4 hr exposure period. There was 20.4% and 29.6% induction of early apoptosis in cells which were exposed to *Z*-venusol 150 µg/mL for 24 hr (Figure 3.17c, LRQ) and 48 hr (Figure 3.17d, LRQ) respectively. For both exposure periods, the difference between cells undergoing early apoptosis (that is Figure 3.17c, LRQ and Figure 3.17d, LRQ) and those undergoing early necrosis (that is Figure 3.17c, ULQ and Figure 3.17d, ULQ) was statistically significant in favour of apoptosis.

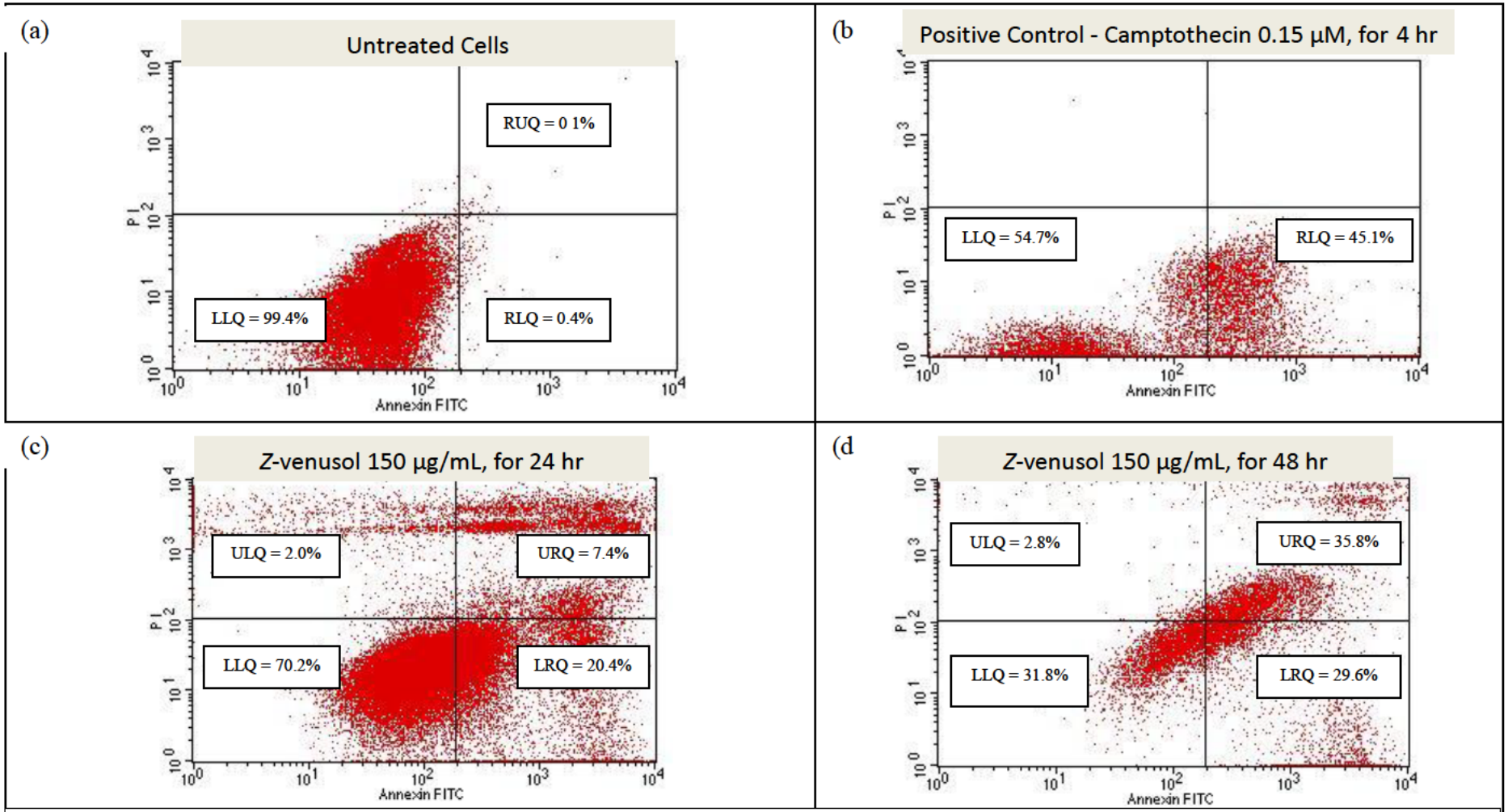


Figure 3.17: Scatter plots showing MCF-7 cells undergoing apoptotic or necrotic cell death after 24 hr and 48 hr treatment with *Z*-venusol 150 µg/mL and camptothecin 1.5 µM treatment over 4hrs. Cells in the lower left quadrants (LLQs) are viable; cells in the lower right quadrants (LRQs) are showing early apoptosis as FITC Annexin V binds to the phosphatidylserine (PS) protein exposed to the outer surface of the cell membrane; cells in the upper left quadrants (ULQs) are showing necrosis as cells absorb propidium iodide (PI) due to breakdown of cell membrane; and cells in in upper right quadrants (ULQs) are showing late apoptosis

3.4.2 Exclusion of Necrotic Pathway Using LDH Assay

As was expected, there was a significant ($p < 0.01$) 70% induction of LDH activity in MCF-7 cells after 4 hr exposure to a high or necrotic concentration (4 µM) of camptothecin (Figure 3:18). However, the highest concentration (150 µg/mL) of *Z*-venusol used in this assay resulted in 23% ($p = 0.31$) and 9% induction ($p = 0.72$) of LDH activity in these cells after 24 hr and 48 hr exposure, respectively.

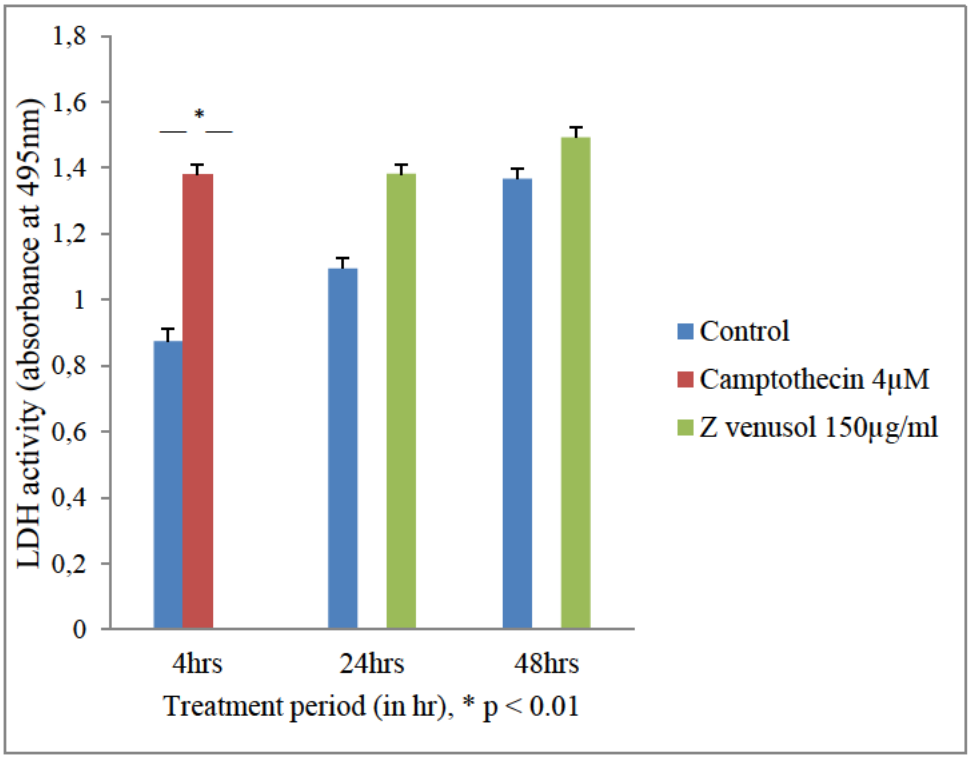


Figure 3.18: Lactate dehydrogenase (LDH) activity in MCF-7 cells after 24 hr, 48 hr treatment with *Z*-venusol 150 µg/mL and necrotic concentration (4 µM) of camptothecin for 4 hr. The LDH enzyme is released to the extra-cellular surface when the cell membrane breaks. Therefore, the higher the LDH activity, as compared to the untreated cell (control), is an indication of a necrotic cell death.

3.5 Mechanism of Action

3.5.1 Effects of Z-venusol on IL-6 Activity

Z-venusol caused a concentration-depended inhibition of IL-6 activity in MCF-7s. As can be seen in Figure 3.19, the highest concentration (75 µg/mL) of *Z*-venusol used in this study produced a 51% inhibition ($p < 0.01$) of the IL-6 activity in these cells. As was expected, the highest concentration (1.5 µM) of pitovastatin which was used as a positive control, resulted in a statistically significant ($p < 0.01$) 49% inhibition of IL-6 activity in the MCF-7s after 48 hr exposure.

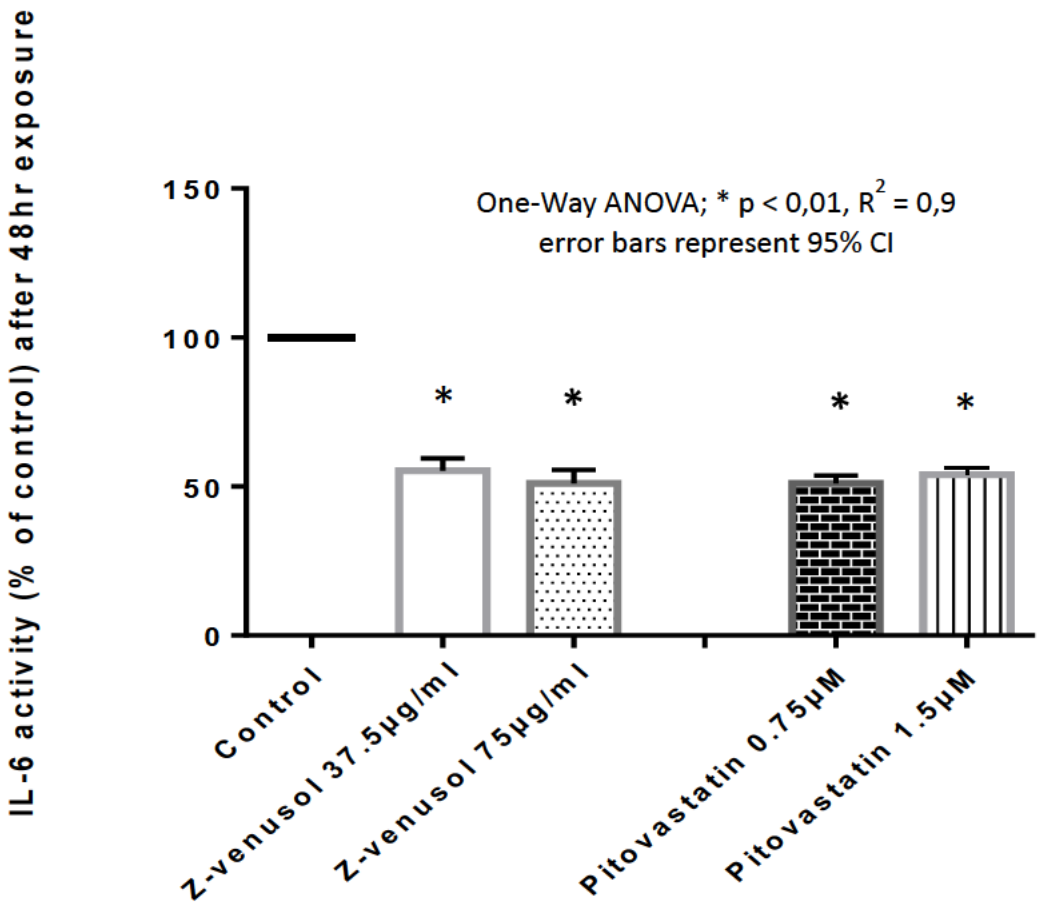


Figure 3.19: IL-6 activity (absorbance at 595 nm wavelengths) of breast cancer cells (MCF-7s) after 48 hr exposure to *Z*-venusol and pitovastatin.

3.5.2 Effects of *Z*-venusol on cAMP Activity

Low concentrations (37, 5 and 75 µg/mL) of *Z*-venusol decreased cAMP, was not statistically significant (Figure 3.20). Surprisingly, a combination of the highest concentration (*Z*-venusol 150 µg/mL) with propranolol 10 µM caused a 34% elevation of cAMP activity ($p < 0.05$) and epinephrine 10 µM caused a 17% reduction of cAMP activity in MCF-7s. However, on its own, *Z*-venusol did not show any significant effect on cAMP activity in these breast cancer cells.

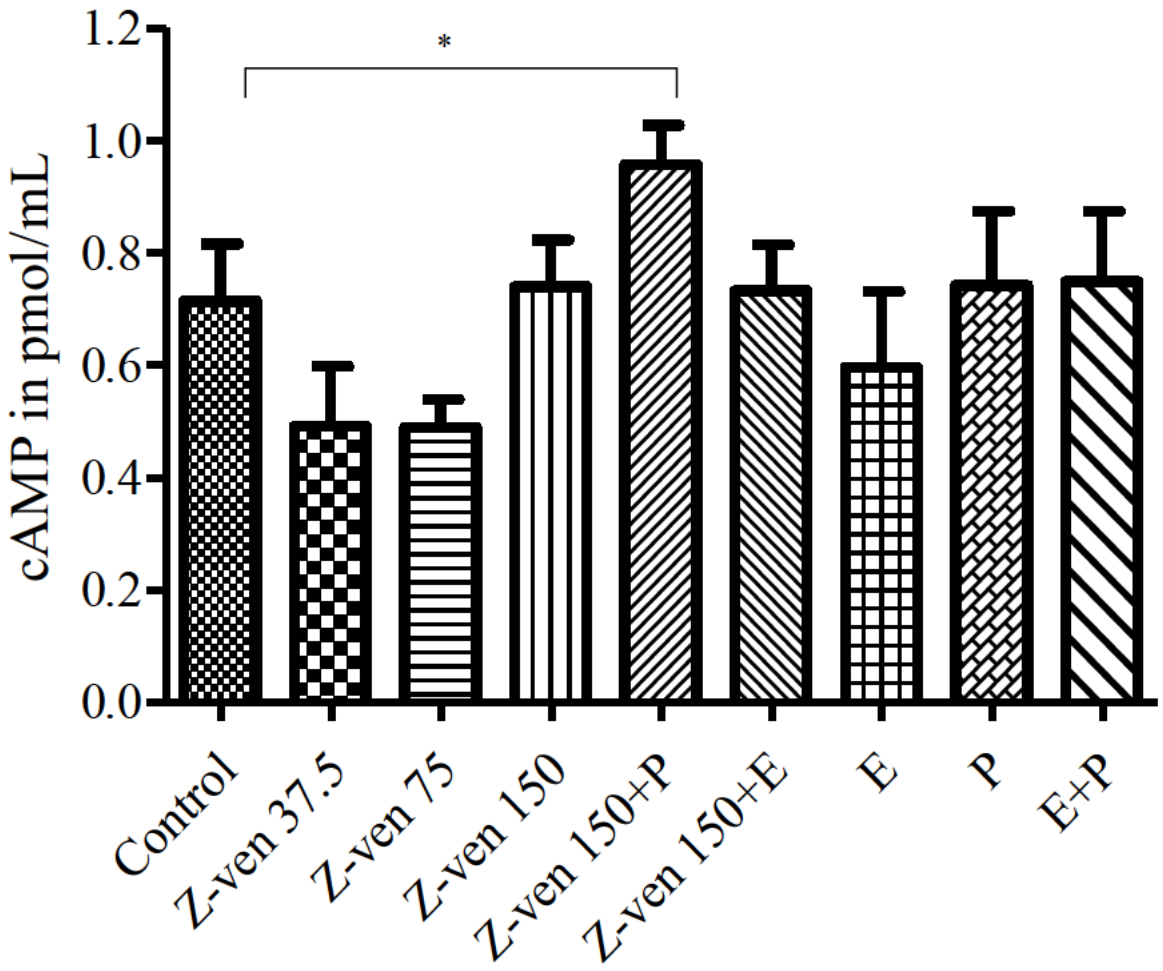


Figure 3.20: cAMP activity (amount in pmol/mL) in MCF-7 breast cancer cells exposed to *Z*-venusol (37.5, 75 & 150 µg/mL), *Z*-venusol 150 µg/mL + propranolol 10 µM (P), *Z*-venusol 150 µg/mL + epinephrine 10 µM (E), epinephrine 10 µM (E), propranolol 10 µM and epinephrine 10 µM + propranolol 10 µM (EP) for 48 hr. Blue dotted line represent a comparative cAMP concentration in untreated (control) cells). *$p \leq 0.028$.

3.5.3 Effects of *Z*-venusol on Activity of Caspase 2, 3, 8 & 9 in MCF-7s

As can be seen in Figure 5. 21, generally, *Z*-venusol caused a decrease in activity of caspases 2, 3 and 9 in MCF-7s after 48 hr exposure. However, none of these results were statistically significant. Surprisingly, there was a 40% and 100% increase in caspase 8 activity in MCF-7s which were exposed to *Z*-venusol 600 µg/mL and 1200 µg/mL, respectively. Unfortunately, these findings were not reproducible.

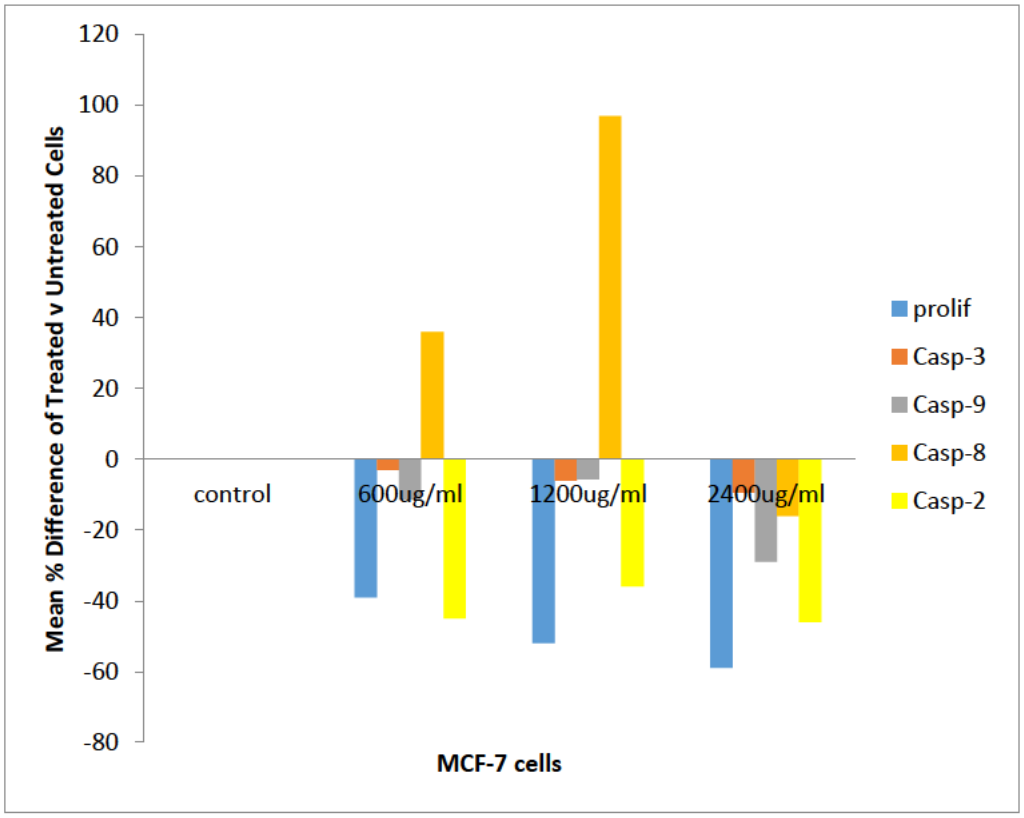


Figure 5.21: Percentage mean difference of caspase activity on MCF-7 cancer cells after 48 hr exposure to different concentrations (µg/mL) of *Z*-venusol for 48 hr *in vitro*.

CHAPTER 4: Discussion, Recommendations and Conclusion

4.1 Discussion of Major Findings

Of the compounds screened during this investigation, the first two (hypoxoside and *ent*-Beyer-15-en-19-ol) were disappointing from the objective of hoping to find potentially useful cancer cures. In particular, hypoxoside was found, at high concentrations (2400 µg/mL), to increase proliferation by 65% in breast and by 25% in cervical cancer cells, suggesting that it would be likely to cause or exacerbate cancer instead of curing it. This was contrary to several other studies that have consistently reported that hypoxoside was pro-apoptotic and that it inhibits cell proliferation. For example; Boukes (2010) reported that it inhibits proliferation of cervical and breast cancer cells; Ali-Azouaou *et al*. (2014) reported that hypoxoside and its derivatives had activated p53 and it increased expression of caspase 3 in human embryonal teratocarcinoma stem cells (the NT2/D1 cells). Similarly, Drewes *et al*. (2008) reported that hypoxoside was active against both malignant and benign prostate cancer cells. However, in support of the current findings, Xulu (2013) also reported that hypoxoside, and its active derivative known as rooperol, significantly increases cell proliferation of both cancer and normal mammary cells *in vitro*. Further, at a clinical level, Smit *et al*. (1995) reported that lung cancer patients who took hypoxoside 1200 mg to 3200 mg per day did not show any remission of the disease and 79% (19 out of 24 patients) died within four months.

There is some evidence of hypoxoside having an effect against human pathogens such as *Staphylococcus aureus* and *Escherichia coli* (Laporta *et al*., 2007) and acting as an antioxidant (Boukes & van de Venter, 2012; Okem *et al*., 2015). Antioxidants do have the ability to scavenge reactive oxygen/nitrogen species (ROS/RNS) and increase survival of human cells (Lu *et al*., 2010) but also decrease the chance of oncogenic mutations (Diplock *et al*., 1998). Therefore, the effects of hypoxoside in cancer remain unclear.

Unfortunately, there are no published studies on *ent*-Beyer-15-en-19-ol for a direct comparison with the findings of the current research. The findings of the current study suggest that it seems to play no role in the treatment of breast cancer and prostate cancer because it showed no influence on proliferation of MCF-7s and DU-145s. However, its ability to inhibit cell proliferation in HeLa lines by almost 50% at concentrations as low as 9.4 µg/mL offers an opportunity for further studies. Further, the differences in expression of the vimentin gene, which is a part of intermediate filaments (IFs) group occurring in the cytoplasm of many human cell, could be used to explain why *ent*-Beyer-15-en-19-ol showed activity on proliferation of HeLa cells and not on proliferation of MCF-7s and DU-145s.

A possible explanation for the different effects on breast and cervical tumours may relate to the vimentin gene. This gene is known to induce cell proliferation in cultured cells by generating polymeric fibres between microfilament and microtubules during mitosis (Oshima, 2002; Satelli & Li, 2011). However, a number of studies have reported that MCF-7s lack vimentin gene (Pieper *et al*., 1992), which is incidentally expressed in large quantities in HeLa cells (Seegers *et al*., 1989; Sarria *et al*., 1990; Yue *et al*., 2016). Therefore, it could be possible that *ent*-Beyer-15-en-19-ol inhibits proliferation of HeLa cell lines by inhibiting the activity of vimentin in these cells while showing low cytotoxicity in the MCF-7s. Other critical proliferation genes which are found in low quantities in MCF7s as compared to high quantities in HeLa cell line are the Ki67 proteins and the HGF cytokine factors such as claudin (Kiessling *et al*., 1993; Boros & Miller, 1995; MacCallum & Hall, 1999; Martin *et al*., 2004; Holliday & Speirs, 2011; Ma *et al*., 2015; Zhou *et al*., 2015).

The most exciting and novel compound discovered during the screening process was *Z*-venusol, which was isolated from *G. perpensa*. This compound significantly inhibited proliferation of

breast cancer cells, after 72 hr exposure, in a concentration-dependent manner. There are no studies with which to directly compare the findings of these results. However, reports on effects of the extracts of *G. perpensa* on various other cancer cell lines provide an opportunity for comparison. For instance, the results of this research support the findings of Simelane and colleagues, who recently reported that *G. perpensa* extracts inhibit cell proliferation (Simelane *et al*., 2012). They reported that crude extracts of the same plant inhibited proliferation of hepatocellular carcinoma cells (HepG2) with an IC_{50} of 222.33 μg/mL and human embryonic kidney 293 (HEK293) cells with an IC_{50} of 279.43 μg/mL both after 48 hr of treatment. By comparison, the pure compound, *Z*-venusol, was more potent against normal cells in the present study as it inhibited proliferation of the normal breast cell line (the MCF-12s) with an IC_{50} of 51.1 μg/mL after 48 hr exposure. However, there was no significant inhibition of the malignant MCF-7s after 48 hr exposure. Direct comparisons are not possible as not only do the cell lines differ, but also the exact content of the crude extract is unknown.

Inhibition of cancer cell proliferation with *Z*-venusol was seen at 72 hr exposure of MCF-7s. The IC_{50} was 53.7 μg/mL. This is slightly more than that specified by the American National Cancer Institute guidelines, which sets the acceptable limit of activity for crude plant extracts at 30 μg/mL or less (Suffness *et al*., 1990). Thus, in this setting *Z*-venusol is unfortunately less potent than the ANCI requires for a crude extract. Expressed as μM, the IC_{50} for *Z*-venusol is 165.6 μM compared to 1.2 μM for cisplatin in the same cancer cell line. This means that, clinically, higher concentrations of *Z*-venusol would be needed compared with existing chemotherapy medicines such as cisplatin, which causes inhibition of cell proliferation at much lower concentrations. Increasing the concentration of *Z*-venusol would be possible as there was no plateau of inhibition of proliferation in MCF-7s. It was only in primary normal human mammary epithelial cells that formation of "plateaus" was observed.

This means that if *Z*-venusol was ever able to be used clinically, the dose might be increased higher enough for the attainment of desired therapeutic effects with minimal cytotoxicity on normal cells. This is in contrast to a plant-derived drug called vinorelbine, isolated from *Catharanthus roseus*, which Bergh and colleagues, have reported to form "plateaus" as the concentration increases beyond a certain point (Bergh *et al*., 2001). It is also in contrast to cisplatin which has a narrow therapeutic index and many other existing chemotherapy drugs that are cytotoxic to normal cells (Lee *et al*., 1992; Hesketh *et al*., 2002. In clinical settings many chemotherapy drugs, such as cisplatin, cause unbearable side effects as the dose is increased (Hudis, 1996; McGuire, 1998).

In this study, *Z*-venusol was more cytotoxic to MCF-12s than to the other to normal cells in the study, i.e. the primary human mammary epithelial cells (HMECs). Tetraethylammonium (TEA), a non-selective potassium channel blocker across the cell membrane, increases cell proliferation and is involved in tumour formation (Marino *et al*., 1994; Levin, 2012; Berzingi *et al*., 2016). Various studies, however, have reported that TEA expression across normal human mammary and breast cancer cell lines varies. For example, Kimura and colleagues have reported that HMECs are trypsin-resistant and that they have a very low uptake and a low expression of TEA (Dimri *et al*., 2005; Kimura *et al*., 2006). On the other hand, there is high expression of TEA in MCF-7s (Berzingi *et al*., 2016) and in MCF-12s (Dhillon, 1999). The difference in the uptake of TEA in the HMECs and in the transformed MCF-7s as well as in MCF-12s may explain why *Z*-venusol is less cytotoxic against the primary human mammary epithelial cells as seen in this study. Therefore, it may be hypothesised that the activities of *Z*-venusol are mammary cell sub-type specific. The mechanism by which it inhibits cell proliferation and causes cell death, may involve blockage of passage of potassium cations during membrane potential depolarisation.

Cell death can happen by apoptosis (the so called “good” death), necrosis (the so called “bad” death), or by a combination of both processes (Karantza & White, 2011; Newton *et al*., 2014). In this study, majority of cancer cells underwent apoptotic cell-death due to exposure to *Z*-venusol. Suggesting that this compound interacts with certain cellular proteins and mediators in a programmed manner in the demise of cancer cells. Other potential plant-derived compounds which have been reported to cause apoptotic cell death in MCF-7s are those extracted from *Aloe vera* (Hussain *et al*., 2015), plumbagin (Sagar *et al*., 2014) as well as parthenolide isolated from *Tanacetum parthenium* (Wyrębska *et al*., 2013). In contrast, Atmaca and colleagues have recently used the Annexin V assay, as was done in this study, and reported that crude extracts of *Galium aparine* caused necrotic, instead of apoptotic cell death in MCF-7s (Atmaca *et al*., 2016). Numerous recent studies have postulated that both necrotic and apoptotic cell death are “programmed” (Galluzzi *et al*., 2008; Kroemer *et al*., 2009; Sun *et al*., 2009; Newton *et al*., 2014). Therefore, knowing the type of cell death that occurred as a result of an exposure to compounds is no longer only important for describing the demise as “good” or “bad” (Galluzzi *et al*., 2008; Sun & Peng, 2009). However, identification of the type of cell death can also help with selection of an appropriate pathway(s) to follow and to choose plausible mediators and proteins to investigate when the exact mechanism of action is being explored.

Elucidation of molecular mechanism of action of many drugs is often an arduous and very complex process which usually takes years of consistent research to accomplish. Even for a well-known drug such as paracetamol, which has been widely used for over 100 years, its mechanism of action has not been fully explained until recently (Graham *et al*., 1999; Botting & Ayoub, 2005; Graham & Scott, 2005; Ayoub *et al*., 2006). Therefore, it was exciting to

discover that the observed apoptotic breast cancer cell death, which occurred as a result of exposure to *Z*-venusol, was mediated directly or indirectly by inhibition of IL-6.

Interleukin-6 is one of many pro-inflammation cytokines secreted by macrophages and T-cells to stimulate immune response (Scheller *et al*., 2011). Over-expression of IL-6 has been implicated in the pathology of cancer (Krueger *et al*., 1991; Simpson *et al*., 1997). Clinically, high serum levels of IL-6 is strongly associated with poor prognosis in patients with melanoma (Rutkowski, *et al*., 2002) and metastatic breast cancer (Zhang & Adachi, 1999; Knüpfer & Preiss, 2010). Traditional uses of extracts from *G. perpensa* include management of pain and inflammatory conditions such as haemorrhoids and endometritis (McGaw *et al*., 2005). Therefore, the initial suggestion that *Z*-venusol causes apoptotic cell death by inhibition of IL-6 correlates well with both the theory of inflammation as a hallmark of cancer pathogenesis and with traditional uses of extracts from *G. perpensa*. Crude extracts from other plants that have been recently shown to cause apoptotic cell death by suppression of IL-6 in MCF-7s are those from an indigenous Taiwanese *Ipomoea batatas* L. Lam herb, commonly called the "Purple-Fleshed Sweet Potatoes" (Sugata *et al*., 2015). Also, as an indirect corroboration of the findings of this study, Costantini and colleagues have reported that extracts from *Punica granatum* L. seeds causes apoptosis in these breast cancers cells by inhibition of IL-6 (Costantini *at al*., 2014).

Since IL-6 is an up-stream protein, considered to play multifunctional roles in the tumour micro-environment, then it triggers numerous middle and downstream mediators to bring about uncontrollable cancer cell proliferation (Sağlam *et al*., 2015). For example, uncontrollable proliferation happens when IL-6-stimulation causes activation of the janus kinase (JAK) tyrosine kinase super family members; and then JAK-stimulation results in the activation of

the signal transducers and activators of transcription 3, i.e., STAT3 (Guo *et al*., 2012). Other down-stream proteins that are activated by IL-6-stimulation are the mitogen-activated protein kinase (MAPK) and the phosphoinositide 3-kinase (PI3K), which function as the major effector of the Ras oncoprotein (Mendoza *et al*., 2011). Lee and colleagues have recently reported that, as a result of an up-stream IL-6-stimulation, withaferin A, isolated from *Withania somnifera* plant, induced apoptosis in MCF-7s through down-stream activation of STAT3 (Lee *et al*., 2010). Therefore, in-depth investigations are needed to assess how *Z*-venusol's IL-6-inhibition affects the STAT3, MAPK, PI3K , Rho kinases and NF-kappaB proteins or even the cAMP signalling "cross talk" described by Irvin *et al.* (2001).

In this study, initial attempts were made to assess the effects of *Z*-venusol on cAMP in MCF-7s. The findings were peculiar and interesting. Several, both *in vitro* and *in vivo*, studies have reported that cAMP is upregulated in cancer cells (Lang *et al*., 2004; Powe *et al*., 2011). Also, Pasquier and colleagues have reported that propranolol, a β-adrenergic blocker, improves survival in patients with breast cancer (Pasquier *et al*., 2011). Further, the traditional use of *G. perpensa* has been reported to cause uterine contraction (Varga & Veale, 1997; Kaido, *et al*., 1997); possibly by inhibition of β-adrenergic receptors, because β-adrenergic agonist relaxes the uterus and increases cAMP. Therefore, we hypothesised that apoptotic cell death in MCF-7s was as a result of inhibition of cAMP in these cells. Thus, we expected *Z*-venusol to act like β-adrenergic blockers since it contracts the uterus and causes apoptotic cell death in MCF7s. However, *Z*-venusol, in combination with propranolol, resulted in a significant elevation of cAMP.

The fact that *Z*-venusol did not behave like a β-adrenergic agonist in these breast cancer cells is consistent with previous reports and reveals the multi-faceted nature of expression of cAMP

in various cancer cell lines. Recently, it has been reported that varying β-adrenergic receptor density in various breast cancer cells determine whether there is an increase or a decrease of cAMP when stimulated or blocked with drugs with an affinity for the β-adrenergic receptors. For example, in MCF-7s that are known to have a very low β-adrenergic receptor density, an agonist such as adrenaline has no effect on cAMP levels (Madden *et al*., 2011; Ganz & Cole., 2011). Also, Vázquez and colleagues have reported that epinephrine decreases the levels of cAMP in the MCF-7s through the α_2 adrenergic receptor signalling (Vázquez *et al*., 1999).

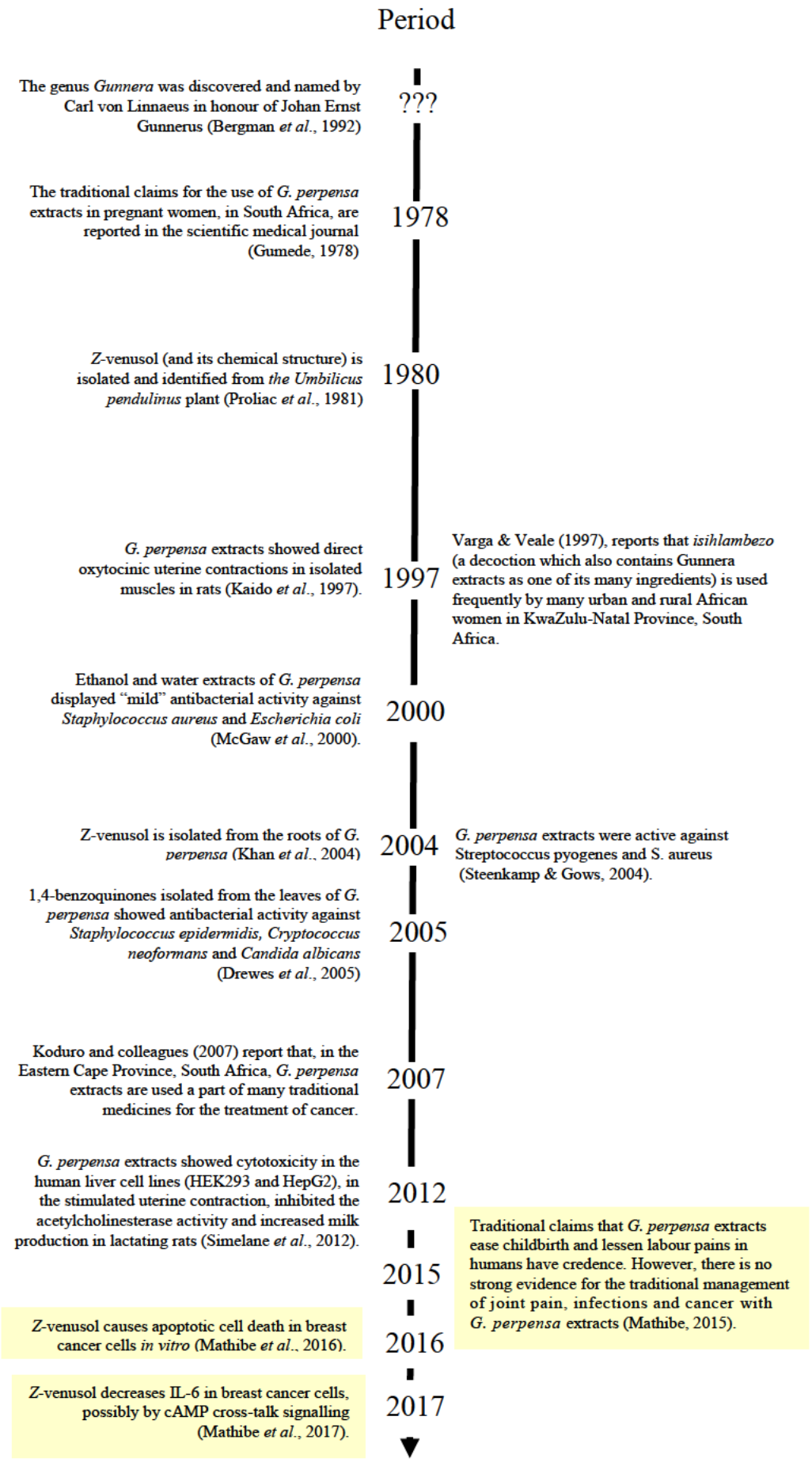
At a clinical level too, various studies have reported conflicting findings with regards to the benefits of β-adrenergic blockers in patients with breast cancer. For example, other researchers have reported no benefits (Shah *et al*., 2011; Sendur *et al*., 2012), while some reported that these drugs do improve survival (Powe *et al*., 2010; Barron *et al*., 2011; Powe *et al*., 2011). Therefore, it is still unclear what role β-adrenergic receptor-stimulation/inhibition and cAMP may have played in apoptotic cell death which was induced by *Z*-venusol in MCF-7s. There may even be "cross talk" (Rezzonico *et al*., 1995; Irvin *et al*., 2001; Hershko *et al*., 2002; Zivadinovic *et al*., 2005) between IL-6, which was inhibited, and cAMP, which was elevated, in this study.

4.2 Time-line for the Discovery of *Z*-venusol

This research provides a significant contribution to the growing knowledge on the use of *G. perpensa*. As can be seen in Figure 4.1, there are many other researcher whose work was used as invaluable stepping stones for the current research. Briefly, Gumede (1978), provided an invaluable record of the maternal use of *G. perpensa* in the scientific literature as far back as 1978 (Gumede, 1978). About ten years later, Kaido and colleagues conducted the first

scientific study and showed that the traditional claims supporting the traditional use of *G. perpensa* extract in pregnant women had some credence (Kaido *et al*., 1997).

Figure 4.1: Time line for recorded traditional usage and basic scientific evidence on the effects of *G. perpensa* extracts and the discovery of *Z*-venusol.



However, a milestone was reached in 2004, when *Z*-venusol was isolated from *G. perpensa* by Khan *et al*. (2004). Also, in 2004, other researchers such as Steenkamp and colleagues as well as Drewes and colleagues reported that *G. perpensa* extracts showed some activity against human pathogens *in vitro* (Steenkamp *et al*., 2004; Drewes *et al*., 2005). The extracts from *G. perpensa* continue to form part of many decoctions and are used widely for traditional management of a variety of ailments in KwaZulu-Natal Province, Africa. However, scientific evidence to support possible benefits of extracts from this indigenous southern African plant against cancer remained tenuous until the current research was carried out and the findings presented at national and international conferences, and published in reputable peer-reviewed South African journals.

4.3 Originality and Methodological Strength of this Study

This was the first study to have investigated the effects of hypoxoside, *ent*-Beyer-15-en-19-ol and *Z*-venusol, pure compounds isolated from indigenous southern African plants called *H. hemerocallidea, H. tenax, G. perpensa,* respectively, in various human cancer and normal cells *in vitro*. The following findings were significant and original:

a) This is the first study to report that *Z*-venusol causes apoptotic cell death in human breast cancer cells.
b) This is the first study to report that *Z*-venusol is less toxic to the primary human mammary epithelial cells (the HMECs) and had no significant effect of proliferation of dMVEC.
c) This is the first study to report that *Z*-venusol inhibits IL-6 activity and that it may act in a "cross-talk" signalling with cAMP in causing apoptotic cell death in MCF-7.

d) This is the first study to report that *ent*-Beyer-15-en-19-ol inhibits proliferation of human cervical cancer cells.

e) This is the first study to report that a widely used decoction, named Re-Joovena™, which claims to contain extracts from *G. perpensa*, has no effect on proliferation of human breast cancer cells.

f) The findings of the present study showed that cell cultures tested negative for mycoplasma contamination and cell viability at the beginning of each experiment was above 98%. This is a measure of how healthy cells are at the beginning of the experiment and cell viability of more than 95% is usually considered acceptable (Abcam Plc., 2016; Gibco, 2016). Mycoplasma contamination negatively affects cell culture (Uphoff & Drexler, 2011). As a result, numerous guidelines, for the use of cell lines in biomedical research strongly recommend that it should not be ignored (Geraghty *et al*., 2014). Therefore, observed effects on proliferation of various human cells, in this study, can be attributed directly to products investigated.

g) Initially, the tetrazolium-based MTT assay was used in this study for the purpose of screening the effects of various compounds on a number of human cancer and normal cells. However, some MTT experiments in this study were not reproducible. Therefore, to strengthen reproducibility of the main findings of this research (McNutt, 2014), subsequent assays were carried out using SRB methodology (Skehan *et al*., 1990). This assay has been reported to be more sensitive and reliable especially with screening of plant-derived compounds in cancer (Sodde *et al*., 2015; van Tonder *et al*., 2015).

h) To ensure reproducibility, parts of this research was carried out at two different institutions of higher learning.

Therefore, it can be concluded that the objectives of this study have, by a large degree, been achieved, notwithstanding for few study limitations which were beyond practical control.

4.4 Study Limitations

a) Research on hypoxoside, *ent*-Beyer-15-en-19-ol was limited by the availability of these compounds.
b) Initial investigations for possible mechanism of action of active compound, *Z*-venusol, was carried-out using a single cell line, MCF-7. Since breast cancer is heterogeneous (Holliday & Speirs, 2011), the conclusions of this study could be considered limited and narrow.
c) The findings on caspase profiling activity were inconsistent and not reproducible. This narrowed the investigation of possible mechanism of action to only two biomarkers, among many.

4.5 Recommendations for Further Research

a) More investigations are needed to assess the effects of *Z*-venusol, e.g.,

    - On other breast cancer cell lines, which are known to cause a more metastatic breast cancer and are difficult to kill, such as the triple-negative MB-231 cell line.
    - Since *Z*-venusol inhibited up-stream mediators (e.g., IL-6), in-depth investigations are needed to assess how it affects downstream mediators such as the STAT3, MAPK, PI3K, Rho kinases and NF-kappaB proteins.

b) *In vivo* studies, such as using the nude mice model, are needed to confirm the *in vitro* results and provide an insight into the benefits of *Z*-venusol in a living systems.

4.6 Recommendations for Change in Public Practice

The findings of the current research, although significant, promising and novel, are, at this stage, adding new knowledge towards the understanding of the effects of substances used for inhibition of cell proliferation, cytotoxicity and the basic science of cancer. They are not yet sufficient enough to:

- Recommend clinical studies to determine the effects of either *Z*-venusol or *G. perpensa* extracts in human beings,
- Encourage consumption/sale of *G. perpensa* extracts or *Z*-venusol compound for the treatment (including the so called "traditional treatment") of cancer.

More importantly, the interpretation of these findings, for now, cannot be extrapolated to clinical or "traditional" use settings.

4.7 Conclusion

The idiosyncrasy and heterogeneity of cancer cells transcends into the clinical setting where experts differ sharply on the progress made with regards to the fight against cancer. Some experts are doubtful (Disis, 2013) while others are very optimistic (Evan, 2015; DeVita & DeVita-Raeburn, 2015) about the benefits of chemotherapeutic agents in the battle against this disease. An enquiry that transcended into the biology of cell death has unearthed many reasons

for one to be optimistic about the prospects of the many indigenous African medicinal plants' place in the frontlines of the war against cancer. However, it is only "quality science", which often requires many years of hard-work and dedication that can turn cynicism, naïve optimism as well as prospects into a reality.

In its own unique way, this study has indeed extended the on-going timeline of unearthing the story of the benefits of *G. perpensa* (in particular). It is anticipated that, in time, the story of *Z*-venusol (isolated from *G. perpensa)* will emulate (Luna *et al*., 2015) that of camptothecin (isolated from *Camptotheca acumenata)*, as was narrated by Wall *et al* (1972), Oberlies & Kroll (2004), and Liu *et al* (2015). There are now firm reasons to believe that this compound (too) will eventually find its place in the list of highly effective and less toxic chemotherapeutic agents used to treat cancer. The objectives of this study have, therefore, not only been fulfilled, but this research has continued where other eminent researcher, in this field have relented.

"The little things we do every day adds up to big breakthroughs" (GENTECH Inc., 2016)

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Appendix A: Copies of Published Peer-Reviewed Papers

Publication 1: A Peer-Reviewed Commentary

South African Family Practice

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The strength of Gunnera perpensa's "evidence of traditional use"

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FORUM

The strength of *Gunnera perpensa*'s "evidence of traditional use"

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In South Africa, and in several countries in the world, several traditional medicines are used often, although their pharmacology is not yet clear and the extent, if any, of their benefit is not scientifically documented. The Australian's "Therapeutic Goods Administration" guidelines for the use of traditional medicines, non-registerable medicines, and other listed medicines, is used in this paper to assess the strength of the traditional-use evidence for the utilisation of *Gunnera perpensa*.

Keywords: evidence-based medicine, *Gunnera perpensa*, Therapeutic Goods Administration, traditional medicines

In the allopathic medicine, indications or therapeutic uses and claims are based on scientific evidence. The evidence-based medicine (EBM) or practice, as was championed by Sackett et al.[1,2] provided a model for "a judicious use of current best evidence in making decisions about the care of individual patients". One of the objectives of EBM is to afford critical methodology for the usage of an ever-growing body of knowledge and to apply the available best evidence in answering clinical and healthcare related questions. This high volume of knowledge, the so called "information overload", necessitated adoption of pyramidal or hierarchical levels, which placed "Systematic Reviews" (SR) or "meta analyses" of "Randomised Controlled Trials" (RCTs) at the top and observational studies and opinions of experts are at the bottom. Cook et al.[3] outlined the following levels of evidence, to be used in grading evidence and in appraisal of research papers:

Level 1: Systematic Review of Randomised Controlled Trials (RCTs)
RCTs with Narrow Confidence Interval
Level 2: Systematic Review Cohort Studies
Cohort Study/Low Quality RCT
Outcomes Research
Systematic Review of Case-Controlled Studies
Level 3: Case-Controlled Study
Level 4: Case Series, Poor Cohort Case Controlled
Level 5: Expert Opinion

However, due to many reasons, which are not the object of this paper, it is still difficult, especially in South Africa, to apply rigorous principles of EBM or "evidenced-based healthcare" (EBHC) to assess the claims made by those who use or sell African traditional and/or complementary medicines. As part of recognising the value of traditional medicines (TMs) and for the fact that TMs are used by many people all over the world, the Australian's "Therapeutic Goods Administration" (TGA) has adopted guidelines to be used for traditional medicines, non-registerable medicines, and other listed medicines.[4]

The TGA defines the "evidence of traditional-use" or "use in traditional practice" as availability of "documentary evidence that a substance has been used over three or more generations of recorded use for a specific health-related or medical purpose", and when "traditional-use has been recorded as an oral rather than written history, the evidence of such should be obtained from the appropriate practitioner or indigenous group(s), who maintain such a history".[4] Thus, various claims could be categorised into "two levels".

Medium Level: Evidence required to support claims include at least TWO of the following:

- medicine is contained in the TGA approved Pharmacopoeia or
- it has TGA approved monograph;
- there are at least THREE independent written histories of use in the classical or traditional medical literature; or
- the medicine is available through any country's government public dispensaries for the indication claimed.

General Level: Evidence required to support the claims include at least ONE of the aforementioned supporting documents/ evidence.

The TGA approved Pharmacopoeia include the Australian Pharmacopoeia, the Aboriginal Pharmacopoeia, The British Pharmacopoeia (BP), the Extra Pharmacopoeia (Martindale), the United State Pharmacopoeia, the European Pharmacopoeia, as well as the World Health Organisation's World Pharmacopoeia.[5] These world acclaimed and TGA approved pharmacopoeias, and the African Pharmacopoeia, which was commissioned by the then Organisation of African Unity in 1984, document very few indigenous South African plants. As a result, the South African Traditional Medicines Research Group (SATMeRG) was established in 1997 to create a truly South Africa pharmacopoeia of indigenous plants – referred to as the "UWC Pharmacopoeia Monograph Project", and the digital version of the African Traditional Medicines is under construction.[6] Therefore, in the absence of a legal framework, in South Africa, to assist family practitioners and many other healthcare providers, whose patients regularly use traditional medicines, the TGA's model might be helpful in assessing the strength of evidence often attributed to many preparations which contain extracts of indigenous South African plants.

Gunnera perpensa, also known as River Pumpkin, *ughobo* or *uklenza* (isiZulu), *ipuzi lomlambo* (isiXhosa), and *qobo* (Sesotho), grows well along the river banks in many parts of South Africa.[6] African traditional healers use it for the treatment of many maternal/foetal ailments, and other conditions such as painful joints, cancerous sores, rheumatism, and psoriasis.[7]

The benefits of traditional use of various decoctions containing *G. perpensa* extracts, in pregnancy, have been reported in scientific literature.[8] However, it was Kaido et al.[9] who confirmed that *G. perpensa* extracts have oxytocic-like effects on an isolated rat uterus. Khan et al.[10] identified *Z*-venusol, a phenylpropanoid glycoside as the major constituent of *G. perpensa*, which caused "state of spontaneous" uterine muscle contractility. Therefore, a "traditional claim" that *G. perpensa* extracts are beneficial has credence and can be catogorised into TGA's "Medium Level". Furthermore, Brookes et al.'s[11] *in vitro* study has reported that *G. perpensa* extracts (up to concentrations of 500 µg/ml) were not toxic to monkey vero cells and human fibroblast cells. However, animals or *in vivo* studies followed by human clinical trials are needed before family practitioners and gynecologists could recommend the use of *G. perpensa*-containing preparations to ease childbirth and labour pains as opposed to what traditional doctors and many 'health shop' keepers are currently doing.

The "traditional-use" of *G. perpensa* extracts as an effective anti-infective agent has been documented.[7] However, Lall and Meyer[12] reported that the highest concentration (5.0 mg/ml) of *G. perpensa* extracts they used was not active against both drug-sensitive and drug-resistant strains of *Mycobacterium tuberculosis*. The crude extracts of *G. perpensa*, and 5 µg/ml concentration (which is less toxic to the peripheral blood mononuclear cells) of *Z*-venusol, one of many active ingredients of *G. perpensa*, failed to inhibit HIV-1 replication in either chronically or acutely infected cells *in vitro*.[13] On the contrary, Drewes et al.[14] found that a 1,4 benzoquinone (isolated from the leaves and stems of *G. perpensa*) inhibited the *in vitro* growth of the human pathogens *Staphylococcus epidermidis*, and *Staphylococcus aureus*. In the study by Koduru et al.,[15] in which information was collected from herbalists, traditional healers, and rural dwellers, *G. perpensa* is reported as one of the medicinal plants used for treatment of cancer in the Eastern Cape Province, South Africa. Iwalewa et al.[16] listed *G. perpensa* among other herbs used in management of joint pain and rheumatism. However, Hutchings et al.[7] were the only original source that supported this claim. More disappointingly, there are neither *in vivo* nor *in vitro* studies that support Hutching et al.'s claim. Therefore, the evidence for traditional-use or for the claims that the extracts or pure compounds extracted from *G. perpensa* are beneficial in the management of joint pain, rheumatism, infections, and cancer are speculative and require more research. Thus, all these claims fall under the TGA's "general level".

In conclusion, one could suggest that 'evidence of traditional use' is equivalent to "expert opinion" level, as used in allopathic or western medicine. In this regard, traditional doctors and individuals who have been using traditional medicines for decades provide "opinions", or their own personal experiences or testimonies of the usefulness of these medicines. However, the disadvantage of an opinion, whether it comes from an expert or from a patient, will, especially in as far as the dominant epistemological demands set by EBM, remain guilty of bias until proven otherwise through an RCT. The landscape for research is still vast, but available evidence of traditional-use should be used as the first step for those who are willing to climb the "pyramid" to place the use of traditional medicines right at the top. It is indeed the responsibility of ALL, including Family Practitioners, who are somehow involved in traditional and complementary medicines, to put ALL claims supported by the "evidence of traditional-use" to the test; in the laboratory (*in vitro*), in animals (*in vivo*), and then in human beings (clinical trials). No medicine, traditional or western, should be parachuted to the top of the "pyramid" before rigorous scientific research is done – we ALL have to work hard and take a long walk way up, even if it seems like being in the wilderness for more than 40 years.

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Publication 2: A Full-Length Original Research Paper

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Z-venusol, from *Gunnera perpensa*, induces apoptotic cell death in breast cancer cells *in vitro*☆

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ABSTRACT

Many African traditional medicines are used although their pharmacology is unclear. In this study, we investigated the *in vitro* anti-tumour effects of pure *Z*-venusol, a compound isolated from an indigenous South African plant called *Gunnera perpensa*. We also compared these to those of a commercially available concoction purported to contain crude extracts of *G. perpensa*.

Pure *Z*-venusol, previously isolated from the roots of *G. perpensa*, was incubated with human breast (the MCF-7 s) cancer cells and human mammary epithelial cells (HMECs). Proliferation was assessed using the sulforhodamine B (SRB) assays. The fluorescein isothiocyanate (FITC) Annexin V and the lactate dehydrogenase (LDH) activity assays were conducted to determine whether cell death, if any, was apoptotic or necrotic. The drugs used as positive controls included cisplatin and camptothecin. Re-Joovena™, a concoction claiming to contain *G. perpensa* (0.3 mg/ml) and *Ocotea bullata* (0.3 mg/ml), was also investigated.

Z-venusol demonstrated a statistically significant, concentration-dependent, apoptotic inhibitory effect on proliferation of MCF-7 cells, with an IC_{50} of 53.7 μg/ml (95% CI, 29.7–96.9.9 μg/ml) after 72 hr exposure, while the highest concentration (250 μg/ml) used resulted in 69% inhibition. The FITC Annexin V and LDH results suggested that apoptosis contributed most of the effect observed. Further, there was insignificant inhibition (20%) of HMEC proliferation observed when the concentration of *Z*-venusol was increased beyond 16.6 μg/ml. The highest concentration used resulted in only 27% inhibition of proliferation of HMEC cells. None of the Re-Joovena™ concentrations tested showed any significant effect. As was expected, cisplatin-induced inhibition of cell proliferation and camptothecin, at lower concentration (0.15 μM), resulted in apoptotic cell death, and at high (≥4 μM) concentrations, it induced necrotic cell death.

Our results suggest that *Z*-venusol is cytotoxic to human breast tumour cells *in vitro*. While some cell death follows a necrotic pathway, the major mechanism of death appears to be apoptosis. The molecular mechanism of the observed effects is currently being investigated, in particular the relationship between *Z*-venusol and β-adrenergic receptors.

1. Introduction

It is estimated that in many Asian and African countries, numerous people rely on traditional medicines for their primary health-care needs (WHO, 2008). However, there is insufficient information regarding the pharmacology, effectiveness, and safety of many unregistered herbal remedies (Street et al., 2008). Nonetheless, these products are available commercially and are advertised aggressively by a variety of means. Often, advertisements of herbal and traditional African medicines placed in mass media are not based on scientific evidence and they "do more harm than good" (Munyaradzi, 2011). Because these products are not properly regulated by law in South Africa, it often falls to the Advertising Standards Authority of South Africa (ASASA) to prevent unsubstantiated promotion of these products [ASASA Ruling, 2014].

Gunnera perpensa, also known as river-pumpkin, *ughobo* or *uklenza* (isiZulu language), *ipuzi lomlambo* (isiXhosa language), and *qobo* (Sesotho language), is one of the many indigenous South African plants whose extracts continue to form part of unregistered herbal products and decoctions used in South Africa (Van Wyk, 1997; Van Wyk, 2008). There are reports suggesting that extracts of *G. perpensa* facilitate speedy and painless vaginal delivery during labour (Varga and Veale, 1997) and that they are also used for the treatment of cancer in the Eastern Cape Province South Africa (Koduru et al., 2007). Recently, it has

☆ Declaration: This manuscript has not been published and is not under consideration in the same or in substantially similar form in any other journal. All those listed as authors are qualified for authorship, and all who qualify to be authors are listed as authors. All listed authors have read and approved the final manuscript. To the best of the authors' knowledge, no conflict of interest, whether financial or others, exists. Funds to conduct the study were received from the University of KwaZulu-Natal, and from the South African NRF (Ref No.: 74267).

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been reported that *G. perpensa* extracts increase milk production in rats and are cytotoxic *in vitro* to both normal human embryonic kidney cells (HEK293) and human liver carcinoma cells (HepG2) (Simelane et al., 2012).

Although extracts are frequently used to investigate the *in vitro* anticancer (Fouche et al., 2008; Steenkamp and Gouws, 2006), antibacterial (Steenkamp et al., (2004), antiviral (Basson, 2005) and antifungal (Ndhlala et al., 2011) effects of plants, very few pure active compounds have been isolated, characterised, and studied. Drewes et al. (2005) found that a 1,4 benzoquinone, isolated from the leaves and stems of *G. perpensa*, inhibited the *in vitro* growth of the human pathogens *Staphylococcus epidermidis* and *Staphylococcus aureus* (Drewes et al., 2005). However, the anticancer potential of *Z*-venusol, a phenylpropanoid glycoside, which has been identified as the major constituent of *G. perpensa*, (Khan et al., 2004), has not been investigated.

Thus, the current study aimed to investigate some fundamental *in vitro* effects of *Z*-venusol on cultured human epithelial breast cancer cells (MCF-7) using normal human mammary epithelial cells (HMECs) as controls. The objectives of the study were to assess the influence of *Z*-venusol on proliferation and, if any, to determine whether *Z*-venusol induced necrosis or apoptosis. Further, we tested the effects of Re-Joovena™ on proliferation of tumour and normal breast cell lines. The common traditional uses of this commercially available concoction, mainly used in KwaZulu-Natal province in South Africa, include traditional treatment of haemorrhoids (*umhluma* in isiZulu language), pregnancy-related complications, painful breasts during menstruation, and the management of many inflammatory disorders (Fig. 1). The label claims that the product contains *G. perpensa* 0.3 mg/ml, *Ocotea bullata* 0.3 mg/ml, as well as unspecified quantities of vitamin E.

2. Material and methods

2.1. Preparation of plant material

Isolation, purification, and characterisation of *Z*-venusol to verify that it was indeed the main active chemical constituent found in the dried roots of the *G. perpensa* were carried out in an assay-guided isolation as reported by Khan et al., (2004). A pure compound, in dry powder form, was supplied by Professor Siegfried Drewes, Department of Chemistry, University of KwaZulu-Natal. The stock solutions of *Z*-venusol powder, and all other powdered drugs used as positive controls, were prepared by dissolving them in dimethyl sulphoxide (DMSO), taking care that the final concentration of DMSO that would be in contact with cells was equal or less than 0.5% (Van Tonder et al., 2014). Aliquots of all stock solutions were stored at −20 °C until used. Cell-specific media were used to dilute the *Z*-venusol stock solutions as well as the Re-Joovena™ on the day of the experiment. The final working concentrations for *Z*-venusol, which were based on unpublished cell viability experiments, were 1.9, 3.9, 7.8, 15.6, 31.3, 65.5, 125, and 250 μg/ml. A similar concentration-range of the Re-Jooneva™ mixture, which was calculated using information on the product label, was also used. After using the GraphPad Prism v5.0 software to determine the IC_{50}, more assays were conducted using the final *Z*-venusol working concentration of 150 μg/ml. The final working concentration for a platinum-based chemotherapy drug used as a positive control, cisplatin, was 4 μM.

2.2. Cell lines, media, and mycoplasma detection

Epithelial human breast cancer cells, the Michigan Cancer Foundation-7 (MCF-7s), and normal human mammary epithelial cells (HMECs) were used. These are well characterised and frequently used in *in vitro* models that investigate the effects of plant extracts on breast cancers (Engel et al., 2014). The MCF-7s were obtained from Highveld Biological, National Repository of the Cancer Association of South Africa, while the HMECs were purchased from the Lonza, Walkerville, USA.

MCF-7 cells were grown in DMEM (Biowhittaker, USA, catalog no. BE12-604 F) supplemented with 10% foetal bovine serum (FBS), 1.0% L-glutamine, and 1.0% Penicillin Streptomycin Amphotericin B® (PSA) and 1.0% sodium pyruvate, purchased from Sigma, St. Louis, USA. HMECs were grown in MEBM (Biowhittaker, USA, cat no. CC-2551) supplemented with 0.2% bovine pituitary extract (BPE), 0.05% human epidermal growth factor recombinant (hEGF), 0.05% human insulin recombinant, 0.05% hydrocortisone, 0.05% gentamycin sulphate amphotericin-B (GA) purchased from BioWhittaker, USA. To determine the yield of healthy cells, we utilised the trypan blue exclusion dye technique. This is a method of determining cell viability, which allows for the direct counting of healthy cells using haemocytometer. In order to control for mycoplasma contamination in the cell cultures,

Fig. 1. The Re-Joovena mixture, purchased at a pharmacy in Umbilo, Durban, South Africa, at R150 (about 15 US $) in March 2014.

the Myco®Alert Mycosplama Detection Kit, purchased from Biotech, was used regularly.

2.3. Sulforhodamine B (SRB) proliferation assay

A conventional SRB assay (Vichai and Kirtikara, 2006) was used to assess the effects of test compounds on proliferation of the human normal and tumour cells. Briefly, cells were harvested and 80 µl of the cell suspension was seeded into 96-well microplates at a density of 2000 cells/well. Cells were then incubated for 24 hr, at 37 °C/5% CO_2 and 100% relative humidity to allow for attachment and acclimatisation. Thereafter, appropriate wells were exposed to various concentrations of Z-venusol, Re-Joovena™, DMSO (less than 0.5%), and 4 µM cisplatin for 24, 48, and 72 hr. Control cells were incubated with media only. At the end of each treatment period, 80 µl of trichloroacetic acid (30%) was added to each well to fix the cells at the bottom of the 96 well plates and were left in the fridge (at 4 °C) for 3 days. Thereafter, the plates were washed three times with distilled water and left to dry in the oven (at 40 °C) for about 2 hr. Thereafter, 100 µl of SRB dye (0.057% in 1% acetic acid) was added to each well and plates were left at room temperature (protected from light) for 30 min. Then plates were washed, three times, with 400 µl of 1% acetic acid and left in the oven (at 40 °C) for about 2 hr. Finally, 200 µl of tris buffer (10 mM, pH 10.5) was added and afterwards plates were placed on the shaker to dissolve the contents of the wells. Absorbance was read using the BioRad microplate reader (Model 3550), at 595 nm. Three independent experiments were carried out in quadruplicate (n = 12) and no outliers were removed.

2.4. The fluorescein isothiocyanate (FITC) Annexin V apoptosis detection assay

The FITC Annexin V apoptosis detection assays (BD Biosciences) were conducted as per manufacturer's protocol. Briefly, MCF-7s were harvested and 80 µl of the cell suspension was seeded at a density of 5×10^4 per well in 12-well culture plates containing 2 ml of medium/well. After 24 hr incubation, test wells were exposed to 150 µg/ml Z-venusol (for 24 hr and 48 hr). Cells were also exposed for 4 hr to an apoptotic concentration (0.15 µM) of camptothecin (Del Bino et al., 1999), which thus served as a positive control. The control wells were not treated, while others contained only medium. Afterwards, treated and untreated cells were detached using 2 ml of Accutase™ cell detachment solution, washed with 2 ml of HBSS, and then suspended in 1X binding buffer solution. Subsequently, 100 µl of cell suspension (with density of not less than 5×10^4 cells) was transferred into 5 ml culture tubes. Thereafter, 5 µl of FITC Annexin V and 5 µl of propidium iodide (PI) dyes were added. These suspensions were analysed using a flow cytometer (the FACS Calibre "JO" E5637), which were verified by an application specialist from the BD Biosciences. Two independent experiments in duplicate were carried out. Regarding interpretation of the scatter plots, cells with intact cell membrane (viable cells) are scattered in the left lower quadrant of the scatter plot. As the process of death begins, cells will take up both dyes to varying extents and the predominant uptake of one dye indicates the particular mechanism of demise; that is, cells undergoing early necrosis absorb only the PI dye and are therefore scattered in the upper left quadrant, while cells undergoing early apoptosis are in the lower right quadrant as the FITC Annexin V dye attaches to the phosphatidylserine (PS) protein exposed to the outer surface of the cell membrane. Dead cells, that is, cells in late apoptosis and/or necrosis, are scattered into the right upper quadrant because they absorb both the PI and the FITC Annexin V dyes. Apoptotic (lower) concentration (0.15 µM) of camptothecin, which was originally isolated from the tree *Campotheca acuminate* by Wall and colleagues (Wall et al., 1966), were used as a positive control.

2.5. The lactate dehydrogenase (LDH) activity assay

The LDH cytotoxicity experiment was done as per manufacturer's (Clontech Laboratories, Inc.) instructions. The LDH enzyme is released to the extracellular environment when the cell membrane breaks. Therefore, the higher the LDH activity, as compared to the untreated cells (control), the more the necrotic cell death. Briefly, cells were harvested and 80 µl were seeded into 96-well plates at a density of 2000 cells/well. After 24 hr incubation, appropriate wells were exposed to 150 µl Z-venusol and necrosis-inducing camptothecin concentration (4 µM) for 4 hr. After centrifuging the plates at 250 *g* for 10 min, 100 µl of supernatant were transferred to corresponding optically clear, flat-bottom 96-well plates. Thereafter, 100 µl of a freshly prepared enzymatic reaction mixture containing diaphorase enzyme and tetrazolium salt was added to each well. After 30 min incubation (protected from light) at room temperature, absorbance was measured at 495 nm using the Biorad Model 3550 microplate reader. Three independent experiments were carried out in quadruplicate (n = 12). Necrotic concentrations (4 µM) of camptothecin were used as positive control.

2.6. Statistical analyses and ethics approval

GraphPad Prism v5.0 (Rv2.13.1 software) was used for statistical manipulations and analyses. To determine the IC_{50} of test compounds, concentrations were converted into $\log_{10}$ values to produce non-linear regression concentration–response curves. To determine whether any observable differences between the treatment group means (with the ± SEM) were statistically significant, either Student's t-tests (for normally distributed data) or Mann–Whitney tests (for non-normal distributed data) were applied. Relevant blanks and vehicle-treated controls (DMSO 0.5% or less) were included. Where appropriate, possible background noise was deducted from experimental values. The protocol was approved by the Biomedical Research Ethics Committee, University of KwaZulu-Natal (UKZN), South Africa (Ref.: BE061/09), and all statistical analyses and interpretations were done with assistance of a qualified UKZN School of Health Sciences' bio-statistician.

3. Results and discussion

3.1. Proliferation assays

The cell cultures showed negative mycoplasma contamination throughout the study and the yield of healthy cells, for both cell lines, ranged from 97.5% to 99.2%. As can be seen in Fig. 2, Z-venusol caused a statistically significant ($p < 0.05$) inhibition of proliferation in human breast cancer cells after 72 hr treatment, with an IC_{50} of 53.7 µg/ml (95% CI 29.7–96.9.9 µg/ml). Inhibition was achieved at an IC_{50} concentration slightly more than that specified by the American National Cancer Institute guidelines, which sets the limit of activity for crude extracts ≤ 30 µg/ml (Suffness and Pezzuto, 1990). The highest Z-venusol concentration (250 µg/ml) used resulted in 69% inhibition of MCF-7 cell proliferation. After 72 hr exposure, there was a modest inhibition (20%) of HMECs proliferation when the concentration of Z-venusol reached 15.6 µg/ml [Fig. 2 (a & b)]. Beyond this concentration of Z-venusol, further increases in concentration did not lead to more inhibition of proliferation. Another plant-derived drug called vinolrebine, which was isolated from *Catharanthus roseus*, is cytotoxic, and is used in cancer chemotherapy, also "plateaus" as the concentration increases beyond a certain point (Bergh et al., 2001). It was, therefore, interesting to note that Z-venusol had an early plateau effect on normal breast cells. Thus, if this product were ever able to be used clinically, it might allow one to increase the concentration for the desired cytotoxic effect while preserving normal cells at higher concentrations.

Regardless of treatment period and concentration, the Re-Joovena™ showed no inhibitory effect on proliferation of tumour cells [the effect after 72 hr is shown in Fig. 2 (c & d)]. One may therefore speculate

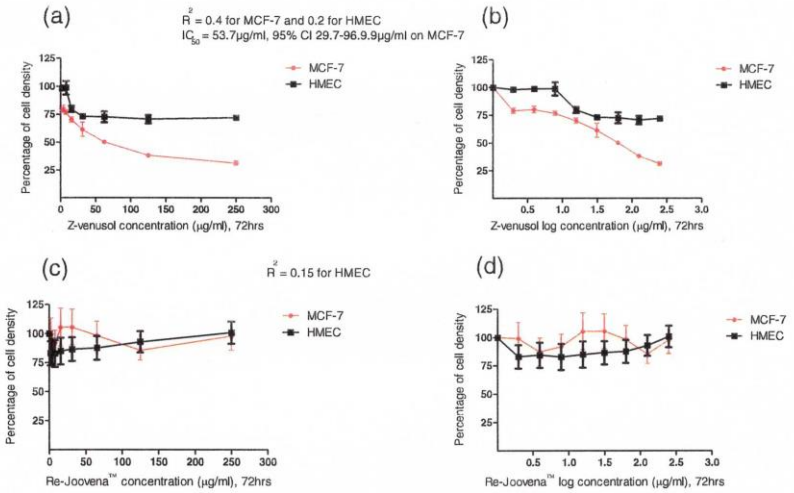

Fig. 2. Cell density (as percentage of control) following 72 hr exposure to Z-venusol (a and b) and Re-Joveena™ (c and d). Concentration–response curves (a and c) and non-linear regression dose–response curves (X axes = $\log_{10}$ concentrations) (c and d). R^2 = co-efficient of determination.

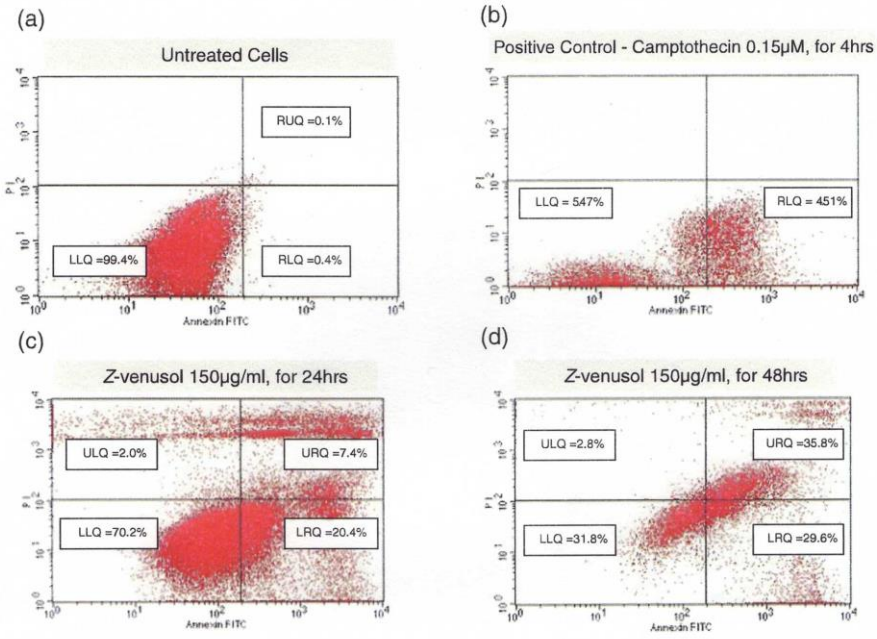

Fig. 3. Scatter plots showing various cells undergoing apoptotic and necrotic cell death after 24 hr and 48 hr treatment with Z-venusol 150 µg/ml and camptothecin 1.5 µM treatment over 4 hr. Cells in the lower left quadrants (LLQs) are viable; cells in the lower right quadrants (LRQs) are showing early apoptosis as FITC Annexin V binds to the phosphatidylserine (PS) protein exposed to the outer surface of the cell membrane; cells in the upper left quadrants (ULQs) are showing necrosis as cells absorb propidium iodide (PI) due to breakdown of cell membrane; and cells in upper right quadrants (ULQs) are showing late apoptosis.

that either the Re-Joovena™ mixture does not contain *G. perpensa* extracts or the quantity as specified on the label is incorrect. It is also possible that active ingredients of *G. perpensa* were destroyed during manufacturing process of Re-Joovena™. As Zschocke and colleagues have reported, some active volatile ingredients of medicinal plants such as *Ocotea bullata* lose their potency during traditional methods of extraction (Zschocke et al., 2000). However, there was some induction of proliferation of normal breast cells seen after 24 hr and 48 hr of treatment. Strangely, this growth advantage was conferred only on normal cells and not on tumour cells. As expected, our positive control, cisplatin, showed significant cytotoxicity to both normal (after 72 hr treatment only) and tumour cells, after 48 hr and 72 hr exposure.

Since there were no similar studies for direct comparison, we chose to relate our result to other studies which assessed the effects of *G. perpensa*'s crude extracts on other cell lines. As expected, the pure compound *Z*-venusol showed a greater cytotoxic effect on MCF-7s than what Simelane et al. (2012) reported for crude extracts of the same plant in human hepatocellular carcinoma cell (HepG2). They reported that *G. perpensa* extracts showed inhibition (IC_{50}) of 222.33 μg/ml and 279.43 μg/ml on the HepG2s and the human embryonic kidney 293 (HEK293) cells after 48 hr of treatment, respectively (Simelane et al., 2012).

3.2. Apoptosis assays

As shown in Fig. 3c and d, *Z*-venusol induced cell death by apoptotic pathway, which was similar to that of the positive control, 0.15 μM camptothecin (Fig. 3b). Our results indicate that observable process of cell death had begun after 24 hr (Fig. 3c, LRQ) and 48 hr (Fig. 3d, LRQ) of exposure. Similarly, in support of our SRB proliferation assay, approximately only 7% (Fig. 3c, URQ) and 35% (Fig. 3d, URQ) of cells were dead after 24 and 48 hr, respectively. The LDH activity assay (Fig. 4) also indicated that most of the observed cell deaths were not by necrosis, as there was no statistically significant difference in LDH activity between cells treated with *Z*-venusol and untreated cells.

Cells death has for many years been divided into two pathways: apoptosis and necrosis (Bonfoco et al., 1995). Apoptosis is a programmed cell death and was for many years considered as a "good way" to die; necrotic cell death, however, was considered as passive, "messy," and as a "bad-way" to die (Nanji and Hiller-Sturmhöfel, 1997; Sun and Peng, 2009). However, other studies have reported on regulated or programmed necrotic cell death, or necroptosis (Sperandio et al., 2000; Wyllie and Golstein, 2001). Recently, Newton et al. (2014) have reported that both necrotic and apoptotic cell-death pathways involving the receptor-interacting protein kinase 3 (RIPK3) intersect, and that disturbing one pathway can stimulate death by the other (Newton et al., 2014; Zhang and Chan, 2014). Similarly, in our study, it would appear that *Z*-venusol can cause cells to die by both necrosis and apoptosis, the latter being the predominant mechanism.

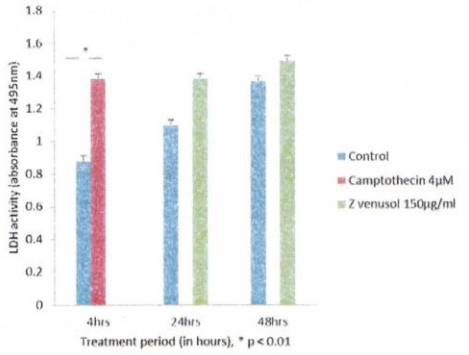

Fig. 4. Lactate dehydrogenase (LDH) activity in MCF-7 cells after 24 hr, 48 hr treatment with *Z*-venusol 150 μg/ml and necrotic concentration (4 μM) of camptothecin for 4 hr. The LDH enzyme is released to the extracellular surface when the cell membrane breaks. Therefore, the higher the LDH activity, as compared to the untreated cell (control), the more the necrotic cell death.

The present study is the first to report that *Z*-venusol inhibits proliferation of cancer cells *in vitro* and to propose the main pathway by which it kills the cells. Accordingly, there are no direct studies with which to compare our findings. As yet, there is no clear explanation for the molecular mechanism of the effects observed. However, there are various receptors which are known to be involved in cancer cell proliferation and which may be affected by plant extracts. Taylor and colleagues have reported that oxytocin induces breast cancer cell proliferation (Taylor et al., 1990) and oxytocin receptor (OTR) mRNA was demonstrated in the majority (80%) of breast lesions using reverse transcription-polymerase chain reaction (Bussolati et al., 1996). Since aqueous extracts of *G. perpensa* have been shown to produce oxytocin-like contractions of the uterus (Kaido et al., 1997), one might expect that, if it were working on oxytocin receptors, *Z*-venusol would promote proliferation rather than inhibit it as was observed in the present study.

There is also a suggestion that COX-2 over-expression may be associated with carcinogenesis (M´eric et al., 2006); over-expression has been observed in carcinomas of human breast, colon, and lung (Soslow et al., 2000), prostate (Fujita et al., 2002 & Edwards et al., 2004), brain (Bodey et al., 2006), uterus and cervix (Fujimoto et al., 2006). Since extracts of *G. perpensa* contract the uterus, an effect associated with prostaglandins (PGs) and over-activity of COX-2 receptors, it is unlikely that the inhibitory effects of *Z*-venusol in our experiments are linked to PGs.

It is also possible that neither oxytocin nor PGs, but the blockade of β-adrenergic receptors, is responsible for the cancer cell inhibition seen in our experiments, and the uterine contractions observed, by others, with extracts of *G. perpensa*. Blockade of β-adrenergic receptors is known to cause uterine contraction (Bardon and Stander, 1968 & Kashanian et al., 2008), and recently, β-adrenergic receptors blockers, such as propranolol, have been reported to improve the outcomes in breast cancer patients when taken concurrently with chemotherapy (Barron et al., 2011 & Pasquier et al., 2011). Thus, at present, we are exploring possible involvement of β-adrenergic receptors in the effects of *Z*-venusol observed in this study.

4. Conclusion

Z-venusol was found to inhibit the proliferation of the human breast cancer cells *in vitro* in a concentration-dependent manner, while there was limited toxicity to normal human breast cells. Cell death was by both necrosis and apoptosis but mainly the latter. Constituents of Re-Joovena™, at the concentrations studied, showed no effect on either normal or tumour breast cell lines. Our findings are largely descriptive, and much extensive work, such as the assessment of the exact apoptotic pathways triggered by the *Z*-venusol, still needs to be done.

5. Authors' contribution

LJM conceived and participated in the design of the study. He also carried out all the assays, performed the statistical analysis and interpretation of the results, and drafted the manuscript. JB & SN helped with the study design, analysis, and interpretation of the results and also performed substantial corrections of the draft manuscript.

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Effects of *Z*-venusol, isolated from *Gunnera perpensa* L., on IL-6 and cAMP activity in human breast cancer cells *in vitro*☆

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Mechanism of action

ABSTRACT

Medicinal plants continue to play an important role in the management of many diseases, including cancer. While numerous scientific studies have reported on the effects of crude extracts, very few have isolated and investigated pure-compounds from indigenous South African plants. Isolation and studying of pure compounds extends the exploration of traditional medicines in that, once the chemical structure of the active compounds is known, changes can be made to synthesise derivatives with enhanced effectiveness and/or fewer adverse effects. This study, reports on the effects of *Z*-venusol, a phenylpropanoid glycoside which was isolated from an indigenous South African plant, *Gunnera perpensa* L., on interleukin-6 (IL-6) and on cyclic adenosine monophosphate (cAMP) activity in the breast cancer cells *in vitro*.

Various concentrations of pure *Z*-venusol, previously isolated from the roots of *G. perpensa* L., were incubated for 48 h with human breast cancer cells (MCF-7s). Conventional IL-6 and cAMP activity assays were used as per manufacturers' protocols. Drugs used as positive controls included pitovastatin (which was expected to inhibit IL-6 activity) and epinephrine and propranolol, which were expected to increase and decrease cAMP levels respectively.

The highest concentration (150 μg/mL) of *Z*-venusol used resulted in 51% inhibition of IL-6 activity in MCF-7s ($p < 0.01$). None of the *Z*-venusol concentrations, either alone or in combination with epinephrine, an agonist at adrenergic receptors, showed any statistically significant effect on cAMP levels. Surprisingly, there was a 34% elevation of cAMP levels ($p \leq 0.028$) in cells which were exposed to a combination of 150 μg/mL *Z*-venusol and propranolol 10 μM. We hypothesise that low β-adrenergic receptor signalling in MCF-7 cells, as reported in the literature, may explain our peculiar findings.

The previously reported apoptotic cell death, caused by *Z*-venusol, may be related to inhibition of IL-6. A possible role for cAMP is less clear although there may be "cross talk" between it and IL-6. More investigations are needed to confirm our findings and establish which other mediators may also be involved.

1. Introduction

Anti-cancer drugs initially discovered from plants include vinblastine (isolated from *Catharanthus roseus* L.), etoposide (isolated from *Podophyllum peltatum* L.), paclitaxel (isolated from *Taxus brevifolia* Nutt.), topotecan and camptothecin (isolated from *Camptotheca acumenata* Decne.). Therefore, medicinal plants continue to play a role in the management of cancer.

Cancer growth can be viewed as cell development and evolution that went astray (Komarova, 2015). Generally, there is fatal, uncontrollable proliferation and spread of the body's own malignant cells. The WHO's International Agency for Research on Cancer has reported that cancer is one of the leading causes of death worldwide with about 14.2 million new cancer cases and 8.2 million cancer deaths occurring in 2012 worldwide. Breast cancer continues to be the most common cancer in women, while lung cancer, which is the most common cancer among males, accounts for the most deaths due to any cancer type globally (GLOBOCAN, 2012). Although various combinations of drugs increase survival rates in patients with cancer, many chemotherapeutic agents are expensive and cause severe side-effects which are also costly to manage (Ubel et al., 2013; Meisenberg, 2015). Therefore, globally, there is a need for cheaper, more effective and less toxic medicines for the treatment of cancer (Kawalec et al., 2015).

Plant extracts are frequently used to investigate the *in vitro* anti-cancer (Fouche et al., 2008), antibacterial (Steenkamp and Gouws, 2006) and antifungal (Ndhlala et al., 2011) effects of plants, but very

☆ Declaration: This manuscript has not been published and is not under consideration in the same or in substantially similar form in any other journal. All those listed as authors are qualified for authorship, and all who qualify to be authors are listed as authors. All listed authors have read and approved the final manuscript. To the best of the authors' knowledge, no conflict of interest, whether financial or other, exists. Funds to conduct the study were received from the University of KwaZulu-Natal and from the NRF (Ref No.: 74267).

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0254-6299/

few pure active compounds have been isolated, characterized and studied (Rates, 2001). The advantages of assessing pure and characterized compounds, whose molecular structure is known are numerous. For example, the chemical structures can be altered to synthesize more pharmacologically-active derivatives. This occurred with the Nobel Prize winning discovery and isolation of an antimalarial drug called artemisinin from an indigenous Chinese plant, *Artemisia annua* L. (Normile, 2015), as well as with camptothecin, an anticancer drug (Wall et al., 1972; Oberlies and Kroll, 2004; Liu et al., 2015). Thus, recently it was reported that *Z*-venusol, a phenylpropanoid alkaloid pure compound, isolated from an indigenous southern African plant known as *Gunnera perpensa* L., also known as River Pumpkin, *ughobo* or *uklenza* (isiZulu language), *ipuzi lomLambo* (isiXhosa language) and *qobo* (Sesotho language), causes controlled cell-death in tumour cells, while being less toxic to normal human mammary epithelial cells *in vitro* (Mathibe et al., 2016). However, the exact molecular mechanism of action, among a multitude of possible pathways, by which *Z*-venusol causes controlled-cell death is not known. Therefore, in this study we investigated the effects of this compound on cyclic adenosine monophosphate (cAMP) and interleukin-6 (IL-6) activity in MCF-7 breast cancer cells *in vitro*. Cyclic adenosine monophosphate is reported to be upregulated in cancer cells, both *in vitro* and *in vivo* (Lang et al., 2004; Powe et al., 2011). We also chose cAMP because of the traditional use of *G. perpensa* in causing uterine contraction (Kaido et al., 1997; Varga and Veale, 1997). As β-adrenergic agonist action relaxes the uterus and increases cAMP, *Z*-venusol may possibly contract the uterus by blocking an epinephrine stimulated increase in cAMP. Also, propranolol, a β-adrenergic blocker, has been shown to improve survival in patients with breast cancer (Pasquier et al., 2011). Therefore, we expected that *Z*-venusol may decrease cAMP and behave like a β-adrenergic blocker since it contracts the uterus and causes apoptotic cell death in MCF-7s (Mathibe et al., 2016).

We also investigated the effects of *Z*-venusol on interleukin-6 (IL-6) activity in MCF-7 cells *in vitro*. Interleukin-6 is an inflammatory-associated cytokine strongly linked to different features of cancer biology like metastasis and decreased survival rates (Bachelot et al., 2003; Knüpfer and Preiss, 2010). Traditional uses of extracts of *G. perpensa* L. include inflammatory conditions such as rheumatoid arthritis and haemorrhoids.

2. Material and methods

2.1. Preparation of plant material

Isolation, purification and characterization of *Z*-venusol to justify that it was indeed the main active chemical constituent found in the dried roots of the *G. perpensa* L., was carried out in an assay-guided isolation as reported by Khan et al. (2004). Pure compound, in dry powder form, was supplied by Professor Siegfried Drewes, Department of Chemistry, University of KwaZulu-Natal. Stock solutions of *Z*-venusol powder, and all other powdered drugs used as positive controls, were prepared by dissolving them in dimethyl sulphoxide (DMSO), taking care that the final concentration of DMSO to be in contact with cells was equal or less than 0.5% (Van Tonder et al., 2014). Aliquots of all stock solutions were stored at −10 °C until used. Cell specific media were used to further dilute the *Z*-venusol 150 μg/mL stock solutions on the day of the experiment. The cells were exposed for 48 h to various working concentrations *Z*-venusol; that is, 37.5 and 75 μg/mL, which were based on unpublished pilot cell viability experiments, and at the end of the exposure time, supernatants (*i.e.*, samples) were store at −10 °C until used.

2.2. Cell line and medium

Epithelial human breast cancer cells, the Michigan Cancer Foundation-7 (MCF-7s), obtained from Highveld Biological, National Repository of the Cancer Association of South Africa, were used. These cells were grown in DMEM (Biowhittaker, USA) supplemented with 10% foetal bovine serum (FBS), L-glutamine and Penicillin Streptomycin Fungizone® (PSF) and 0.1% sodium pyruvate, all purchased from Sigma, St. Louis, USA.

2.3. Human interleukin-6 (IL-6) activity assay

The human IL-6 sR ELISA kits (Lot # 0317C0367), purchased from Sigma, were used as per manufacturer's protocol. Briefly, all reagents were brought to room temperature. Thereafter, 100 μL of each standard and sample was added into appropriate wells coated with human IL-6 sR antibodies, covered and incubated at 37 °C/5% CO_2 and 100% relative humidity. On the following day, solutions were discarded and each well was washed 4 times with 300 μL of 1× Wash Solution. Then, 100 μL of prepared biotinylated detection antibody solution was added to each well and incubated on the gentle shaker at room temperature for 1 h. Thereafter, the solution was discarded and each well was washed with 1× Wash Solution as described above. Then, 100 μL of prepared HRP-Streptavidin solution was added to each well, and incubated with gentle shake at room temperature for 45 min. Thereafter, the solution was discarded and each well was washed with 1× Wash Solution as described above. Then, 100 μL of ELISA colourimetric tetramethylbenzidine (TMB) reagent was added to each well, and incubated in the dark with gentle shake at room temperature for 30 min. Finally, 50 μL Stop Solution was added to each well and absorbance was read using the BioRad (Model 3550) microplate reader, at 595 nm wavelengths. The highest final working concentration for a drug used as a positive control, pitovastatin, was 1.5 μM (Wang and Kitajima, 2007). Three independent experiments were carried out in quadruplicate (n = 12) and outliers were removed.

2.4. The direct acetylated cyclic adenosine monophosphate (cAMP) assay

The direct cAMP enzyme immunoassay kits (Lot # SLBM17230), purchased from Sigma were used as per manufacturer's protocol. Briefly, all reagents were thawed to room temperature. The acetylation reagent (or solution) was prepared by adding 0.5 mL of acetic anhydride to 1 mL of triethylamine. Subsequently, 10 μL of acetylation solution was added to every 200 μL of standard and sample solutions to enhance the detection of cAMP activity, because there is a low expression of β-adrenergic receptors in MCF-7s (Madden et al., 2011). Various concentrations (0.078, 0.312, 1.25, 5 and 20 pmol/mL) of standard solution were prepared by serial dilution of cAMP solution with 0.1 M HCl. Then, 50 μL of neutralising reagent was added into each well of the 96-well-plate coated with goat anti-rabbit IgG antibodies. Thereafter, 100 μL of standard solutions and 100 μL of sample solutions added to appropriate wells. This was followed by addition of 50 μL of the blue cAMP-Alkaline Phosphatase Conjugate into appropriated wells and inoculation of 50 μL of the yellow cAMP EIA antibody solution. Then, the plate was incubated on a gentle shaker at room temperature for 2 h. Thereafter, the contents were emptied and the wells were washed three times with a freshly prepared 10% wash buffer solution. Then, 200 μL of the p-nitrophenyl phosphatase conjugate substrate solution was added to each well and thereafter the plate was incubated at room temperature, protected from light, for 1 h. Finally, 50 μL Stop Solution was added to each well and absorbance was read using the BioRad (Model 3550) microplate reader, at 415 nm wavelengths. The measured optical intensity is inversely proportional to the concentration of cAMP in either the samples or the standards. Epinephrine (10 μM) and propranolol (10 μM), both purchased from Sigma, were used separately, in combination and added to the highest concentration of Z-venusol for comparison (Kelly et al., 2009; Madden et al., 2011). Four independent experiments were carried out in duplicates (n = 8) and five outliers (representing 5.2% of the data), which could not fit into the standard curve were removed.

2.5. Statistical analyses and ethics approval

GraphPad Prism v5.0 with the freeware package R v2.13.1 was used for statistical manipulations and analyses. Relevant blanks and vehicle-treated controls (DMSO 0.5% or less) were included. Where appropriate, possible background noise (the mean) was deducted from experimental values. The protocol was given full ethics approval by the Biomedical Research Ethics Committee, University of KwaZulu-Natal, South Africa (Ref.: BE061/09).

3. Results and discussion

The cell cultures showed no contamination by mycoplasma throughout the study and the yield, of viable cells, ranged from 98.5% to 99.0%. As can be seen in Fig. 1, the highest concentration of Z-venusol used in this study, and the pitovastatin which was used as a positive control, resulted in a statistically significant ($p < 0.01$) 51% inhibition of IL-6 activity in the MCF-7 after 48 h exposure. As IL-6 is a pro-inflammatory cytokine (Schaper and Rose-John, 2015), we had hypothesised that its inhibition may be responsible for the alleged effects of *G. perpensa* L. in the traditional treatment of inflammatory conditions such as haemorrhoids (Hutchings et al., 1996; Mathibe et al., 2016). High activity and expression of IL-6 is associated with proliferation of breast cancer cells (Adams et al., 1991; Jiang et al., 2011; Guo et al., 2012) and is also involved in tumorigenesis (Bachelot et al., 2003). Thus the fact that it was suppressed in this experiment indicates that the apoptotic effects of Z-venusol in MCF-7, as reported recently (Mathibe et al., 2016), may be related to the suppression of IL-6 activity. However, as Hanahan and Weinberg (2000) have demonstrated that IL-6 is involved at the initial stages of cell demise, the exact downstream pathway may involve other mediators such as the Rho kinases, NF-kappaB (Wang and Kitajima, 2007), or the cAMP signalling "cross talk" described by Irvin et al. (2001). Therefore, more studies are needed to investigate whether Z-venusol has any effect on other downstream mediators and proteins.

One would expect that epinephrine, a β-adrenergic agonist, would increase cAMP. However, the fact that it did not do so in this experiment (Fig. 2) is consistent with previous reports and probably involves the complex nature of expression of cAMP in cancer cells. It has recently been reported that in breast cancer cells with a very low β-adrenergic receptor density, such as MCF-7s, an agonist, such as epinephrine (adrenaline), has no effect on cAMP levels (Ganz and Cole, 2011; Madden et al., 2011). It has also been reported, by Vázquez et al. (1999), that epinephrine decreases the levels of cAMP in the MCF-7 cells *via* the α_2 adrenergic receptor signalling. This would be consistent

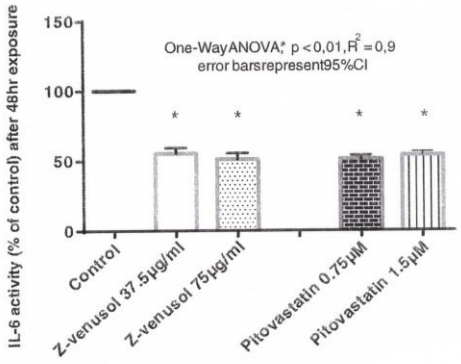

Fig. 1. IL-6 activity (absorbance at 595 nm wavelengths) of breast cancer cells (MCF-7s) after 48 h exposure to Z-venusol and pitovastatin. Overexpression and activity of IL-6 is associated with cell proliferation and tumorigenesis (Jiang et al., 2011; Guo et al., 2012).

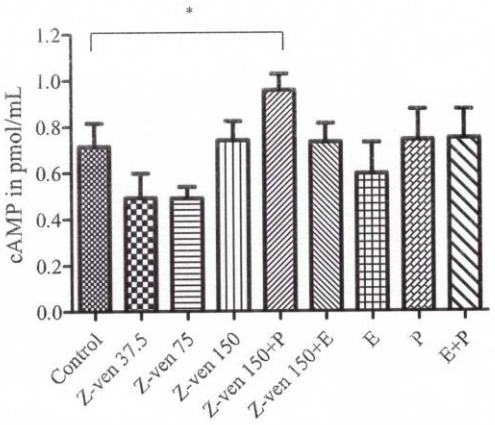

Fig. 2. cAMP activity (amount in pmol/mL) in MCF-7 breast cancer cells exposed to Z-venusol (37.5, 75 & 150 µg/mL), Z-venusol 150 µg/mL + propranolol 10 µM (P), Z-venusol 150 µg/mL + epinephrine 10 µM (E), epinephrine 10 µM (E), propranolol 10 µM and epinephrine 10 µM + propranolol 10 µM (EP) for 48 h. *$p \leq 0.028$.

with our findings and further investigation is needed to find out whether Z-venusol acts through these receptors as well.

Likewise, at a clinical level, the relationships between various cancer cells and β-adrenergic agonists, antagonists and cAMP are also complex. Various studies report conflicting results in terms of the benefits of β-adrenergic blockers in patients with breast cancer (Powe et al., 2010; Barron et al., 2011; Powe et al., 2011; Sendur et al., 2012). These contradictions are mainly due to the fact that an induction or inhibition of cAMP expression can cause proliferation or apoptosis of cancer cells because of divergent β-adrenergic receptor density (Brent and Pang, 1995; Zivadinovic et al., 2005; Follin-Arbelet et al., 2011; Madden et al., 2011; Pasquier et al., 2011). In our study, propranolol was without effect. Although low concentrations of Z-venusol decreased cAMP ($p > 0.05$), a combination of the highest concentration with propranolol caused a 34% elevation in cAMP levels ($p \leq 0.028$). So it is unclear what role, if any, cAMP may play in our previously observed apoptosis in MCF-7s. There may even be "cross talk" between IL-6, which was inhibited in our study, and cAMP (Irvin et al., 2001).

4. Limitations of this study

In this study, only positive controls for endpoints measured were included. However, in our recently published study (Mathibe et al., 2016), cisplatin and camptothecin (at high concentrations) were used and were shown, as expected, to induce apoptosis and necrosis respectively.

5. Conclusion

This is the first study to report on, and provide initial evidence for, a possible molecular mechanism for the apoptotic cell death caused by Z-venusol in MCF-7s. While it seems likely that inhibition of IL-6 is involved, the role of cAMP is less certain and more investigations are needed to further clarify the complex pathways involved.

Authors' contributions

LJM conceived of the study. He also carried out all the assays, performed the statistical analysis, interpretation of the results and drafted the manuscript. *JB & SN* helped with study design, analysis

and interpretation of the results and performed substantial corrections to the draft manuscript.

Conflict of interest statement

The authors declare that there is no conflict of interest in this research.

Acknowledgements

We are indebted to Professor Drewes for providing us with pure Z-venusol powder which was used in this study; thank you to the UKZN and the South African NRF for funding part(s) of this project.

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Appendix B: Ethics and Postgraduate Committee Approval

UNIVERSITY OF
KWAZULU-NATAL

08 December 2009

Dr S Naidoo
Department of Therapeutics & Medicines Management
Nelson R Mandela School of Medicine

naidoot@ukzn.ac.za

Dear Dr Naidoo

PROTOCOL PhD:"The effects of certain indigenous South African plants on tumorigenesis and anglogenesis" (Therapeutics & Medicines Management) L.J. Mathibe, Student number 200500883.

The Postgraduate Education Committee ratified the approval of the abovementioned study on 08 December 2009

Please note:

- The Postgraduate Education Committee must review any changes made to this study.
- The study may not begin without the approval of the Biomedical Research Ethics Committee.

May I take this opportunity to wish the student every success with the study.

Yours sincerely

[redacted]

Professor SJ Botha
Chair Postgraduate Education Committee

Cc Rev L J Mathibe
mathibel@ukzn.ac.za

Ms D Ramnarain
Biomedical Research Ethics Committee
Westville Campus

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Website: http://research.ukzn.ac.za/ResearchEthics11415.aspx

15 July 2009

Rev. L Mathibe
Department of Therapeutics & medicines Management
Nelson R Mandela School of Medicine

Dear Rev Mathibe

PROTOCOL: The effects of certain indigenous South African plants on tumorigenesis and angiogenesis. REF: BE061/09

EXPEDITED APPLICATION - RATIFICATION

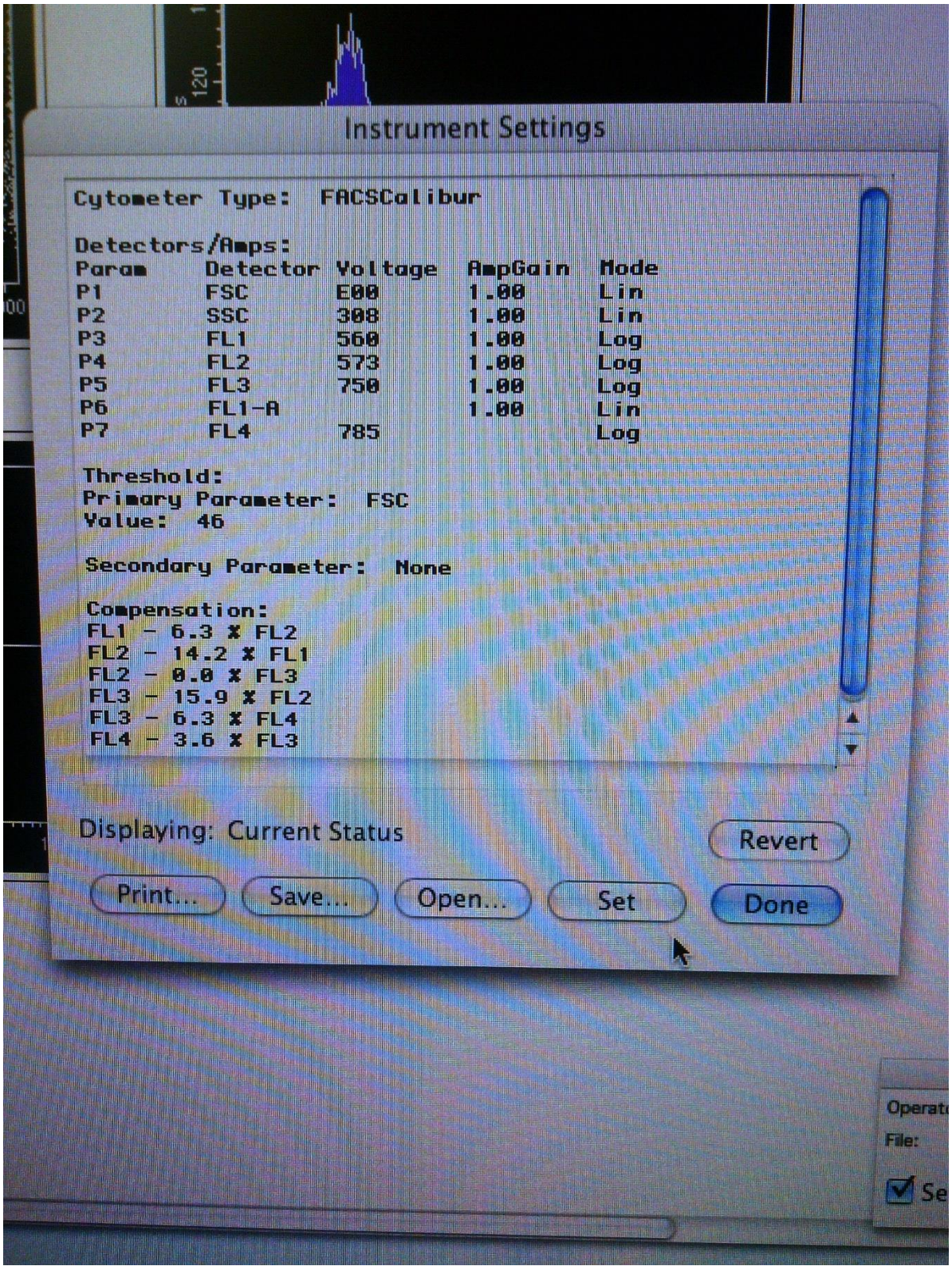
This letter serves to notify you that at a full sitting of the Biomedical Research Ethics Committee meeting held on **14 July 2009**, the Committee **RATIFIED** the sub-committee's decision to approve the above study.

Yours sincerely

[redacted]

Ms D Ramnarain
Senior Administrator: Biomedical Research Ethics
DR/dl

Appendix C: FACS Calibur Instrument Settings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Cytometer Type: FACSCalibur
Detectors/Amps:
Param Detector Voltage AmpGain Mode
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P5 FL3 750 1.00 Log
P6 FL1-A 1.00 Lin
P7 FL4 785 Log
Threshold:
Primary Parameter: FSC
Value: 46
Secondary Parameter: None
Compensation:
FL1 - 6.3 % FL2
FL2 - 14.2 % FL1
FL2 - 0.0 % FL3
FL3 - 15.9 % FL2
FL3 - 6.3 % FL4
FL4 - 3.6 % FL3
Displaying: Current Status
Revert
Print...
Save...
Open...
Set
Done