In vitro evaluation of the efficacy of selected medicinal plant extracts against multidrug resistant cancer cells

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Die kinders van God.

1 Johannes 3:1

Kyk wat ‘n groot liefde die Vader aan ons bewys het, dat ons kinders van God genoem kan word! Om hierde rede ken die wêreld ons nie, omdat dit Hom nie geken het nie.

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Abstract

Cancer is ranked as one of the leading causes of death globally, and a significant number of these diagnosed malignancies are found in lung tissue. Small cell lung cancer (SCLC) is a high grade neuroendocrine cancer, which is responsible for high mortality rates worldwide. The failure of therapeutic regimes in cancer management can be ascribed to increased cancer metastasis and the occurrence of multidrug resistance (MDR). MDR in SCLC is often the result of hyperexpression of several adenosine triphosphates (ATP)-binding cassette (ABC) efflux transporters in these tumours, which can decrease the intracellular accumulation of chemotherapeutic drugs, resulting in sub-therapeutic levels. P-glycoprotein (P-gp), multidrug resistance-associated protein 1 (MRP1) and breast cancer resistance protein (BCRP) are some of the most widely studied efflux transporters involved in cancer MDR.

There is an urgent need to identify novel treatment approaches to combat MDR in cancer, and various traditionally used medicinal plants are believed to cure, prevent or manage cancer. In this study, *Aloe vera* gel material and precipitated polysaccharides, *Sutherlandia frutescens* and *Xysmalobium undulatum* were investigated as an ethno-medicinal approach to combat MDR in cancer. This was done through evaluation of their potential *in vitro* anticancer efficacy against selected chemosensitive and chemoresistant SCLC cell models. These SCLC cell models included a chemosensitive line (H69V), a multidrug resistant line with hyperexpressed MRP1 efflux transporters (H69AR), as well as the multidrug resistant NCI-H69/LX4 line with hyperexpressed P-gp transporters. A porcine kidney non-tumorigenic cell line (LLC-PK1) was also included to evaluate the cytotoxic effects of the selected plant materials.

The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay was used as a preliminary screening method to determine the relative reduction in cell viability (IC) of each selected SCLC cell line, after a 96 h exposure to the various plant materials. Subsequently, the effect of the selected plant materials on intracellular ATP and extracellular adenylate kinase (AK) levels of the different SCLC and LLC-PK1 cells were measured to establish their anticancer efficacy and cytotoxic potential more accurately.

All the selected plant materials investigated in this study resulted in a statistically significant reduction in cell viability for all of the SCLC cell lines ($p<0.05$), although a measure of resistance was observed in the chemoresistant cell lines. The anticancer phytochemicals in the crude extracts are therefore most probably substrates for MRP1 and P-gp related efflux. The aqueous *S. frutescens* extract was found to possibly induce necrosis in the MRP1 hyperexpressive SCLC cell line at 1.406 mg/ml, while the MTT data suggested that *S. frutescens* extract had the highest cancer selectivity ratio in P-glycoprotein (P-gp) hyperexpressive SCLC management of all the materials investigated. The

cytotoxic effect of the *X. undulatum* extract on non-cancerous cells (LLC-PK1 cell line) contradicts its use in SCLC management.

The selected medicinal plant materials significantly altered both the intracellular ATP and extracellular AK levels of the chemosensitive and chemoresistant SCLC cell lines, indicating anticancer effects against SCLC cells. However, indications of cytotoxicity to some extent were also detected in a non-cancerous LLC-PK1 cell line for all of the plant materials.

The affinity of phytochemicals in the plant materials for the efflux transporters may be put to use though combination with standard anticancer drugs with an affinity for these efflux transporters. The phytochemicals may enhance intracellular drug accumulation by saturating the transporter binding sites, or competing with the standard drugs for binding.

Key words:

ABC efflux transporters, *Aloe vera,* anticancer efficacy, cytotoxicity, herbal medicine, multidrug resistance, small cell lung cancer, *Sutherlandia frutescens, Xysmalobium undulatum*.

Opsomming

Kanker word universeel beskou as een van die hoofoorsake van sterftes, waarvan ’n baie groot persentasie diagnoses toegeskryf word aan longkanker. Klein sel longkanker (KSLK), ’n ernstig neuro-endokriene kanker, is veral verantwoordelik vir ’n hoë aantal sterftes wêreldwyd. Swak terapeutiese uitkomstes tydens kanker behandeling kan toegeskryf word aan verhoogde kanker metastase, asook veelvoudige geneesmiddel weerstandbiedendheid (VGW). VGW in KSLK word merendeels geassosieer met die verhoogde uitdrukking van verskeie adenosien trifosfaat (ATP)-bindingskasset (ABK) efluks pompe in hierdie tipe karsinoom, wat daartoe lei dat sub-terapeutiese geneesmiddel vlakke bereik word weens die verlaagde intrasellulêre akkumulasie van chemoterapeutiese middels. In terme van VGW in kanker is P-glikoproteïen (P-gp), veelvoudige geneesmiddel weerstandbiedendheid-geassosieerde proteïen 1 (MRP1) en bors kanker weerstandbiedendheidsproteïen (BKRP) van die efluks pompe wat al die meeste ondersoek is.

Daar is dus ’n dringende behoefte om innoverende terapeutiese benaderings te identifiseer om VGW in kanker te oorkom. Daar word ook geglo dat verskeie medisinale plante wat tradisioneel gebruik word kanker kan genees, voorkom of help bestuur. In hierdie studie is die gebruik van *Aloe vera* jel materiaal en ’n gepresipiteerde polisakkaried fraksie, *Sutherlandia frutescens* en *Xysmalobium undulatum* ondersoek as ’n moontlike plant-gebaseerde benadering om kanker te behandel. Die moontlike *in vitro* antikanker eienskappe van die gekose plante was ondersoek in beide chemosensitiewe en chemoweerstandbiedende KSLK selkultuurmodelle. Hierdie KSLK modelle het ’n chemosensitiewe sellyn (H69V), ’n veelvoudige weerstandbiedende sellyn met verhoogde MRP1 uitdrukking (H69AR), asook ’n veelvoudige geneesmiddel weerstandbiedende sellyn met verhoogde P-gp uitdrukking (NCI-H69V/LX4), ingesluit. Om die moontlike sitotoksiese effekte van die gekose plantmateriaal te ondersoek, is daar ook ’n nie-kanker vark nier sellyn (LLC-PK1) gebruik.

’n Voorlopige analise van die antikanker effektiwiteit van die gekose plantmateriaal is uitgevoer d.m.v. die 3-[4,5-dimetieltiasol-2-iel]-2,5-difeniel tetrazolium bromied (MTT) toets, na blootstelling vir 96 uur. Dit is gedoen deur die relatiewe afname in lewensvatbaarheid van die selkulture (IC) te bepaal vir elke KSLK sellyn na behandeling met elke ekstrak. Beide die antikanker, asook die sitotoksiese potensiaal van die plantmateriaal is daarna verder ondersoek deur hulle effek op die vlakke van intrasellulêre ATP en ekstrasellulêre adenilaat kinase (AK) te bepaal in die verskeie KSLK sellyne, asook die LLC-PK1 selle.

Daar is gevind dat al die gekose plantmateriaal bestudeer in hierdie studie die lewensvatbaarheid van al die KSLK sellyne statisties betekenisvol verlaag het ($p<0.05$), alhoewel daar ’n mate van weerstandigheid teen die behandeling waargeneem is in die chemoweerstandige selkulture. Die

fitochemiese komponente in die plantmateriaal wat die antikanker effekte veroorsaak het, kan dus moontlik as substrate dien vir die MRP1 en P-gp pompe. Die waterige *S. frutescens* ekstrak het moontlik nekrose veroorsaak in die weerstandige KSLK selkultuur met verhoogde MRP1 uitdrukking teen 1.406 mg/ml, terwyl die MTT data aangedui het dat die *S. frutescens* ekstrak die hoogste kanker selektiwiteit verhouding in die behandeling van KSLK selle met verhoogde P-gp uitdrukking getoon het. Die gebruik van die *X. undulatum* ekstrak vir die behandeling van KSLK is egter weerspreek deur die sitotoksisiteit waargeneem in die nie-kanker selle (LLC-PK1).

Al die medisinale plantmateriaal gebruik in hierdie studie het beide die intrasellulêre ATP en ekstrasellulêre AK vlakke statisties betekenisvol verander, in beide die chemosensitiewe en die chemoweerstandige KSLK sellyne. Dit dui dus op ’n antikanker effek teen KSLK. Daar was egter ook ’n mate van sitotoksisiteit waargeneem in die nie-kanker (LLC-PK1) sellyn na behandeling met al die geselekteerde plantmateriaal.

Die fitochemiese komponente in die plantmateriaal se affiniteit vir die efluks pompe mag egter positief gebruik word deur dit te kombineer met standaard antikanker geneesmiddels wat ook affiniteit toon vir hierdie pompe. Gevolglik mag die fitochemiese komponente die intrasellulêre geneesmiddel akkumulasie verhoog deur die pompe se bindingsetels te versadig of deur te kompeteer met die geneesmiddels om te bind aan die bindingsetels van die pompe.

Sleutel terme:

ABC efluks pompe, *Aloe vera*, antikanker aktiwiteit, klein sel longkanker, medisinale plante, *Sutherlandia frutescens*, veelvoudige weerstandigheid, *Xysmalobium undulatum*.

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Lists of Symbols

A	Alpha
Å	Angstrom
B	Beta
Cm	Centimetre
Da	Dalton
^{o}C	Degree Celsius
Δ	Delta *or* change
γ	Gamma
G	Gram
H	Hour
kDa	Kilo Dalton
L	Litre
l/h	Litre per hour
m/z	Mass-to-charge ratio
M	Meter
µg/mg	Microgram per milligram
µg/ml	Microgram per millilitre
Ml	Microlitre
µl/well	Microlitre per well
Mm	Micrometre
Mg	Milligram
mg/ml	Milligram per millilitre
Ml	Millilitre
ml/min	Millilitre per minute
Mm	Millimetre
Min	Minute
nm	Nanometre
nM	Nano Molar
%	Percentage
PDA	Photodiode Array
G	Relative centrifugal force
Sec	Second
mm^2	Square millimetre
U/ml	Units per millilitre

V	Voltage
v/v	Volume per volume

List of Abbreviations

A	
A549	Human non-small cell lung carcinoma
ABC	ATP-binding cassette
ABCA-G	Subfamilies of ABC
ABCB1	P-glycoprotein
ABCC1	Multidrug resistance-associated protein 1
ABCG2	Breast cancer resistance protein
abcg2	*Breast cancer resistance protein* (gene)
ABK	Adenosien trifosfaat (ATP)-bindingskasset (ABK) efluks pompe
ADME	Absorption, distribution, metabolism and excretion
AGS	Human Caucasian gastric adenocarcinoma
AIDS	Acquired immune deficiency syndrome
AK	Adenylate kinase
ATCC	The American Tissue Culture Collection
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
B	
143B	Osteosarcoma cell line
Balb/c	Nude mice
BCRP	Breast cancer resistance protein
BKRP	Bors kanker weerstandbiedendheidsproteïen
C	
C	Carbon
Caco-2	Human Caucasian colon adenocarcinoma
CBT-01®	Natural alkaloid (tetrandrine)
CCRF-CEM	Leukaemia cell line
CHO	Chinese hamster ovary cell
CO_2	Carbon dioxide
COLO 320 DM	Human colon cancer
CS	Collateral sensitivity
D	
DMEM	Dulbecco's Modified Eagle's medium
DMSO	Dimethyl sulfoxide

DNA	Deoxyribonucleic acid
DOX	Doxorubicin hydrochloride
E	
E	Ethylenediaminetetraacetic acid
ECACC	The European Collection of Authenticated Cell Cultures
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
e.g.	For example
ES	Estrone 3-sulfate
ET	Etoposide
F	
FBS	Foetal bovine serum
FD18	Flavonoid dimer
FVB	Albino inbred mouse strain susceptibile to Friend leukemia virus B
G	
GABA	γ-aminobutyric acid
GSH	Glutathione
GSSG	Glutathione disulfide
GSTs	Glutathione *S*-transferases
GS-X	Drug conjugated glutathione
H	
H	Hydrogen
H_2O	Hydrogen oxide (water)
H_2O_2	Hydrogen peroxide
H69	Classic human SCLC cell line
H69AR	Hyperexpressed MRP1 SCLC cell line
H69CIS200	Cisplatin deviant cell lines
H69/LX4	Hyperexpressed P-gp SCLC cell line
H69OX400	Oxaliplatin deviant cell lines
H69V	Chemosensitive SCLC cell line
H82	Variant SCLC cell lines
HEK293	Human embryonic kidney cells
Hep3B	Human liver cancer cell line
HepG2	Human liver cancer cell line
HepG2/C3A	Hepatocellular carcinoma cell line
HIV	Human Immunodeficiency Virus
HL60	Human promyelocyte cells

HM30181	Derivate of known P-gp inhibitor
H-NMR	Nuclear magnetic resonance spectroscopy
I	
IC	Cell viability
IC_{50}	Concentrations reducing cell viability by 50%, relative to an untreated control
IC_{75}	Concentrations reducing cell viability by 75%, relative to an untreated control
K	
K+	Potassium ion
KSLK	Klein sel longkanker
L	
LCC6MDR	Human breast cancer cell line
LC-MS	Liquid chromatography-mass spectrometry
LLC-PK1	Porcine kidney non-tumorigenic cell line/ nie-kanker vark nier sellyn
LS174T	Human colon cancer cell line
M	
MCF-7	Breast cancer cell line
MCF-7/ADR	Drug resistance breast cancer cell line
MCF-12A	Non-tumorigenic breast cell line
MDA435/LCC6	Hyperexpressed P-g cancer cell line
MDA-MB-468	Human mammary adenocarcinoma
MDR	Multidrug resistant/ Multidrug resistance
miRNA	Micro ribonucleic acid
mrp	*Multidrug resistance protein* (gene)
MRP1	Multidrug resistance-associated protein 1/ veelvoudige geneesmiddel weerstandbiedendheid-geassosieerde proteïen 1
MRP2	Multidrug resistance-associated protein 2
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5- diphenyl tetrazolium bromide/3-[4,5-dimetieltiasol-2-iel]-2,5-difeniel tetrazolium bromied
N	
Na+	Sodium ion
NADP	Nicotinamide adenine dinucleotide phosphate
NBD	Nucleotide binding domains
NCI	National cancer institute
NCI-H187	Variant SCLC cell lines
NCI-N417	Variant SCLC cell lines

NCI-H526	Variant SCLC cell lines
NCI-H69	Classic human SCLC cell line
NCI-H69AR	Resistant SCLC cell lines
NCI-H69/LX4	Hyperexpressed P-gp suspension SCLC cell line
NCI-H69/LX10	Hyperexpressed P-gp adherent SCLC cell line
NEAA	Non-essential amino acids
NSCLC	Non-small cell lung cancer
O	
-OH	Hydroxyl group
P	
Pen/Strep	Penicillin/streptomycin
PBS	Phosphate-buffered saline
Pg	Page
P-gp	P-glycoprotein/ P-glikoproteïen
Pi/ADP	Inorganic phosphate adenosine 5'-diphosphate complex
PX-478	Small-molecule inhibitor of hypoxia-inducible factor 1α
R	
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute 1640
S	
SCLC	Small cell lung cancer
SFFW	*Sutherlandia frutescens*
siRNA	Small interfering RNA
SU1	Cycloartane-type triterpene glycoside
T	
TMD	Transmembrane domains
TNM	Primary tumour (T), the regional lymph nodes (N) and distant metastasis (M)
U	
UK	United kingdom
UN	Untreated
UPLC	Ultra-performance liquid chromatography
US	United States
USA	United States of America

V

VP	Verapamil
VGW	Veelvoudige geneesmiddel weerstandbiedendheid

W

WHO	World Health Organization

X

X	Drug
XU174	*Xysmalobium undulatum*

Chapter 1
Introduction

1.1. Background

An astonishing one in every six reported deaths globally are caused by cancer (WHO, 2018). Lung cancer is one of the most diagnosed cancers in both male and female patients, which highlights its high prevalence in the population (Ahmed *et al.*, 2017). Small cell lung cancer (SCLC) is a type of lung cancer defined by a rapid proliferation rate and early metastasis (Tartarone *et al.*, 2017). The treatment approaches for SCLC have not changed in the last three decades, which involve platinum combined chemotherapy, prophylactic cranial irradiation of brain metastases or hyperfractionation (Paumier & Le Péchoux, 2010; Kaur *et al.*, 2016). Tumour cells can have primary resistance to chemotherapy prior to drug exposure. On the other hand, acquired resistance can also develop after exposure to a chemotherapeutic drug (Wu *et al.*, 2014). Cancer cells demonstrating drug resistance towards structurally and functionally unrelated compounds are described as multidrug resistant (Saraswathy & Gong, 2013). Efflux transporter mediated resistance is a well-known mechanism of multidrug resistance (MDR), which decreases the intracellular drug concentration in the cancer cells and leads to a reduction of the therapeutic effect (Triller *et al.*, 2006).

Transporter mediated resistance in cancer tissue is often associated with a group of adenosine triphosphate (ATP)-binding cassette (ABC) transporters, acting as large membrane proteins responsible for transporting different substrates across the cell membrane (Fletcher *et al.*, 2016). These transporters include P-glycoprotein (P-gp), multidrug resistance-associated protein 1 (MRP1) and breast cancer resistance protein (BCRP) (Ozben, 2006). Several known anticancer drugs (e.g. doxorubicin and vincristine) are substrates for some of the ABC transporters (Cole, 2014). Inhibition of these efflux transporters to reduce anticancer drug efflux is a possible mechanism to combat cancer MDR (Amaral *et al.*, 2016). Another avenue being explored to combat MDR in cancer is the use of medicinal plants, which consist of abundant phytochemical constituents that can act by different mechanisms in an additive or synergistic way (Mbaveng *et al.*, 2018).

A survey summarising indigenous medicinal knowledge in the southern Karoo region of South Africa identified three medicinal plants traditionally used in cancer treatment, namely *Sutherlandia microphylla*, *Withania somnifera* and *Dicoma capensis* (Van Wyk *et al.*, 2008). Another traditionally used anticancer plant, named *Sutherlandia frutescens* (cancer bush), is found in some of the Cape provinces and the KwaZulu-Natal region (Aboyade *et al.*, 2014). Several *in vitro* studies have established the anticancer efficacy of *S. frutescens* against prostate, breast and cervical cancer (Chinkwo, 2005; Vorster *et al.*, 2012; Lu *et al.*, 2015). Furthermore, several phytochemicals found

in *Aloe vera* and *Xysmalobium undulatum* are also purported to have anticancer efficacy (Choi & Chung, 2003; Krishna *et al.*, 2015). It is important to keep in mind that several allopathic chemotherapeutic drugs, such as vinca alkaloids, paclitaxel and camptothecin, initially originated from plants. Therefore, natural flora are still one of the cornerstone resources in finding alternative cancer treatments (Efferth *et al.*, 2017).

1.2. Research problem

The occurrence of multidrug resistant cancer due to the hyperexpression of several ABC transporter proteins causes a reduced chemotherapeutic treatment outcome. Thus, there is an urgent need to explore ways to combat MDR. Worldwide, and especially in Souhern Africa, various medicinal plants are commonly used as alternative and more cost-effective anticancer treatment options. Several traditional healing practices include the use of medicinal plants for their anticancer activity, therefore, it is necessary to scientifically investigate their anticancer potential using appropriate preclinical screening models and assays.

1.3. Aim and objectives

The aim of this study is to evaluate the possible anticancer efficacy of three selected medicinal plants (including *Aloe vera*, *Sutherlandia frutescens* and *Xysmalobium undulatum*) using a panel of chemosensitive and chemoresistant SCLC cell models. A non-tumorigenic cell line was also included to assess the possible cytotoxicity of the selected plants.

To accomplish the aim, the following objectives were set:

- Preparation and chemical fingerprinting of crude aqueous extracts from dried *S. frutescens* and *X. undulatum* plant materials.
- Chemical fingerprinting of *Aloe vera* gel material and precipitated polysaccharide materials.
- Culturing human SCLC cell lines consisting of H69V (chemosensitive), H69AR (MRP1 hyperexpressive) and NCI-H69/LX4 (P-gp hyperexpressive); as well as a non-tumorigenic porcine kidney cell line (LLC-PK1).
- Preliminary screening of the selected medicinal plant materials for anticancer potential by means of a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay on the selected SCLC and LLC-PK1 cell models to estimate inhibitory concentration (IC_{50}) values.
- Advanced evaluation of the anticancer potential by determining the influence of selected concentrations of the plant materials on extracellular adenylate kinase (AK) and intracellular ATP levels in the SCLC and LLC-PK1 cell models.

1.4. Structure of dissertation

This dissertation consists of an introductory chapter (Chapter 1), which gives a condensed background of the challenges regarding cancer treatment, and a summary of the research problem, aims and objectives, the study outline, publication status and collaborations. Chapter 2 presents a literature review as background for the current study. Chapter 3 consists of a review manuscript, discussing the different models currently available to screen treatment for efflux-based MDR in cancer. This manuscript has been submitted for publication. The methods and results of the MTT assays are discussed in Chapter 4, while Chapter 5 consists of a research article describing the advanced screening of the anticancer efficacy of the three plants on the SCLC and LLC-PK1 cells. This manuscript has been prepared for submission for publication. Finally, the concluding remarks and future recommendations are stated in Chapter 6. Figure 1.1 illustrates the experimental design for the study.

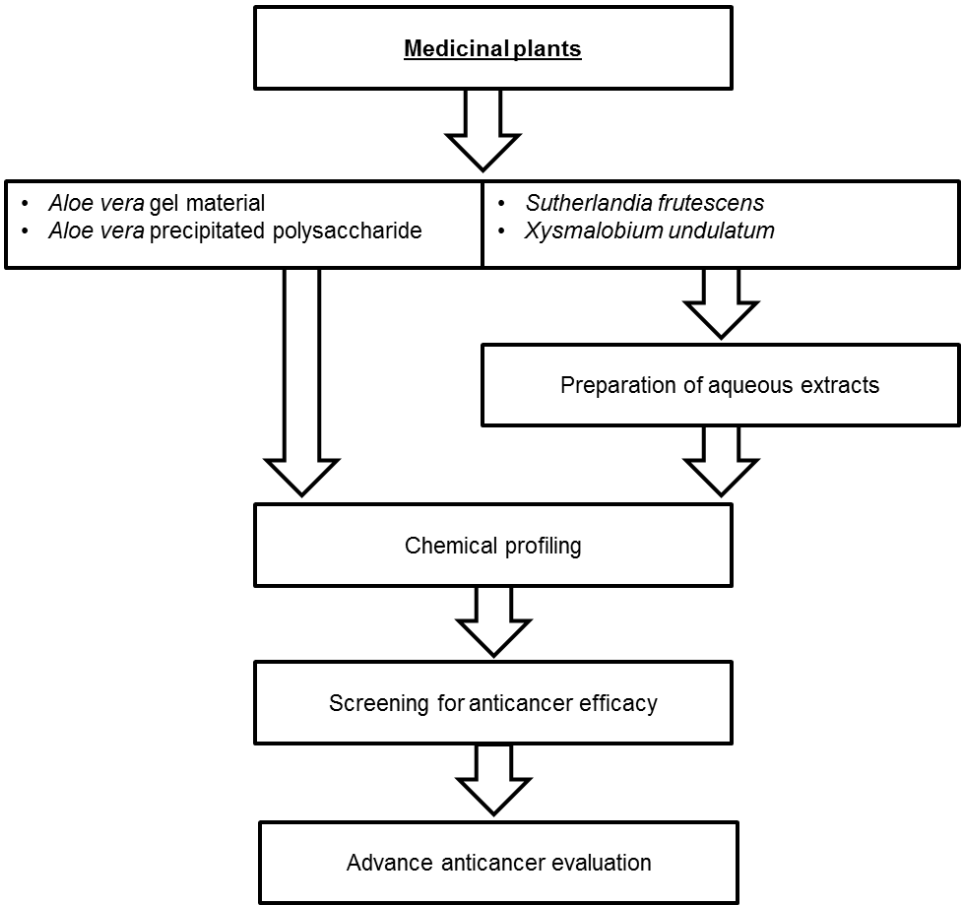


Figure 1.1: The experimental design included the preparation of crude aqueous extracts from the dried plant materials of *S. frutescens* and *X. undulatum*. The plant material and extracts were chemically profiled to confirm the presence of specific marker molecules. The preliminary anticancer screening, using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay, was used to calculate a 50% viability reduction concentration (IC_{50}) for each crude extract in each cell line. These values were then used in the advanced anticancer efficacy evaluations.

1.5. Publication status of research

Supplementary to the literature overview (Chapter 2), a review article was prepared in which models contributing to efflux-based cancer treatment screening were presented. This manuscript was included in Chapter 3, and has been submitted to the journal *Current Cancer Drug Targets* for publication. The first three authors contributed equally to the content of the manuscript.

- Willers, C., Rossouw, M.J., Swanepoel, R.A., Svitina, H., Hamman, J.H., & Gouws, C. Efflux-based models for multidrug resistant cancer treatment screening. *Current Cancer Drug Targets.* Under review.

The results of the advanced anticancer efficacy screening of the selected plant materials on the different SCLC cell lines were also prepared as a research article. This manuscript was included in Chapter 5, and is to be submitted for publication to the *Journal of Ethnopharmacology*. The contribution of the authers are stated in the manuscript.

- Swanepoel, R.A., Willers, C., Calitz, C., Viljoen, A.M., Hamman, J.H. & Gouws, C. *In vitro* screening of selected medicinal plants as treatment for drug resistant small cell lung cancer. To be submitted.

The results from the current study were also presented at the First Conference of Biomedical and Natural Sciences and Therapeutics (CoBNeST) in Stellenbosch, 7-10 October 2018 (See Appendix F).

- Swanepoel, RA., Willers, C., Calitz, C., Hamman, JH. & Gouws, C. 2018. *In vitro* efficacy evaluation of selected medicinal plant extracts against multidrug resistant small cell lung cancer. First Conference of Biomedical and Natural Sciences and Therapeutics (CoBNeST). (Oral presentation: APSSA Young Scientist competition).

1.6. Collaboration

The current study formed part of a larger study, and the validation of the SCLC models used formed part of another MSc study (Rossouw, 2018). The current study, therefore, did not include validation of the suitability of the various SCLC cell lines as screening models, but only used them to evaluate the anticancer potential of the three selected medicinal plants.

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Chapter 2
Literature overview

2.1. Introduction

Chemotherapy is considered the leading practice in cancer management (Zhang *et al.*, 2017). Unfortunately, multidrug resistance (MDR) can be acquired when chemotherapy treatment is administered continuously or due to primarily intrinsic resistance before treatment (Lopes-Rodrigues *et al.*, 2017). Contributing to MDR is the adenosine triphosphate (ATP)-binding cassette (ABC) transporter protein family consisting of 48 known transporters (Köhler *et al.*, 2016). The hyperexpressed MDR proteins in cancer cells are located on the cytoplasmic cell surface and can actively transport anticancer drugs or their metabolites to the extracellular environment, affecting the therapeutic outcome of cancer management (Wu *et al.*, 2014; Błauż & Rychlik, 2017). Thus, the ABC transporter family is a possible target for inhibition in an attempt to enhance the response to chemotherapy and to reduce MDR facilitated by anticancer drug efflux. Currently, ethno-medical approaches in treating a wide variety of health conditions are increasing due to its low cost, decreased side effects and traditional beliefs regarding health benefits (Oga *et al.*, 2016). Therefore, evaluating the anticancer properties of selected medicinal plants which are traditionally used to treat cancer may result in an innovative therapeutic approach towards MDR.

2.2. Cancer

2.2.1. Introduction

The World Health Organization (WHO, 2018) reported cancer as the second major cause of death in 2018 based on 9.6 million cancer associated deaths and an estimated mortality rate of 1:6 worldwide. In the United States (US), 14.8 per 100 000 of the US population died due to colorectal cancer in the period from 2010 - 2014 (Siegel *et al.*, 2017). In South Africa, the Eastern Cape Province population-based cancer registry documented a high prevalence of oesophageal cancer in men between 1998 and 2012 (Somdyala *et al.*, 2015). Metastasis to lung tissue is reported to occur in 25% of patients living with oesophageal cancer (Tu *et al.*, 2014). Hence, cancer is classified as a fatal disease in the modern world and is defined by irregular cell development and uncontrollable cell growth not restricted to one part of the human body (Singh, 2016; Bhatta, 2017). The major difference in cellular behaviour between benign (non-cancerous) cells and malignant (cancerous) cells is listed in Table 2.1. Cancer cells have the ability to alter their metabolism to sustain cell proliferation through the production of biomolecules and energy (Pattni *et al.*, 2017). Rapidly proliferating cancer cells are known to have an increased glucose consumption rate, recognised as

the Warburg effect, compared to the normal cellular glucose consumption in benign tissues (DeBerardinis *et al.*, 2008). Furthermore, cancer cells are capable of metastasis which is known as the ability of cancer cells to invade neighbouring or distant organs (Bhatta, 2017).

The invasion process occurs when the cancer cells develop a migration path by degrading their extracellular matrix (ECM) environment (Paul *et al.*, 2017). The ECM of cancer cells consist of a network of macromolecules having biomechanical, physical and biochemical features (Lu *et al.*, 2012). Irregular functionality of the ECM, due to proteolytic degradation, has the ability to enhance cancer progression (Lu *et al.*, 2012; Seguin *et al.*, 2015).

Table 2.1: Differences in the cellular characteristics of benign and malignant cells

Characteristics	Benign cells	Malignant cells	References
Deoxyribonucleic acid (DNA) composition	Normal DNA methylation	Abnormal DNA methylation	(Siebenkäs *et al.*, 2017; Wang *et al.*, 2017)
Glucose metabolism	Decreased glucose uptake compared to cancer cells	Conform to the Warburg effect	(Zhang *et al.*, 2014; Devic, 2016)
Cell replication	Replication is terminated through cell contact inhibition	Fast replication which is uninhibited through cell-to-cell contact	(Kalyanaraman, 2017)
Intracellular nutrient concentrations	Increased	Decreased by adapted metabolism	(Birsoy *et al.*, 2014)
ATP production	Lower rate of ATP production	Abundant ATP production	(DeBerardinis *et al.*, 2008)
Blood supply	Organised blood vessel orientation	Hypoxia occurs due to disorganised blood vessel orientation	(Hsu & Sabatini, 2008)

Cancer is divided into sarcomas, carcinomas and lymphomas or leukaemia, where carcinomas (malignancies of epithelial cells) are purported to show a higher prevalence in humans (Sengupta *et al.*, 2017). Therefore, in this study small cell lung cancer (SCLC) was used as a carcinoma model for the evaluation of the anticancer properties of selected medicinal plants.

2.2.2. Small cell lung cancer

Lung cancer is considered one of the leading causes of cancer deaths globally, accounting for an estimated 1.76 million deaths in 2018, according to the World Health Organization (WHO, 2018). Histologically, lung cancer can be divided into non-small cell (NSCLC) and small cell (SCLC) lung cancer (Hodkinson *et al.*, 2007). The clinical development of SCLC, distinguishing this lung cancer from NSCLC, is a rapid doubling time and a higher incidence of metastases (Govindan *et al.*, 2006). The distinct SCLC entity will be the main focus for this study, and is responsible for approximately 13% of reported lung cancers (Ahmed *et al.*, 2017). In the absence of SCLC therapy, the median

survival period from diagnosis is approximately 5 weeks to 3 months, however, the treatment of SCLC is claimed to extend the survival median to 8 months (Buttery *et al.*, 2004; Hodkinson *et al.*, 2007). Although SCLC is sensitive to first line platinum combined therapy, recurrence of SCLC still occurs and less than 5% of patients with recurrent malignancy live longer than 5 years (Gomez-Casal *et al.*, 2016; Kaur *et al.*, 2016). The destructive clinical progression of SCLC arises from the widespread metastasis, enhanced cell proliferation, poor prognosis and recurrence (Tartarone *et al.*, 2017). Lung cancer is categorised according to the TNM classification which defines the primary tumour (T), the regional lymph nodes (N) and distant metastasis (M), and can help to determine the therapeutic approach to be followed (Goldstraw *et al.*, 2007).

2.2.3. Current treatment options for small cell lung cancer

The treatment objectives for SCLC malignancy are based on curing SCLC or extending the survival period through several combinations of anticancer drugs (Wells *et al.*, 2012). The commonly prescribed treatments for SCLC include prophylactic cranial irradiation, platinum combined therapy and hyperfractionated thoracic radiation (Kaur *et al.*, 2016). Multimodality therapy of etoposide with carboplatin or cisplatinum (also known as cisplatin) extended the survival period in clinical trials (phase III) (Parsons *et al.*, 2014). A new approach involving the combination of standard platinum treatment with pravastatin showed no value in treating SCLC (Seckl *et al.*, 2017). Topotecan is the preferred second-line treatment option; however, amrubicin can also be considered (Rossi *et al.*, 2016). During stage one SCLC, the recommended treatment approach for a T1-2, N0, M0 classified tumour (tumour size between 3 – 7 cm, no metastasis detected in the lymph nodes and no distant metastasis), is surgery with adjuvant cancer treatment afterwards (Jazieh, 2012; Ricciuti *et al.*, 2017). Some SCLC tumours are intrinsically drug resistant and will respond poorly to cytotoxic drugs, whereas other SCLC tumours can acquire resistance during the prolonged treatment period (Triller *et al.*, 2006).

The anticancer agent etoposide induces cell death by damaging the cellular DNA through modifying DNA topoisomerase IIα. However, one of the mechanisms known to induce resistance against etoposide is the occurrence of mutations in DNA topoisomerase IIα (Kreisholt *et al.*, 1998). Furthermore, the cellular communication between the SCLC cells and the ECM (known as integrin-mediated cell-ECM binding) can also contribute to the occurrence of drug resistance (Buttery *et al.*, 2004). Integrins are present on the cell surface and can be defined as heterodimeric glycoproteins responsible for cell survival, migration, cellular adhesion and differentiation (Hodkinson *et al.*, 2007; McHugh *et al.*, 2012). The attachment of SCLC cells to parts of the ECM, namely collagen IV, laminin and fibronectin by means of β1 integrins, can inhibit the therapeutic response to cisplatinum, etoposide and adriamycin (Rintoul & Sethi, 2002). In SCLC cells, β1 integrin activation subsequently activates phosphoinositide-3-OH kinase, which results in intercepting etoposide-induced caspase-3 activation and cellular apoptosis (Hodkinson *et al.*, 2007).

Etoposide treatment options are limited in MDR caused by the hyperexpression of P-glycoprotein (P-gp) and multidrug resistant protein (MRP) (Keilholz *et al.*, 2017). Hyperexpressed P-gp and MRP1 in SCLC cells were shown to decrease the response rate of anticancer therapy and contribute to cross-resistance between colchicines, taxanes, epipodophyllotoxins, antracyclines and vinka alcaloids, even though they differ functionally and structurally from one another (Triller *et al.*, 2006). Cellular transport of cisplatin can change by means of reduced uptake and/or an increased drug efflux, which can influence the intracellular accumulation and result in cisplatin resistance (Köberle *et al.*, 2010). Efflux transporters also influence drug delivery to internal organelles, including the mitochondria and nuclei, causing anticancer drugs to not reach their intracellular target e.g. deoxyribonucleic acid (DNA) (Yuan *et al.*, 2015). The major cause of treatment failure in SCLC is the occurrence of MDR. Thus, the urgent need for new treatment strategies against MDR is crucial (Rossi *et al.*, 2016).

2.2.4. Models to investigate small cell lung cancer and related multidrug resistance

The models generally used to study drug resistance in cancer can be broadly divided into *in vitro* (conventional and advanced cell culture models) and *in vivo* (e.g. xenografts), as described extensively in Chapter 3.

The classic H69 human SCLC conventional cell line has been used widely to develop numerous sub-clone cell models for the evaluation of MDR SCLC. These models include, but are not restricted to, the H69V (drug sensitive), H69AR (hyperexpressing MRP1), NCI-H69/LX4 (hyperexpressing P-gp) and NCI-H69/LX10 (hyperexpressing P-gp) cell lines (Wishart *et al.*, 1990; Sadava & Winesburg, 2005; Sandvold *et al.*, 2010; Stefan *et al.*, 2017). The technique used to develop the H69AR MDR variant cell line included exposing the H69 model to increased doxorubicin concentrations for 14 months (Mirski *et al.*, 1987). These cell culture models can further be used to develop an *in vivo* SCLC model, as done by Moreira *et al.* (2001), where the subcutaneous inoculation of the H69 cell line resulted in a Balb/c nude mice SCLC xenograft model. The application of the classic and variant cell models are commonly used in *in vitro* cancer studies.

The original H69 human SCLC cell line was used in the evaluation of the progression of platinum drug (oxaliplatin and cisplatin) resistance, during which two further cell lines (H69OX400 and H69CIS200) were developed after treatment with the studied drugs (Stordal *et al.*, 2006). The elucidation of the molecular MDR mechanism in SCLC was possible through evaluation of 856 micro ribonucleic acid (miRNA) expression in NCI-H69AR and NCI-H69 cell lines. This demonstrated a specific miRNA correlation with SCLC drug resistance (Guo *et al.*, 2010). The H69/LX4 model, exposed to doxorubicin for 6 - 9 months, was used in a drug sensitisation study and demonstrated the interaction between nanoparticle formulations and P-gp receptors (Puranik *et al.*, 2016). Two SCLC cell lines (H82 and H69) were exposed to

epirubicin, resulting in two extended multidrug resistant cell lines, used to evaluate the potential of paclitaxel to treat refractory SCLC (Su *et al.*, 1998). Additional advanced cancer models entail the injection of SCLC (NCI-H187 and NCI-N417) cells into organs (including the lungs of an animal model), to investigate the effect of PX-478 on lung tumours (Jacoby *et al.*, 2010). An NCI-H526 (SCLC) xenograft model was also established inside a chick chorioallantoic membrane for the evaluation of drug resistance (Chin *et al.*, 2007).

2.2.5. Drug resistance in cancer

The occurrence of tumour metastasis and drug resistance are responsible for a decreased therapeutic effect and a high prevalence of cancer mortality (Yang *et al.*, 2003). Drug resistance is branched into intrinsic resistance, occurring long before the treatment with chemotherapy, and acquired resistance which develop during the course of the cancer treatment (Majidinia & Yousefi, 2016). Cancer cells are capable of becoming concurrently resistant to a varied range of structurally unrelated cancer drugs and this phenomenon is known as MDR (Gottesman *et al.*, 2002; Singh *et al.*, 2017). Chemoresistance in cancer cells can develop by means of decreased cellular apoptosis, drug target modification, adaptive DNA repair mechanisms and the occurrence of transporter-associated drug efflux (Cree & Charlton, 2017). However, the hyperexpression of efflux transporters such as the ABC family in cancer cells is claimed to be a major barrier contributing to drug resistance (Zhang *et al.*, 2017).

The following studies targeted transporter-associated drug efflux, to alter drug resistance by increasing the intracellular drug concentration. The inhibition of apoptosis and MDR was shown to enhance the anticancer activity of doxorubicin through a liposomal drug delivery system (Pakunlu *et al.*, 2003). Verapamil, a known calcium channel blocker, increased the intracellular accumulation of doxorubicin through possible efflux inhibition (Harker *et al.*, 1986). The first generation MDR modulator, Cyclosporin A, reversed MDR although this reversed effect was detected at a high dosage and subsequently led to an immunosuppressant effect (Krishna & Mayer, 2000). Multidrug resistance was bridged in murine colon carcinoma cells during the simultaneous administration of doxorubicin and a P-gp inhibitor as a combined polymeric conjugate (Sivak *et al.*, 2017). Several phytochemicals in plants which inhibit the ABC transporters can increase the cytotoxic effect of cancer drugs when used in combination therapy (Molnar *et al.*, 2010). Natural alkaloids were reviewed in demonstrating their MDR reversal activity, of which tetrandrine/CBT-01® was found to be tested in clinical trials for inhibition of P-gp in cancer (Joshi *et al.*, 2017).

2.3. Multidrug resistance

2.3.1. Introduction

Cells exposed to a single anticancer drug can develop a broad cellular resistance towards other structurally, mechanistically and functionally unrelated drugs, which is known as multidrug resistance (MDR) and may further be associated with ATP dependent drug efflux (Ford & Hait, 1990; Yu *et al.*, 2016b). In humans, there are 48 distinctive ABC efflux transporters that are subdivided into seven families (ABCA-G) according to different structural features and size (Taylor *et al.*, 2017). The basic fundamental components of the ABC transporter family are illustrated in Figure 2.1. The ABC transporter family effluxes substrates actively from the intracellular to the extracellular environment by using the energy produced during the hydrolysis of ATP to adenosine diphosphate (ADP), resulting in the detoxification of the cancer cell (Lopes-Rodrigues *et al.*, 2017). The following members of the ABC transporter family namely P-glycoprotein (P-gp, ABCB1), breast cancer resistance protein (BCRP, ABCG2) and multidrug resistance-associated protein 1 (MRP1, ABCC1) are associated with MDR and can display hyperexpression in MDR carcinomas (Köhler *et al.*, 2016).

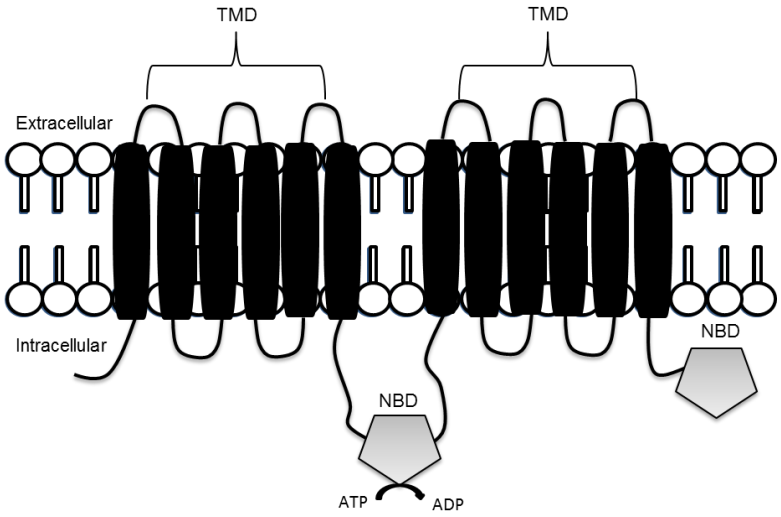


Figure 2.1: The minimum structural features of the ABC transporter family include two nucleotide binding domains (NBD), responsible for ATP hydrolysis, and a permeation pathway consisting of two transmembrane domains (TMD) (adapted from Sharom, 2008 and Tarapcsák *et al.*, 2017).

2.3.2. P-glycoprotein (P-gp)

The identification of P-gp was primarily done in multidrug resistant Chinese hamster ovary cells (Ling & Thompson, 1974). This 170 kDa protein structure are made up of 1 280 amino acids and two homologous halves, both consisting of a transmembrane domain (TMD), six α–helix segments and an intracellular nucleotide binding domain (NBD) (Loo & Clarke, 2005; Pluchino *et al.*, 2016). The P-gp transporter protein depends on the basal ATPase activity (which can be stimulated through

various substrates) and the hydrolysis of ATP as the efflux power supply (Sharom, 2014). Furthermore, the attachment of the two ATP molecules at the NBDs is the driving force behind the conformational alterations of the TMDs and changes the whole transporter structure from inward to an outward facing, which results in the opening of the drug-binding pocket (Ward *et al.*, 2013; Wessler *et al.*, 2013). The NBDs (also known as ATP-binding domains) transfer the generated energy to actively efflux the substrates across the lipid bilayers (Dewanjee *et al.*, 2017). The closing of the drug-binding pocket arises after the hydrolysis of the ATP molecule to inorganic phosphate adenosine 5'-diphosphate complex (Pi/ADP) (Ward *et al.*, 2013). The P-gp protein is responsible for maintaining physiological absorption, distribution, metabolism and excretion (ADME) processes in the body, yet, in cancer cells P-gp decreases drug accumulation and reduce the therapeutic effect (Joshi *et al.*, 2014).

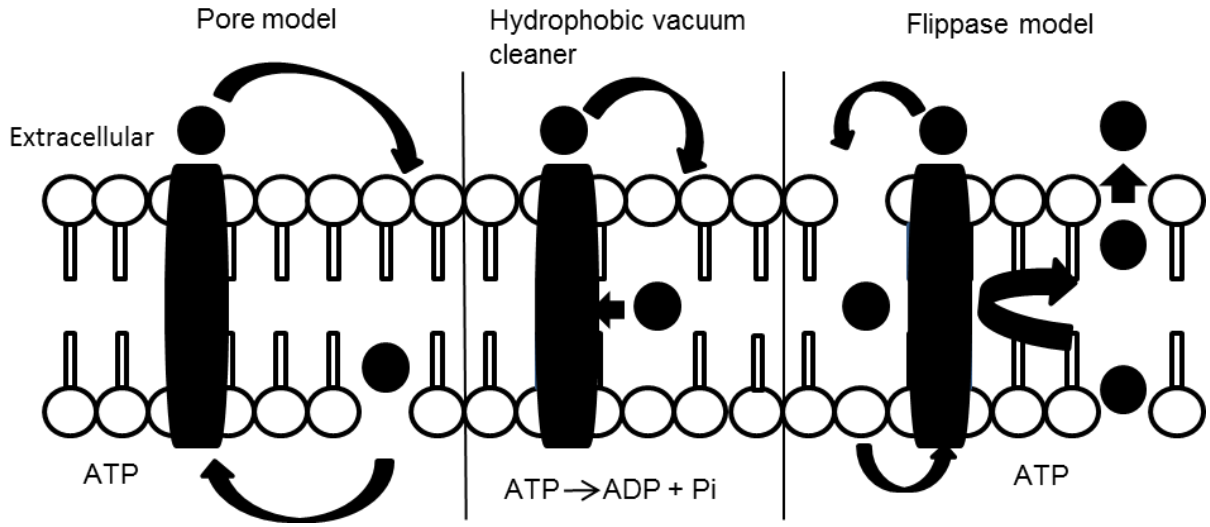


Figure 2.2: A simplistic illustration of the three commonly described P-gp transporter associated efflux models adapted from (Dewanjee *et al.*, 2017).

The three different models as illustrated in Figure 2.2, explain the efflux mechanism of P-gp and include: the pore model, flippase model and the hydrophobic vacuum cleaner (Ferreira *et al.*, 2015; Dewanjee *et al.*, 2017). The classic pore pump mechanism states that the P-gp efflux pathway is hydrophilic and the ejection of the substrate through this pore keeps the substrate from interacting with the hydrophobic lipid part of the membrane (Sharom, 2008). The ability of the P-gp protein to interrupt the substrate permeation through the lipid bilayer, by flipping the substrate to the outer leaflet of the lipid bilayer or instantaneously to the extracellular environment, are taken into account in the flippase model (Hennessy & Spiers, 2007). The uptake of the substrate from the lipid bilayer, rather than from the aqueous cytosol, is described by the hydrophobic vacuum cleaner model (Romsicki & Sharom, 2001).

Structurally, P-gp substrates are commonly grouped as organic molecules consisting of aromatic substitutes with a size parameter between 200 and 1 900 Da (Schinkel & Jonker, 2003). The P-gp

efflux pump is claimed to increase resistance to the following substrates used in cancer management: paclitaxel, anthracyclines, vinca alkaloids, epipodophyllotoxins and camptothecins (Leslie *et al.*, 2005). The clinical use of some P-gp inhibiters such as verapamil (a MDR modulator) are restricted due to several adverse effects, of which verapamil can result in *in vivo* cardio-toxicity at high concentrations (Di Pietro *et al.*, 2002).

2.3.3. Multidrug resistance-associated protein 1 (MRP1)

The C-subfamily of the ABC superfamily consists of nine human multidrug resistance-associated protein (MRP) transporters, of which the first MRP1 transporter with a crystal structure of 22 Å was established during the exposure of the NCI-H69AR (SCLC cell line) to doxorubicin (Cole *et al.*, 1992; Zhang *et al.*, 2015). The gene encoding for the MRP1 190 kDa glycoprotein pump (identified by using HL60 cells resistant to adriamycin) is expressed as *mrp* (multidrug resistance protein*)* (Krishnamachary & Center, 1993). The MRP1 efflux pump positioned on the basolateral layer of the cell membrane consists of twelve TMDs, two ATP binding sites and another five TMDs located at the end of the amino terminal (Wind & Holen, 2011). Structural conformation changes of the protein occur through ATP binding and hydrolysis, yet, MRP1 can also efflux anticancer drugs such as vincristine by means of glutathione (GSH) (Cole, 2014).

The influence of glutathione in MRP1 efflux is illustrated in Figure 2.3. The conjugation of GSH with the drug is catalysed by means of the glutathione *S*-transferases (GSTs) enzyme (Figure 2.3a), resulting in the efflux of the conjugated product (Morrow *et al.*, 1998). The transport of etoposide as a GSH *S*-conjugate is not known and findings rather indicate a co-transportation of etoposide with GSH (Figure 2.3b) (Rappa *et al.*, 1997). The efflux of estrone 3-sulfate, an MRP1 substrate, was found to be improved in the presence of GSH as indicated in Figure 2.3c (Qian *et al.*, 2001). MRP1 transport requires GSH, although, a decreased intracellular GSH accumulation was detected in the presence of verapamil (Figure 2.3d) (Cullen *et al.*, 2001). Glutathione reductase is responsible for the conversion of GSH to glutathione disulfide (GSSG) which is a substrate for MRP1 (Figure 2.3e) (Hirrlinger *et al.*, 2001).

One of the strategies in reducing MRP1 drug efflux was demonstrated by depleting the GSH intracellular concentrations, by mimicking the effect of verapamil through several flavonoids. Chrysin, a flavonoid, was suggested as a compound to consider in MDR cancer therapy (Lorendeau *et al.*, 2014). Numerous methoxylated flavonoids also indicated MRP1 inhibition properties (Van Zanden *et al.*, 2005). A triterpene, namely 3β-acetyl tormentic acid, was found to inhibit MRP1 activity through reducing both the GSH intracellular pool and conjugated drug complexes (Da Graça Rocha *et al.*, 2014). The influence of MRP1 transport on daunorubicin (chemodrug) was evaluated by using different SCLC and NSCLC cell lines and a known MRP1 substrate and chemotherapeutic drug namely vincristine (Renes *et al.*, 1999). The concluding remarks noted daunorubicin as a MRP1

substrate as the transport increased in the presence of GSH. Anticancer drugs such as doxorubicin, etoposide, paclitaxel, epirubicin and vinorelbine are some of the commonly listed MRP1 substrates. Furthermore, the conjugated estrogens chlorambucil and leukotrine C_4 are also considered MRP1 substrates (Leier *et al.*, 1994; Loe *et al.*, 1996; Barnouin *et al.*, 1998; Ozben, 2006).

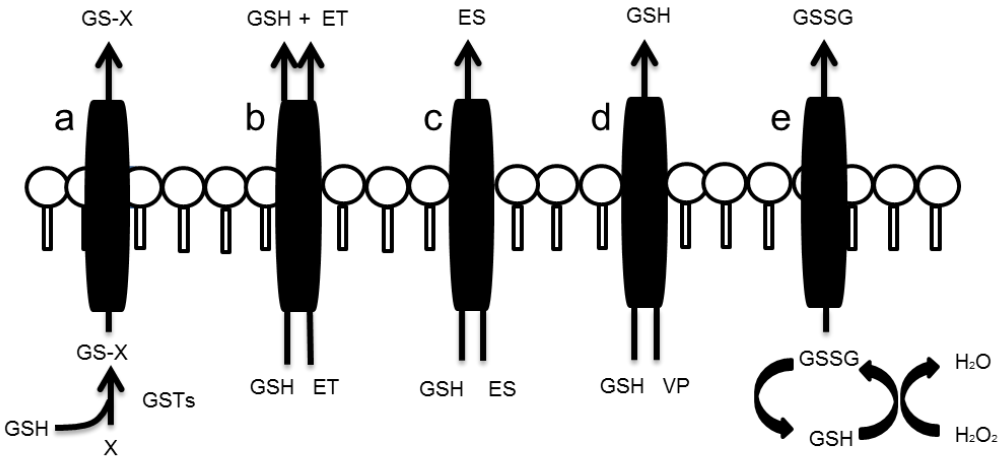


Figure 2.3: An illustration of the mechanistic impact of glutathione on MRP1 efflux, as adapted from (Kruh & Belinsky, 2003). Key to abbreviations: ET - etoposide; ES - estrone 3-sulfate; GSH - glutathione; GS-X - drug conjugated glutathione; GSSG - glutathione disulfide; GSTs - glutathione *S*-transferases; H_20 - water; H_2O_2 - hydrogen peroxide; VP - verapamil and X - drug.

2.3.4. Breast cancer resistance protein (BCRP)

One of the G-subfamily transporters which contribute to the drug resistance mechanism in SCLC is the breast cancer resistance protein (BCRP) (Kim *et al.*, 2009). Retrospectively, the cloning of the *abcg2* gene was done in the late 1990's, from a human breast carcinoma cell line (Doyle *et al.*, 1998). The BCRP structure consists of only one NBD, one TMD with six membrane α-helices and 655 amino acids; signifying why BCRP is referred to as half a transporter (Jani *et al.*, 2014). Furthermore, the BCRP transporter functionality is based on either a homodimer or a tetramer fashion which consists out of disulphide bindings (Staud & Pavek, 2005). Intra- and intermolecular disulphide bindings are considered of utmost importance for optimal functionality (Henriksen *et al.*, 2005). The BCRP efflux protein can be a promising target in SCLC treatment.

The hyperexpression of the BCRP transporter in several cell lines reduces the intracellular accumulation of numerous drugs including topotecan, mitoxantrone and daunorubicin (Litman *et al.*, 2000). The BCRP transporter shows resistance to the following substrates commonly used in cancer treatment irinotecan, gefitinib and imatinib (Maliepaard *et al.*, 2001; Burger *et al.*, 2004; Elkind *et al.*, 2005). However, the intracellular pool of anticancer drugs can increase in the presence of erlotinib, which can reverse both P-gp and BCRP-associated MDR (Shi *et al.*, 2007). The BCRP inhibition

properties of 31 flavonoids were investigated and the founding remarks indicated both ayanin and retusin to be less toxic with BCRP inhibition (Pick *et al.*, 2011). Novel compounds were synthesised from a known third-generation P-gp inhibitor (HM30181), to investigate BCRP inhibition and possible toxicity by performing a cell viability assay. The results indicated that the compounds without the tetrahydroisoquinoline group were selective BCRP inhibitors (Köhler & Wiese, 2015).

2.3.5. Current treatment approaches towards multidrug resistance

The establishment of small interfering ribonucleic acid (siRNA) in 1993, evolved into a prognostic, diagnostic and therapeutic tool in cancer research (Iorio & Croce, 2009). These low molecular RNA structures are taken up by the cancer cell and reduce specific genes' expression which are required for cancer cell proliferation (Weinstein & Joe, 2006). A study combined siRNA and doxorubicin in a polymeric system which reduced P-gp associated doxorubicin resistance (Xiong & Lavasanifar, 2011). In an attempt to improve the cellular uptake of siRNA, a nanocarrier particle consisting of polymers showed effective uptake through endocytosis by the MDA435/LCC6 carcinoma cell line which expressed P-gp efflux transporters (Xiong *et al.*, 2009). A silicon nanostructure capable of delivering siRNA to the cell was capable of down regulating MRP1 expression in glioblastoma multiforme cells (Tong *et al.*, 2018). Although siRNA MDR treatment have shown to be a promising approach, some of the down falls includes ineffective target delivery due to enzyme degradation, reduced cellular influx and clearance (first pass effect) (Patil *et al.*, 2011).

The phenomenon termed collateral sensitivity (CS) defines the ability of an anticancer drug to target a MDR cell line to a greater extent, in comparison to the parental line (Hall *et al.*, 2009). Several ABC transporter substrates, acting as CS agents, can increase reactive oxygen species (ROS), causing the apoptosis of MDR cells as a result of a decreased ATP replenishment (Pluchino *et al.*, 2012). In a strategy to overcome MDR in colon cancer, the phytochemical cryptotanshinone was found to cause autophagic cell death in a ROS dependent manner (Xu *et al.*, 2017). The formulation of nanoparticles consisting of conjugated copper(II)-doxorubicin and ascorbic acid increased intracellular hydrogen peroxide, resulting in ROS production that can be used to overcome MDR (Kankala *et al.*, 2017).

In an overview, seven P-gp inhibitors researched in clinical trials were listed highlighting this as a modern approach strategy to abate MDR (Binkhathlan & Lavasanifar, 2013). The energy source used by the P-gp transporters for active efflux was targeted by developing a cationic water soluble pillar[6]arene complex with ATP (Yu *et al.*, 2016a). Thus, Yu and colleagues evaluated P-gp efflux inhibition by means of decreasing ATP hydrolysis, which resulted in efficient doxorubicin intracellular concentrations in MCF-7/ADR cells. The combination of an ATP inhibitor with paclitaxel in a liposome drug delivery system mechanistically altered the mitochondria, followed by both MDR and P-gp inhibition (Assanhou *et al.*, 2015). Several attempts to increase the therapeutic outcome of

chemotherapy by developing ABC transporter modulators have been studied. However, the therapeutic applications thereof are hindered by pharmacokinetic interfaces and toxicity (Ferreira *et al.*, 2014). Current treatment approaches in MDR cancer is further discussed in the review manuscript presented in Chapter 3.

2.4. Natural remedies

2.4.1. Introduction

An estimated 80% of the world's population living in developing countries rely primarily on ethno-botanic plant remedies for treating primary health care needs (Aziz *et al.*, 2017). Traditional knowledge and the comprehensive availability of medicinal plants contribute to their widespread use in developing countries (Ochwang'i *et al.*, 2018). The traditional use of plants for healing purposes in the Cape region of South Africa was originally initiated by the Khoi-khoi and San community (Van Wyk, 2008b). In the Eastern Cape province, seventeen plant species were found to be used traditionally in cancer management through oral consumption as decoctions or infusions, prepared form different sections of the plant (e.g. roots, bulbs and corms) (Koduru *et al.*, 2007). One known medicinal plant *Hypoxis hemerocallidea* (African potato) have been used in the treatment of cancer, and a traditional preparation technique includes the boiling of chopped corm (20 g) for 20 min to obtain a daily dose of 250 ml for oral consumption (Owira & Ojewole, 2009).

Some well-known traditionally used anticancer plants are available as commercial products, such as African potato as Harzol® (Germany) and *Sutherlandia frutescens* as Phyto nova *Sutherlandia* (Van Wyk, 2011). Some traditional Chinese herbal medicines are suggested to have anticancer properties through modifying epigenetic targets (Zhou *et al.*, 2017). Medicinal plants are considered as reservoirs, consisting of chemical entities which can contribute to the field of cancer research (Iqbal *et al.*, 2017).

The chemical constituents of plants are non-nutritive derivatives (phytochemicals) and are purported to cure or prevent a variety of health conditions (Mugomeri *et al.*, 2014). A wide range of phytochemicals, including lipophilic terpenoids, some alkaloids, steroids and triterpenes are also suggested to inhibit MRP1, P-gp and BRCP in a competitive manner (Wink *et al.*, 2012). Flavonoids and terpenoids are chemical constituents occurring in several plant extracts, which may result in herb-drug interactions through modification or inhibition of P-gp expression (Yu *et al.*, 2016b). The cytotoxicity of ardisiacrispin B, an oleanane-type triterpene saponin, induced apoptosis in CCRF-CEM leukaemia cells which mechanistically involved ROS production and the modification of caspases 8 and 9 (Mbaveng *et al.*, 2018). Sanguiin H-6 was one of six ellagitannins that inhibited cell viability of breast cancer adriamycin resistant cells (Berdowska *et al.*, 2018). The inhibition of P-gp efflux by *Stemona* alkaloids increased cellular sensitivity towards anticancer drugs (Umsumarng *et al.*, 2017).

Herbal medicines are often used in combination with western medicines, which may result in pharmacokinetic and pharmacodynamic herb-drug interactions (Oga *et al.*, 2016; Ondieki *et al.*, 2017). Pharmacokinetic interactions between doxorubicin and both capsaicin and piperine were evaluated *in vivo*, with concluding remarks of capsaicin being able to reverse MDR cancer (Kim *et al.*, 2018). The combination of the flavonoid dimer (FD18), a known P-gp modulator, with paclitaxel reduced the LCC6MDR (human breast cancer cell line) xenograft volume to a greater extent than paclitaxel alone. Furthermore, FD18 is considered a "safe" *in vivo* P-gp modulator (Yan *et al.*, 2015). Three *Aconitum* alkaloids often used in combination with chemical drugs were shown to induce the expression of multidrug resistance-associated protein 2 (MRP2) and BCRP transporters in both the digestive tract of an albino inbred mouse strain susceptibile to Friend leukemia virus B (FVB) mice and *in vitro* cell cultures, such as LS174T (human colon cancer cell line) (Wu *et al.*, 2018). Thus, the possibility of reduced efficacy can also arise with the combination of western medicine (especially MRP2 and BCRP substrates) and *Aconitum* alkaloids. In a study done by Saeed *et al.* (2016), seven South African plant extracts inhibited a P-gp hyperexpressive leukaemia cell line. Saeed and colleagues concluded that South African medicinal plants can contribute to the development of new improved strategies in combating MDR.

2.4.2. *Aloe vera*

2.4.2.1. Traditional preparation and use of *Aloe vera*

Aloe vera is also referred to as either *Aloe vulgari* or *Aloe barbadensis* (Richardson *et al.,* 2005). *Aloe vera* is a semi-tropical plant grouped under the *Asphodelaceae* botanic family and is one of approximately 250 *Aloe* species (Gupta *et al.*, 2018). One of the characteristics of the *A. vera* plant is the thick spear shaped leaves (12-16 per plant which can reach a length close to 90 cm) with saw-like diverticula around the edges (Eshun & He, 2004). The *A. vera* leaf is divided into the outer green rind (vascular bundles) and the inner pale parenchyma layer with a gel-like consistency (Dick *et al.*, 2016). The term parenchyma tissue or pulp are used to define the intact fleshy inner layer of the *A. vera* leaf, which consists of both the organelles and cell walls, yet, inside the parenchyma cells a viscous pale liquid is harboured known as the aloe gel (Sharma *et al.*, 2015). The viscosity of the pale liquid can be linked to a phytochemical named mannan (a mannose containing polysaccharide) present in the gel (Ni *et al.*, 2004). The yellow flowers of the *A. vera* plant are not claimed to have any ethno-medicinal value (Moghaddasi & Verma, 2011).

The three primary processing methods of *A. vera* leaf include the traditional hand-filleting method, whole leaf processing and total processing (Ramachandra & Rao, 2008). The traditional hand-filleting technique of processing the *A. vera* leaves involves the removal of the diverticula, the white part of the base and the pointed tip of the leaf (approximately 2.5 - 5 cm of the extended parts of the leaf) by using a sharp knife. The knife is then used to detach the rind to expose the aloe gel (Dayal

et al., 2018). During whole leaf processing, the leaves are cut into several sections while the base and extended tip segments are removed; followed by the grinding or crushing of the remaining whole *A. vera* leaf sections, producing a liquid soup which is then filtered and stabilised resulting in an increased bio-active aloe juice product (Pandey & Singh, 2016). Total processed *A. vera* product is a combination of the green rinds and the aloe gel which provides a product containing less unwanted laxative anthraquinones, (Ramachandra & Rao, 2008).

The ethno-botanic plant *Aloe vera* has been used as a traditional medicinal remedy for more than 2000 years. However, this plant is still used currently as an important medicinal component in India, Japan and China (Boudreau & Beland, 2006). *A. vera* is considered a "wonder plant" and is deemed to be beneficial for treating diabetes, arthritis, heart diseases and cancer, with additional antioxidant and hepatic protective properties (Nazir & Ahsan, 2017). *A. vera* is used topical to treat abrasions and orally for constipation. Moreover, *A. vera* contributes to the cosmetic industry, with reports claiming the improvement of skin hydration (Dal'Belo *et al.*, 2006; Ünlü *et al.*, 2016). In a single-blind clinical trial, *A. vera* gel was injected into the periodontal area of patients with periodontitis, during which *A. vera* gel was suggested as an adjunctive treatment option (Moghaddam *et al.*, 2017). A crude polysaccharide fraction prepared from *A. vera* indicated antioxidant properties, with suggests a cosmetic application (Kang *et al.*, 2014). The medicinal value of *A. vera* is attributed to the polysaccharides present in the parenchyma, yet, the bioactivity can be apportioned to synergistic activity of different constituents (Radha & Laxmipriya, 2015).

2.4.2.2. Biologically active constituents of *Aloe vera*

Water is a major constituent of *A. vera,* given that it represents 98.8% of the aloe leaf pulp and 99.5% of the aloe gel (Eshun & He, 2004). The most studied chemical components of *A. vera* include polysaccharides, amino acids, vitamins, enzymes, anthraquinones and low molecular weight constituents (Choi & Chung, 2003). The abovementioned constituents are listed in Table 2.2. The gel-like consistency of the leaf is suggested to contain the following: zinc, magnesium, vitamins (A, C, E), saponins, salicylic acid, polysaccharides, amino acids and sterols. However, the latex leaf lining primarily contains several anthraquinone glucosides (Moghaddasi & Verma, 2011).

Table 2.2: A summary of the chemical constituents inside *Aloe vera* leaf (Choi & Chung, 2003; Hamman, 2008)

Chemical constituent	Component
Inorganic components	Magnesium, calcium, copper
Polysaccharides	Acemanna (acetylated mannan), mannose derivatives, pectins
Amino acids	Glutamic acid, histidine, leucine, arginine

Vitamins	Vitamin B(1,2,6), vitamin C, folic acid, α-tocopherol, choline
Enzymes	Catalase, oxidase, lipase, amylase, carboxypeptidase
Anthraquinones	Emodin, aloin A and B, anthranol, aloe-emodin, isobarbaloin
Low molecular weight constituents	Salicylic acid, β-sitosterol, lectin-like substance, lignins, cholesterol

2.4.2.3. Cancer research involving *Aloe vera*

The initiation of apoptosis in a human hepatocellular carcinoma cell line was detected during treatment with *A. vera,* demonstrating the cytotoxic and genotoxic effects of this plant (Shalabi *et al.*, 2015). A possible anticancer mechanism of *A. vera* gel involves the down regulation of new blood vessel formation in tumours, even though no reduction in tumour volume have been found in an *in vivo* induced L-1 sarcoma mouse study (Kocik *et al.*, 2014). *A. vera* was found to have chemopreventive properties that were associated with lectins, glycoproteins and polysaccharides (Singh *et al.*, 2000). In a study, the crude modified *Aloe* polysaccharides of *A. vera* gel showed increased *in vivo* antitumour activity from a 400 – 5 KDa mass (Im *et al.*, 2005). Extracted polysaccharides showed *in vivo* and *in vitro* alteration of benzo[a]pyrene diol epoxide I DNA adduct formation, which suggests that the fractionated polysaccharides are chemopreventive and antigenotoxic (Kim & Lee, 1997). The extracted di(2-ethylhexyl)phthalate component from *A. vera* Linne was found to have antimutagenic activity and reduced the cellular growth of three human leukemic cell lines (Lee *et al.*, 2000).

Emodin, a known anthraquinone also present in Chinese herbal medicine, was found to reduce glutathione, hence, an increased platinum-drug efficacy and reduced MRP1 expression in a human gallbladder cell line were detected (Wang *et al.*, 2010). Emodin used in combination with cisplatin resulted in both increased ROS levels and chemosensitivity in an MDR prostate carcinoma cell line (Huang *et al.*, 2008). In two human liver cancer cell lines (HepG2 and Hep3B), Aloe-emodin induced apoptosis and was further suggested as a chemopreventive phytochemical (Kuo *et al.*, 2002). In another study, Aloe-emodin and emodin demonstrated cytotoxicity through induction of apoptosis in both lung carcinoma and human lung squamous carcinoma cell lines (Lee, 2001). The ability of emodin and Aloe-emodin to alter DNA formation was detected against human tongue cancer cells, and occurred in a dose dependent manner (Chen *et al.*, 2010). Thus the above mentioned studies suggest the phytochemicals present in *A. vera*, including emodin and Aloe-emodin, have promising anticancer effects.

2.4.3. *Sutherlandia frutescens*

2.4.3.1. Traditional preparation and use of *Sutherlandia frutescens*

Sutherlandia frutescens is grouped under the *Fabaceae* botanic family and is indigenously known as “cancer bush” (Van Wyk *et al.*, 2009). *S. frutescens* is a shrub that can reach a height between 0.2-2.5 m, and is recognised by the prominent butterfly-shaped red flowers and hairy leaves with an almost greenish-grey appearance (Van Wyk & Albrecht, 2008; Aboyade *et al.*, 2014). *S. frutescens* is indigenous to South Africa (mainly KwaZulu-Natal and the northern-, western- and eastern Cape), Lesotho, South-Eastern Botswana and southern Namibia (Aboyade *et al.*, 2014; Van der Walt *et al.*, 2016). The wide distribution of *S. frutescens* led to variance in the genetic and chemical makeup of this plant across these geographical areas (Aboyade *et al.*, 2014). Common names given to *S. frutescens* include turkey bush, duckling (English), kortbeenwildekeur, kankerbos (Afrikaans) and phetola (Tswana) (Van Wyk *et al.*, 2008; Aboyade *et al.*, 2014).

The name “cancer bush” originated from traditional beliefs that *S. frutescens* can be used as a treatment option in cancer management (Fasinu *et al.*, 2013). This multi-purpose plant can be used for treating viral diseases including HIV/AIDS, inflammatory conditions, gastritis, diabetes, anxiety, fever and skin disorders (Atawodi, 2005; Van Wyk, 2008a; Müller *et al.*, 2013). Commercial *S. frutescens* capsules filled with 300 mg dried, powdered leaves are recommended to be taken twice a day orally, and are available in herbal shops all over South Africa (Van Wyk & Albrecht, 2008). A clinical study evaluating the safety of powdered *S. frutescens* leave capsules have reported a well-tolerated daily dosage of 800 mg (Fu *et al.*, 2008). Traditionally, a *S. frutescens* tea solution is consumed orally to treat stomach problems (Van Wyk *et al.*, 2008). A decoction prepared by traditional healers can consist of the flowers, stem, leaves and the roots, depending on the patient’s needs and medical condition (Aboyade *et al.*, 2014).

2.4.3.2. Biologically active constituents of *Sutherlandia frutescens*

The leaves of *S. frutescens* contain the following phytochemicals: γ-aminobutyric acid (GABA), L-canavanine, flavonoids, pinitol, triterpenoid saponins and other compounds (Van Wyk & Albrecht, 2008; Van Wyk *et al.*, 2009). In the brain, GABA acts as an inhibitory neurotransmitter that supports the use of *S. frutescens* for anxiety. Furthermore, GABA has been shown to have anticancer properties by inhibiting SW 480 colon carcinoma cell migration (Joseph *et al.*, 2002; Van Wyk & Albrecht, 2008). The analogue form of L-arginine is L-canavanine, a non-protein amino acid which modulates ribosomal protein biosynthesis by means of changing the 3D conformation and functionality of the formed protein (Nurcahyanti & Wink, 2015). Hence, the ability of L-canavanine to alter ribosomal protein biosynthesis inhibits cell growth; leading to cell death (Nurcahyanti & Wink, 2015). The flavonoids (sutherlandins A-D) present in *S. frutescens* are grouped according to their preliminary chemical structure as flavonol glycosides (Van Wyk & Albrecht, 2008; Avula *et al.*, 2010).

Pinitol is claimed to have an insulin-like action by improving glycaemic control and stimulates glucose uptake, which supports the traditional use of *S. frutescens* for diabetes (Bates *et al.*, 2000; Aboyade *et al.*, 2014). The main triterpene glycoside detected in commercial *Sutherlandia* plant material is a cycloartane-type triterpene glycoside known as SU1 (Van Wyk & Albrecht, 2008). The potential of SU1 to act as a chemo-preventive agent in chemical carcinogenesis is result of a hydroxy group on the C-24 atom (Stander *et al.*, 2009). Other phytochemicals including stigmast-4-en-3-one, γ-sitosterol, hexadecanoic acid and unidentified polysaccharides, have also been found in *S. frutescens* (Van Wyk & Albrecht, 2008).

2.4.3.3. Cancer research involving *Sutherlandia frutescens*

The isolation of a single phytochemical responsible for all the multi-purpose medicinal uses of *S. frutescens* has not been reported. However, the cumulative combination of several bioactive phytochemicals in *S. frutescens* are purported to contribute to an antiproliferative effect (Vorster *et al.*, 2012). The antiproliferative effect of *S. frutescens* was shown through *in vitro* studies in human leukemia Jurkat cells, human promyelocyte HL60 cells, human mammary adenocarcinoma MDA-MB-468 and MCF7 cells (Tai *et al.*, 2004). An *S. frutescens* methanol extract impaired the growth of human prostate cancer cells and mouse prostate cancer cells in a time-and-dose dependent manner (Lin *et al.*, 2016). In the same study using the *S. frutescens* methanol extract, a dose dependent impairment of the Gli-reporter activity in TRAMP-C2QGli and Shh Light II cells was shown (Lin *et al.*, 2016). The inhibition of Gli/ hedgehog signalling in prostate cancer cells was found to suppress cancer cell proliferation (Lu *et al.*, 2015).

The anticancer activity of an aqueous *S. frutescens* extract against a non-tumorigenic breast cell line (MCF-12A) and an adenocarcinoma epithelial cell line (MCF-7), was detected as the induction of cellular death through autophagy and apoptosis (Mqoco *et al.*, 2014). Programmed cell death or apoptosis is defined as the activation of the cellular suicide mechanism, which results in the elimination of unwanted cells (Chinkwo, 2005). An *S. frutescens* extract showed induction of apoptosis in Jurkat T lymphoma cells, Chinese hamster ovary (CHO) cells and Caski cells via the flip-flop translocation of the membrane protein phosphatidylserine (Stander *et al.*, 2009). Phosphatidylserine is an amino phospholipid forming part of the cytoplasmic surface of mammillary plasma membranes, hence the flip-flop movement of phosphatidylserine disturbs the asymmetry of the cellular membrane (Sasaki *et al.*, 2004). The effect of the translocation of phosphatidylserine through the flip-flop effect also resulted in the loss of mitochondrial membrane potential (Barroso *et al.*, 2006).

The anticancer treatment of MDR cancer cells may require it to overcome a hyperexpressed barrier of several efflux transporters. An *S. frutescens* extract (IC_{50} of 324.8 µg/ml) contributed to an increased therapeutic effect by inhibiting P-gp efflux (Ondieki *et al.*, 2017). The P-gp efflux inhibitory

effect was also confirmed in a study using a 100 mg/ml aqueous *S. frutescens* extract (Müller *et al.*, 2012). The phytochemical L-canavanine significantly inhibited nevirapine efflux when the transport of this antiretroviral drug was tested in Caco-2 cells (Brown *et al.*, 2008). A triterpenoid glucoside, known as sutherlandioside B, was shown to be an MRP and P-gp substrate with the ability to inhibit both MRP2 and P-gp efflux of the antiretroviral drug atazanavir (Müller *et al.*, 2012).

2.4.4. *Xysmalobium undulatum*

2.4.4.1. Traditional preparation and use of *Xysmalobium undulatum*

Xysmalobium undulatum forms part of the *Apocynaceae* botanic family and is commonly known as uzara, milkwort (English), melkbos, bitterwortel (Afrikaans), ishongwane and iyeza elimhlophe (Xhosa) (Van Wyk *et al.*, 2009; Balogun *et al.*, 2016). The revision of *Apocynaceae* resulted in the inclusion of the *Asclepiadaceae* family into the *Apocynaceae* family (Madani *et al.*, 2017). Geographically *X. undulatum* is distributed in the savannah region of southern Africa and the prevalence of this plant can be seasonal, depending on the regional rainfall patterns (Van Wyk *et al.*, 2009). The identifying characteristics of *X. undulatum* include the green-creamy almost yellow flowers, hairy heart shaped leaves and a rootstock reaching a height between 0.5 - 2 m (Bester, 2009). A decoction of the bitter rootstock is traditionally used to treat afterbirth cramps, diarrhoea and colic (Van Wyk, 2008a). The traditional preparation of *X. undulatum* is done through boiling 125 ml of the powdered rootstock in 1 l water; forming a decoction that can be orally consumed and taken as a 125 ml dosage twice a day (Van Wyk *et al.*, 2009). Powdered rootstock is also traditionally sniffed to treat hysteria and annoyance (headache), or applied topically for wound healing (Van Wyk, 2008a).

2.4.4.2. Biologically active constituents of *Xysmalobium undulatum*

The major glycosides present in the rootstock of *X. undulatum* include xysmalorin, uzarin and their H-17 β-isomers namely alloxysmalorin and allouzarin (Ghorbani *et al.*, 1996). Minor phytochemicals include xysmalogenin and uzarigenin known as cardenolide aglycones (Vermaak *et al.*, 2014). The cardiac glycoside, uzarin, has also been detected in *Gomphocarpus fruticosus* (*Apocynaceae*) and *Asclepias curassavica* (*Asclepiadaceae*) (Roy *et al.*, 2005; Baskar *et al.*, 2012; Krishna *et al.*, 2015). Toxic cardiac glycosides have been reported to occur in *G. fruticosus*, which is responsible for livestock poisoning (Fouché *et al.*, 2006). Cardenolides can inhibit cell death and enhance proliferation in normal cells, nevertheless, the modulation of cancer cells through cardenolides differ and is considered a promising anticancer approach (Krishna *et al.*, 2015). Supporting the proposed effects on cancer cells, the medicinal use of *A. curassavica* include treating abdominal tumours (Baskar *et al.*, 2012).

2.4.4.3. Cancer research involving *Xysmalobium undulatum*

The botanical plant family *Apocynaceae* (*Ascleipiadaceae*) is known to be toxic. The cytotoxic effects of this family is associated with the presence of cardenolide constituents (Mothana *et al.*, 2009; Agrawal *et al.*, 2012). A possible anticancer target of cardenolides includes the sodium-potassium pump (Na+/K+-ATPase), given that cardenolides are a natural Na+/K+-ATPase ligand affecting numerous signalling cascades which can suppress tumour growth (Krishna *et al.*, 2015). Cardenolides inhibit the ubiquitous mammillary Na+/K+-ATPase and disturb the cationic transport of sodium (Na+) and potassium (K+) across the cell membrane, disrupting the membrane potential and secondary cellular transport (Agrawal *et al.*, 2012). Cardenolides can be considered a substrate for P-gp due to the suggested efflux of cardenolides entering intestinal cells (Agrawal *et al.*, 2012). In a study conducted by Delebinski *et al.* (2015), the anticancer property of uzarin was tested at 100 µM, no antiproliferative or apoptotic effect was observed against an osteosarcoma (143B) cell line. A dose- and time dependent antiproliferative effect of *A. curassavica* in an ethyl acetate extract form was detected against several human tumour cells (MCF-7, COLO 320 DM, AGS and A549) (Baskar *et al.*, 2012). A crude *X. undulatum* aqueous extract demonstrated antiproliferating efficacy towards a hepatocellular carcinoma cell line (Calitz *et al.*, 2018). Therefore, the anticancer properties of uzarin still need to be investigated in the plant material of *X. undulatum*, due to the possibility of the *Apocynaceae* family having anticancer effects.

2.5. Summary

One of the major challenges in lung cancer treatment is the potential hyperexpression of ABC efflux transporters. These transporters show cross-resistance to a variety of anticancer drugs, altering their intracellular accumulation and decreasing their therapeutic efficacy. One of the approaches to increase the therapeutic response in cancer patients is to inhibit these efflux transporters in an effort to reduce drug efflux and increase drug accumulation. Several studies included the use of medicinal plants to target efflux transporters in cancer cells. Traditional medicinal remedies, which are claimed to prevent or treat cancer are still widely used in modern society, yet, evidence supporting these beliefs are lacking. To evaluate the potential anticancer and cytotoxic effects of three selected medicinal plants (*A. vera*, *S. frutescens* and *X. undulatum*) a panel of SCLC cell lines with varying resistance profiles, as well as a non-tumorigenic cell line, were used. This panel of SCLC cells consist of a chemosensitive, a MRP1 hyperexpressive and a P-gp hyperexpressive cell line, which were used to determine the ability of the selected plants to reduce MDR in cancer.

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Chapter 3

Review manuscript

Chapter 3 consist of a review article submitted for publication in *Current Cancer Drug Targets*. The manuscript was prepared as prescritbed by the specific author guideline of *Current Cancer Drug Targets,* as presented in Appendix A.

Efflux-Based Models for Multidrug Resistant Cancer Treatment Screening

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Abstract: Efflux transporters of the adenosine triphosphate (ATP)-binding cassette (ABC)-superfamily play an important role in the development of multidrug resistance (MDR) in cancer. The overexpression of these transporters can directly contribute to the failure of chemotherapeutic drugs. Several *in vitro* and *in vivo* models exist to screen for the efficacy of chemotherapeutic drugs against multidrug resistant cancer, facilitated by efflux transporters. This article reviews a range of efflux-based models used to test the efficacy of compounds to overcome MDR in cancer. These models are classified as either *in vitro* or *in vivo* and are categorised from the most basic, conventional models to more complex and advanced systems. Each model's origin, advantages and limitations, as well as specific efflux-based MDR applications are discussed. Accordingly, future modifications to existing models or new research approaches are suggested to develop prototypes that closely resemble the true nature of multidrug resistant cancer in the human body. It is evident from this review that a combination of both *in vitro* and *in vivo* preclinical models can provide a better understanding of cancer itself, than using a single model only.

Keywords: Cancer, efflux transporters, genetically engineered mouse models, multidrug resistance, preclinical screening model, two dimensional and three dimensional cell culture, xenograft.

1. INTRODUCTION

According to the International Agency for Research on Cancer, 18.1 million new cancer cases were registered around the world in 2018. The estimated number of deaths due to cancer in 2018 were approximately 9.6 million and this number is expected to increase to 29.5 million by 2040 (https://gco.iarc.fr/), mainly because of phenomena such as multidrug resistance (MDR). Multidrug resistance is defined as the ability of a living cell to display resistance to multiple unrelated anticancer drugs that are structurally and/or functionally different [1]. To date, MDR has been shown to develop via cell death inhibition, drug inactivation, deoxyribonucleic acid (DNA) damage repair, drug target alteration, the epithelial-mesenchymal transition and epigenetic modifications, but the most widely studied MDR mechanism is that of drug efflux involving adenosine triphosphate (ATP)-binding cassette (ABC) membrane transporters [2-3]. At least 49 structurally related transporters, known collectively as the ABC-superfamily, have been identified and categorised into 7 subfamilies. Of these, 16 are primarily involved in human diseases [4], of which P-glycoprotein (P-gp; MDR1/ABCB1), multidrug resistance-associated protein 1 (MRP1/ABCC1) and breast cancer resistance protein (BCRP1/ABCG2) are the major drug transporters widely implicated in drug resistant cancers [5-6]. P-gp, MRP1 and BCRP1 are present in the epithelial lining of the lungs, testes, skin, kidneys, small intestines and in heart and muscle tissues, acting as a barrier against many xenobiotics [7-8]. Through staining, P-gp and BCRP1 have been observed predominantly in the apical side of certain epithelium, whereas MRP1 has a basolateral cell localisation [9]. All three transporters have significantly overlapping resistance profiles [9], but MRP1 does not confer resistance to taxanes and some classes of tyrosine kinase inhibitors [10-11], while BCRP1 is not inhibited by arylmethyloxy-derivatives [12].

The more ABC transporters are expressed in the cancer tissue, the lower the chance will be to have effective anticancer results with chemotherapy. For example, a recent study on children with acute myeloid leukaemia found a strong association between the relapse of patients and the number of overexpressed ABC transporters such as ABCA3, ABCB1, ABCC3 and ABCG2 in the cancer cells [13]. Furthermore, a compensatory effect between different ABC transporters has been proposed in the sense that the down-regulation of one transporter is interchanged by the up-regulation of another [14-15]. This highlights the challenge with the development of new anticancer drugs that should be able to deal with complex interactions between different efflux transporters.

This review presents and discusses the different *in vitro* and *in vivo* models currently available to evaluate the treatment of efflux-based MDR in cancer. The *in vitro* models that are discussed include both conventional cell culture-based models cultured as basic two-dimensional (2D) flat cultures and more complex cell-based models that make use of specialised matrices or objects to culture non-conventional or three-dimensional (3D) models. The *in vivo* models that are discussed include cell- and patient-derived xenografts as well as genetically engineered mouse models (GEMMs). In addition to the discussions of these selected models, a comprehensive summary of both *in vitro* (Table **1**) and *in vivo* models (Table **2**) are given which have been used in previous studies to investigate the anticancer efficacy of compounds by influencing efflux transporter activity and/or expression. Lastly, a few suggestions are provided on future modifications that can be made to existing models, as well as newly developed approaches, that can accurately imitate the true drug response of tumours occurring in the human body.

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2. MULTIDRUG RESISTANT CANCER TREATMENT APPROACHES

Common treatments of cancer include surgery, radiation therapy and chemotherapy, or combinations thereof [16]. Chemotherapy specifically is important in the treatment of metastatic and haematological malignancies [17]. The phenomenon of MDR is, however, a major challenge causing failures in chemotherapy, but the mechanisms responsible for both intrinsic and acquired MDR also provide potential targets for new anticancer drugs to overcome MDR. Therefore, many innovative therapeutic strategies focus specifically on reversing, suppressing or evading MDR mechanisms in tumours [1, 3, 18].

Inhibition of efflux transporters, specifically P-gp, by co-administration of transporter inhibitors has been explored as a treatment strategy to overcome MDR. Different P-gp inhibitors have been investigated (including first, second and third generation inhibitors) by using *in silico*, *in vitro* and *in vivo* models [2-3,19]. However, many clinical trials that tested the efficacy of co-administration of P-gp inhibitors with anticancer drugs could not provide sufficient evidence that this strategy could overcome MDR in most cancers. Several reasons were given for this outcome such as associated toxicity, poor planning or lack of randomisation in clinical trial design and the fact that little effort was made to determine if the tumours in the subjects did actually express P-gp [20]. Besides competitive and non-competitive inhibition of P-gp, modulation of P-gp expression has also been investigated as a strategy to overcome chemoresistance. Several signalling pathways that modulate P-gp expression in cancer cells have been targeted to regulate P-gp function and expression. However, P-gp modulators appeared to have selective efficacy depending on the specific anticancer drug that it was combined with [21]. Despite the failure to produce significant improvements in the treatment of multidrug resistant cancer with co-administering efflux transporter inhibitors, research relevant to this approach remains ongoing. In a recent study, it was shown that combining an approved anticancer drug that suppresses efflux activity (i.e. regorafenib) with another anticancer drug (i.e. paclitaxel), resulted in increased concentrations of paclitaxel in the tumours and thereby provided a synergistic anti-tumour effect [2].

Some of the other strategies that have been attempted to overcome MDR in chemotherapy, besides efflux inhibition, are briefly outlined here. Collateral sensitivity is the use of compounds that selectively kill multidrug resistant cells without killing the non-resistant parental cells from which they were derived. The genetic alterations accrued by cancer cells to obtain resistance against one compound may provide hypersensitivity towards another compound, which can be exploited. An important aspect of this strategy is that its efficacy is independent of other mechanisms of MDR in cancer cells [17]. Another strategy that was proposed to overcome MDR is ribonucleic acid (RNA) interference (RNAi) therapy. In brief, this approach aims to silence or down-regulate the expression of specific genes by means of destructing mRNA molecules as triggered by other RNA molecules [3]. Metronomic chemotherapy is the long-term administration of low, but active, concentrations of chemotherapeutic drugs on a frequent basis that target endothelial cells and thereby provide an anti-angiogenic effect [22]. The combination of metronomic chemotherapy and immunotherapy exhibited promising results in both preclinical and clinical studies, but more research is necessary to be conclusive [23]. Microparticles have been identified as vectors to spread MDR to drug sensitive cancer cells by means of a non-genetic mechanism [24]. Compounds that are capable of inhibiting formation of microparticles are under investigation to potentially overcome MDR in cancer [25]. Replication competent viruses have been used to kill cancer cells without harming normal cells. In order to improve the anticancer activities of oncolytic viruses, they can be engineered to express exogenous transgenes for tumour proteins (e.g. tumour protein p53) [26]. Several models are available to study the anti-tumour effect of potential compounds in an attempt to overcome MDR caused by efflux transporter up-regulation.

3. *IN VITRO* MODELS FOR MULTIDRUG RESISTANT CANCER TREATMENT SCREENING

For the screening of the effectiveness of anticancer drugs, it is essential to use a viable test system that truly represents the disease as it appears in human subjects. Systems used as preclinical screening models include, among others, *in vitro* approaches such as cultured stem cells or other unicellular or multicellular cell lines [27-29]. Cultured human cancer cells form a strong pillar in anticancer drug screening and an exhaustive number of high-throughput assays and types of cellular material are available for measuring anticancer effects [30-31]. Furthermore, it is relatively easy to perform genetic manipulation of these cells that can provide information on the genetic mutations occurring in cancer cells that correlates with tumour tissues [31-33]. Essentially, *in vitro* approaches refine preclinical results before advancing to animal and human trials, thus reducing ethical concerns, time and cost. *In vitro* cell culture-based models also have certain drawbacks as outlined in the next sections.

Cultured cell models can range from very simple systems to very intricate and complex models consisting of multiple cell types (i.e. co-cultures), additional matrices or mechanical support. For the purpose of this review, the *in vitro* cell-based models used to screen for the anticancer efficacy of efflux-targeting MDR treatments are divided into conventional (basic mammalian cell lines cultured as 2D or flat cultures) and complex (mammalian cell lines cultured with additional matrices, foreign objects or as 3D cultures) cell-based models.

3.1. Conventional cell-based models

For the purpose of this review, “conventional models” include all adherent cell cultures which are grown in an artificial environment in a 2D state as a monolayer or flat culture, although some suspension cells can also be classified as conventional cell-based models. Conventional mammalian cell culture models have served as the primary pillar for the evaluation of tumour biology, drug screening and mechanistic studies [30].

The introduction of cell cultures in 1950 provided an exciting new method for cancer drug screening in an *in vitro* environment [34]. Retrospectively, during the mid-1970's the Chinese hamster ovary cell line was the first cell model to indicate a correlation between the drug-resistant phenotype and the drug efflux P-gp membrane transporter, which was indicated by a change in the drug permeation rate [35]. Drug resistance was induced in the cultured cells through exposure to actinomycin D, which resulted in a 2500-fold increase in resistance compared to the parental cell line [36]. In the 1980's, the United States National Cancer Institute (NCI) listed 60 human tumour cell lines which can be used for *in vitro* drug screening and discovery [37], which is available online on the NCI's website. These cell lines encompass nine different tumour organs/systems including blood, prostate, colon, central nervous system, ovaries, kidney, skin, breast and lung. This NCI-60 cell panel is considered an important platform for high-throughput models for characterisation and a biologically based approach to investigate treatment of malignancies [38].

The initial technique used in developing the drug-resistant Chinese hamster cell model in the mid-1970's is still used in modern cell culture laboratories, which entails continuous exposure of a parental cell line to an anticancer drug that can result in a drug-resistant daughter cell line. Furthermore, conventional efflux cell culture models and modified cell culture models are used together with suitable assays during drug screening to investigate MDR. Determination of P-gp protein levels can be done through a Western blot assay [39]. Several other cell viability, proliferation and cytotoxicity assays (e.g. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)), bidirectional transport studies and clonogenic assays are commonly used to compare the sensitivity or resistance of both the parental and daughter cell lines towards anticancer drugs [40-43]. Furthermore, the inhibition of ABC transporters and the extent of substrate efflux can be determined through fluorescent dye accumulation and vesicular transport assays [44-45].

The effect of the ABC transporter family on chemotherapeutics, and potential inhibitors of these transporters, has been studied using the conventional cell lines as listed by the NCI (see Table **1**). Human leukaemia K562 and K562/Dox cells were used to evaluate the influence of metformin on the P-gp efflux of doxorubicin and the cytotoxicity of doxorubicin was shown to increase in the presence of metformin [43]. A human embryonic kidney (HEK293) cell line was used to modify an inverted membrane vesicle and estradiol-17-β-glucuronide was subsequently identified as a substrate for MRP2, P-gp and BCRP. This shared substrate was then used to determine the transport inhibition ability of nine inhibitors, showing different inhibitory properties towards the studied ABC transporters [46]. The ability of cordycepin, a phytochemical in dietary mushrooms, to modulate the functionality and expression of MRP1, P-gp and BCRP transporter proteins was assessed in the following cell lines: MDR human non-small cell lung carcinoma (NSCLC) cell line NCI-H460/MX20, MDR human hepatocellular carcinoma cell line HepG2-vin and MDR human cervical cancer cell lineKB-vin. The findings purported cordycepin to influence P-gp ATPase activity, resulting in the inhibition of the P-gp transporter and thereby illustrated cordycepin as a promising substance to be used in combination with chemotherapy [47].

In a study conducted by Kim *et al*. [48], a doxorubicin resistant breast cancer cell line (MCF-7/ADR) was obtained by exposing the parental MCF-7 cell line to 0.3 μM doxorubicin (Adriamycin®) over an extended period of time. The combined effect of doxorubicin and resveratrol subsequently decreased the gene expression of MDR1 and MRP1 in this breast cancer model, which simultaneously increased the intracellular doxorubicin concentration. The same MDR MCF-7/ADR model was also used to establish a novel micelle-based drug delivery system to increase the uptake of paclitaxel into the tumour tissue [49]. The MDR MCF-7/ADR cell line is one of the most widely used conventional models in cancer research, although its true origin is still questionable. Therefore, it should rather be used for hormone-responsive breast cancer studies due to its expression of oestrogen and progesterone receptors [50-51].

The cytotoxic ability of fascaplysin (a natural product with anticancer activity) was screened between 134 and 1 740 nM against a panel of cell lines including NSCLC, small cell lung cancer (SCLC), unrelated cell lines and the HEK293 cell line. The concluding remarks suggested the alteration of reactive oxygen species (ROS), topoisomerase I and poly-(adenosine diphosphate (ADP)-ribose) polymerase (PARP) activity through the influence of several cellular mechanisms [42]. Other conventional cell models, including a cell clone of human epidermoid carcinoma (KB-3-1), namely KB-CV60, were used to evaluate the ability of sulindac sulfide to inhibit MRP1 efflux [44]. More recently, it was shown that the efflux of doxorubicin and rhodamine 123, through ABCB1 and ABCG2 transporters, was inhibited by abemaciclib in hyper-expressing cell lines such as KB, MCF-7, the human colon carcinoma cell line S1 and HEK293 cells [52].

Contributing further to the development of drug screening models is the transfection of a parent cell line with microRNA or small interfering RNA (siRNA). These models enable targeted alteration of the transporter expression of the parental line in the hope of modifying the extent of drug efflux, resulting in anticancer drug accumulation in the tumour and consequently increased therapeutic effects [53-54]. However, the shortcomings of using nucleic acid (RNA and DNA) interference therapies include ineffective tumour targeting, reduced RNA stability and short circulation time, which limit its clinical use [55]. In a study conducted by Yang *et al*. [56], a co-delivery system containing both siRNA (to silence MDR1 mRNA) and paclitaxel, showed increased cytotoxicity of paclitaxel in MCF-7 and MCF7/taxol cells. The ovarian carcinoma cell line (SKOV-3) was used to evaluate the effectiveness of a nanoscale metal-organic framework co-delivery system of siRNA and cisplatin, to decrease the expression of MDR genes, and an increased chemotherapeutic efficacy was observed [57]. More recently, a hollow carbon nanosphere particle was used to treat MCF-7 and MCF-7/ADR cells by delivering either cisplatin or doxorubicin, in combination with siRNAs targeting MDR1. The findings included a down regulation

of up to 96% of MDR1 protein expression in the MCF-7/ADR cells [58].

Resistance in a paclitaxel resistant human colon cell line (HCT 116) was reduced through incorporation of paclitaxel into a nanoparticle delivery system [59]. Furthermore, an exosome-encapsulated paclitaxel delivery system was tested in P-gp expressing Madin-Darby canine kidney (MDCK) and RAW 264.7 macrophage cells, indicating a potential strategy for future chemotherapeutic treatment [48]. The screening of a multifunctional nanoparticle containing paclitaxel and curcumin was recently performed on a breast cancer cell line expressing P-gp (MCF-7/ADR). This delivery system was able to inhibit the P-gp mediated efflux of paclitaxel, thus increasing its intracellular accumulation [60].

In a sub-clone of human colorectal adenocarcinoma cells (Caco-2; sub-clone C2BBe1), the zinc finger nuclease-mediated gene knockout approach was used to reduce the extent of transport facilitated by several ABC transporters. This subsequently produced a successful tool for explaining transport interactions [61]. Human cervix carcinoma (HeLa) cells have also been used for transcription activator-like effector nucleases (TALEN)-mediated Nanog gene knockout, resulting in weakened malignancy [62].

Although continuous 2D cell lines are easily maintained and the same cell line can be used for long-term experimental evaluations, these models do not fully correlate with *in vivo* conditions [63]. The monolayer cellular growth pattern of conventional cells is a problem, since few primary tumours can grow as a monolayer *ex vivo* [64]. Other drawbacks of conventional cell models are the absence of 3D architecture, limited cell-cell and cell-extracellular matrix interactions and uncontrolled exponential growth [65-67]. All these drawbacks add to the need for development of more advanced cell culture systems in an attempt to overcome these limitations.

3.2. Complex cell-based models

The lack of models that consider and include the organisational physiological structure of human tissues is deemed the major precursor of clinical drug failure during *in vivo* trials [68-69], while large scale screening of anticancer drug compounds in *in vivo* models (e.g. rodent models) is for the most part unpractical and subject to ethical scrutiny [70-71]. Intrinsic limitations of conventional models cause isolation of certain characteristics, thus ignoring major pharmacokinetic parameters. This inability to accurately extrapolate to humans caused a major void in clinical trials and eventually stimulated the design of more complex systems resulting in advanced *in vitro* models [72]. These models are designed to better reproduce the primary elements of MDR, allowing better screening of drug compounds before they advance to the later stages of clinical trials. For the purpose of this review, advanced cell culture models are defined as mammalian cells incorporated into advanced systems with more realistic intercellular communications and other features that better resemble those of the *in vivo* microenvironment. Table **1** provides some examples of complex cell-based models.

3.2.1. Integrated discrete multiple organ culture (IdMOC) system:

The integrated discrete multiple organ culture (IdMOC) system was designed to consist of individual wells, each containing their own cell culture and growth medium, embedded in a larger outer chamber [73]. Once the cells in each well reached experimental viability, a universal medium containing the test compound is added to such an extent that it overflows into all the wells and fills up the outer chamber, thereby creating an interconnected system. This universal medium serves as a blood surrogate between the different cells in the system, containing metabolites and waste products produced by each organ-representative well, and thereby mimic the *in vivo* state by exposing the blood surrogate to every part of the system. This overcomes the problem of cell type isolation that is present in traditional 2D cell culture studies. The universal medium can be analysed to determine accumulative system metabolism, whereas each individual cell culture can be individually analysed for organ toxicity, bio-accumulation and cytotoxicity [74-77]. The IdMOC system was designed to incorporate primary hepatocytes, cell cultures representing several organs and a cancer cell line to allow more effective testing of potential anticancer drugs on multiple organ systems [78]. This model currently permits for the screening of organ selective toxicity, cytotoxicity, drug-drug interactions and metabolic activation and also enables analyses of pharmacokinetic parameters (e.g. MDR1 and MRP1 influence) [73].

The major advantages of the IdMOC system include the ability to do high-throughput screening assays, retaining the homogeneity of the individual culture conditions of each cell line and the possibility of inter-cell metabolite interactions between each organ-representative. The model also functions as a low cost alternative for screening with an adaptable microenvironment to optimise growth conditions for each individual cell type. A major problem with this system, however, is the elevated risk for cross contamination. The model is also not designed to homogeneously spread universal medium through the system like a classic blood circulatory system, which makes this model vulnerable to criticism. Another restriction lies in the disparity of the metabolic needs of the various cultures, potentially causing saturation or starvation of some of the cells receiving universal medium [77, 79]. Time-dependent interactions between the organs are also not simulated, reducing this model's capability of giving exact quantitative results [80].

The application of the IdMOC system in MDR studies was suggested by Uzgare and Li [79] in a review of the IdMOC model. Targeted disruption of specific transporters responsible for MDR (e.g. MDR1, MRP1, etc.), and the pharmacokinetic consequences thereof can be investigated in this model due to the incorporation of liver cells [79]. A cytotoxicity study on tamoxifen was completed using the IdMOC system incorporating primary cells of different origin namely hepatocytes (liver), cortical cells (kidney), small airway epithelial cells (lung), astrocytes (central nervous system), aortic endothelial cells (blood vessels) and the MCF-7 human breast adenocarcinoma line. This allowed for the effective

quantification of cytotoxicity in normal cells compared to cancer cells. Each individual organ system provided results comparable to those seen in literature during human trial cytotoxicity studies, thus validating this model [79, 81].

3.2.2. Microfluidic channel-based systems:

The idea of microfluidic systems was conceptualised around the design of a system encapsulating complicated, time intensive and expensive laboratory protocols into a relatively inexpensive, portable cell culture model that involves the manipulation of relatively small amounts of fluid (microlitre or picolitre) in a channel-based system on a chip [82-83]. Microfluidic systems were designed to overcome the absence of pharmacokinetic features in *in vitro* cell-based models as well as to refine, automate and reduce the associated costs of current laboratory practises [84]. The incorporation of multiple barriers commonly affecting drug delivery into organ/tissue compartments, as well as an interconnected microfluidic channel arrangement acting as a vascular circulating system, allowed for testing the compounds' permeability capabilities in the *in vivo* setting. Culture medium acts as a blood surrogate in the microfluidic channels, delivering important nutrients into the cells, while carrying metabolites away from the cells. The microfluidic channel model is scaled according to human physiological parameters, which provides a realistic degree of drug exposure to each specific physiological barrier. This also allows a better understanding of pharmacokinetic interactions at each of the barriers, thus increasing predictive capabilities during extrapolation to the *in vivo* situation [85]. Microfluidic chips containing cell cultures are equipped with microphysiometers capable of measuring numerous metabolic parameters (e.g. oxygen consumption, glucose uptake, lactate production) by means of microsensors [86]. The incorporation of multiple-well plates (e.g. 96-well plates) also makes this model a viable option for high-throughput screening [87]. Its dynamic nature ensures the real-time monitoring of cell culture parameters needed for drug development, while minimising standard deviations of results due to the concentrated amounts of reagent used in these studies. Human cell lines can be used in these systems, each chosen to have the desired characteristics of the designated organ portrayed in the model (e.g. multidrug resistant cancer combined with a normal epithelial cell line). A physiologically-based pharmacokinetic model (PBPK) is usually used adjacently in combination with the microfluidic channel systems in order to mathematically translate absorption, distribution, metabolism and excretion (ADME) data obtained from the micro-scale cell culture analogue (μCCA) model into actual *in vivo*-relatable results [88]. The model can also be manipulated to test permeability potential through different barriers by sequential organisation of the tissue chambers (e.g. intestinal lumen before the cancer cells) [89-90]. Microfluidic based systems also have the capability to present drug synergistic effects that cannot be seen in traditional *in vitro* studies and the replicated vascular system allows for different dose exposure parameters, giving results similar to those *in vivo*. Currently, there are vast selections of these chips, each containing unique mechanisms and added protocols. Organ systems included in this design is lung, liver, intestinal, urinary, breast, brain and bone marrow tumours [91-93].

The microfluidic channel system incorporates conventional cell models, which presents its own range of drawbacks including cell changes and differentiations through passages and physiological structure differences compared to cells within organs *in vivo*. Adapting this model for high-throughput screening studies is difficult and expensive because of the expertise needed to maintain viability and sterility for all the different cell cultures in play [89]. Different types of microfluidic channel systems have been developed and used in studies as briefly described in the sections below.

3.2.2.1. Multi-organ co-culture in tumour-on-a-chip system:

The next step in developing advanced organ-on-a-chip systems was to incorporate multiple organ cell cultures in the same system. This creates an *in vivo* like microphysiological system that represents all of the major tissues responsible for metabolic processes, as well as influencing drug pharmacokinetic and pharmacodynamics [94].

One example of a multi-organ co-culture as part of a tumour-on-a-chip design included HepG2/C3A (hepatocellular carcinoma / liver cells), MEG-01 (megakaryoblast / bone marrow), MES-SA (drug sensitive uterine sarcoma) and MES-SA/DX-5 (MDR uterine sarcoma) cell lines. The HepG2/C3A cell line was included to view the effect of the metabolism of doxorubicin towards the less active doxorubicinol. The MEG-01 cell line represented the thrombocytopenic effect of this drug due to bone marrow suppression, while the cancer cell line MES-SA/DX-5 was transfected to over-express P-gp, thereby inducing MDR. This P-gp over-expression allowed the researchers to determine if the required concentration of drug needed to have cytotoxic effects on the resistant cancer cells, was in an acceptable range to avoid systemic toxicity [95].

3.2.2.2. Lung-on-a-chip microfluidic device:

This novel biomimetic device was developed to improve the model by incorporating key characteristics of tissues that were absent from the traditional microfluidic channel system. The lung-on-a-chip microfluidic device was designed to accommodate an extracellular matrix coating (consisting of collagen and fibronectan), whilst human alveolar epithelial cells and human pulmonary microvascular endothelial cells were cultured to confluent monolayers on opposing membrane sides, which allowed airflow between the barriers. This airflow allowed for an air-liquid interface similar to the alveolar airspace found in human lungs. The compartmentalised design enabled controlled delivery of nutrients to the different cell cultures. Two vacuum inducing micro-chambers were installed to mimic sub-atmospheric pressure driven stretching of the membranes to replicate normal breathing. In this model, the researchers attempted to replicate the major factors of pulmonary physiology in order to evaluate how drug compounds may act in these systems and to compare the outcomes to actual *in vivo* results [96].

3.2.2.3. 3D lung cancer microfluidic constructs:

Polydimethylsiloxane (PDMS) round bottom micro-wells were constructed as microchip bases and hydrostatic pressure was used to guide the introduced cancer cell suspension to the membranes. Gravitational forces kept the cells in place and forced the formation of 3D cell culture constructs. The mesothelioma cell line (H2052) and primary cell lines from patients (NSCLC, squamous carcinoma and pericytes) were introduced into the model as mono- and co-cultures to test the effect of stromal cells on MDR, using cisplatin as chemotherapeutic compound. This system allowed viable assays, but a concentration gradient generator was proposed for future, more realistic studies [97].

3.2.3. 3D spheroid cell cultures:

Complex 3D spheroid cell cultures are being incorporated into modern *in vitro* screening models, which attempt to bridge the gap between traditional flat culture shortcomings and the limitations experienced with *in vivo* studies [98-99]. This design more realistically mimics the physiological characteristics of *in vivo* tumours by allowing cell-cell interaction as well as better communication within the microenvironment (e.g. extracellular matrix). Intercellular communication includes the activation of signalling pathways, which mediates a cascade of effects including molecular, subcellular and cellular changes. These changes can include alterations in protein/gene expression, molecular exchanges, enhanced cellular transport effects (endocytosis/exocytosis) and cellular behavioural changes (e.g. apoptosis, proliferation, cell shape, motility) [100-101].

3D spheroid cell culture is seen as the first and oldest 3D cell culture model construct and dates back to the 1970's. This model depends on the tumour cells' capability to differentiate into heterogeneous multi-cellular spheroids by either introducing them into an extracellular matrix (ECM) as a scaffold-dependant (i.e. scaffold-based) construct or by introducing external factors to enforce spheroid growth (non-scaffold based). Scaffold-based cell culture models include matrix-on-top, matrix-embedded, matrix-encapsulation, spinner flasks and the micro-patterned plate technique. Non-scaffold-based cell culture models employ ultra-low attachment plates, hanging drop, microgravity (rotating wall-vessels/bioreactors or clinostat), spontaneous aggregation, magnetic levitation and 3D bioprinting [102]. After the cells start piling up on each other in 3D spheroid cultures, they develop surface receptors known as integrins, which are used to anchor themselves onto the ECM and thereby sustaining tumour growth and enhancing cell-cell communication [103-104].

The connection of these cells to the microenvironment initiates increased protein and gene expression levels of MDR precursors, which are generally limited in conventional models. Changes that were found to occur in these cell structures included metabolic changes, increased responses to cellular stress, cellular structural changes, increased signal transduction, slowing down proliferation cycles (quiescent cells), limiting drug surface contact, inhibiting apoptotic precursors and elevated expression of cellular transporter proteins, all playing a role in the increased resistance of these cell models to cancer treatment [102]. The capability of these cells to use cadherin molecules and integrin surface receptors to mediate resistance against drug treatment, allowed for the design of effective MDR cell culture models [105-107].

The combination of acquired MDR characteristics with surface ABC transporters created a cellular model that more accurately predicted the *in vivo* occurrences of drug interactions. The major advantage of this 3D spheroid cell culture model is the formation of the *in vivo*-like necrotic centres (although recent publications showed some spheroid systems to be devoid of necrotic centres) and an overall heterogeneity [108]. Modern 3D tumour cell model concepts incorporated angiogenesis to recreate the *in vivo* microenvironment even more closely. Vascularisations of breast and prostate 3D spheroid cellular models (MCF-7, MDA-MB-231, LNCaP and PC3) were initiated by incorporating glycosaminoglycan-based hydrogel culture systems containing vascular endothelial growth factor (VEGF), fibroblast growth factor-2 (FGF-2) and stromal cell-derived factor-1 (SDF1), thus creating a multi-parametric bioengineered tumour angiogenesis microenvironment. This vascularisation ultimately resulted in increased chemoresistance as well as tumour regression rates comparable to that observed clinically [109].

Further investigation found that the introduction of an exogenous ECM into the 3D cell culture model design can increase the *in vivo* imitation of cancer cells, ultimately resulting in more predictable screening. This model has the capacity not only for screening of anticancer compounds, but also to investigate protein changes (mRNA inducing protein expression and complex interactions), cellular communication and cellular metabolic and functional processes (ATP production and cell migration/invasion). In most larger 3D cell spheroids (i.e. exceeding 150 µm in diameter), the nutritional starvation and hypoxic conditions of the tumour centre causes necrosis accompanied by micro-metastatic behaviour, thus allowing investigation of anticancer compound evasion mechanisms, as well as the overall influence that these physiological changes may have on tumour biology [110-112].

The superiority of this model lies in its ability to shy away from the exponential growth phase priority that 2D cell cultures exhibit, by re-directing energy to develop advanced tumour-like structures [65]. This mechanism of sophisticated self-assembly occurs because the cells reach a "dynamic equilibrium", and this phenomenon essentially bridges the gap between *in vivo* and *in vitro* screening [65]. The development of 3D multicellular tumour spheroids is also purported to be more time and cost effective in the identification of novel anticancer drugs and produce data of superior quality compared to that of conventional models [113].

Three-dimensional cell models have been shown to have greater chemo- and radiotherapy resistance compared to their 2D counterparts and they show similar characteristics to those expressed by native cancers, including lactate accumulation, oxygen/hypoxia and proliferation. It has also been suggested that 3D cell culture models lead to more aggressive cancer

phenotypes, including sustained proliferative signalling, inducing angiogenesis, resisting cell death, genome instability and mutations, and deregulating cellular energetics [114]. The 3D spheroids can also be used to determine lethal doses closely related to *in vivo* values [115]. It has also been claimed that in 3D cell culture models, the non-hologynic exposure of the cells within the spheroid to the drug being tested and the non-constant proliferation rate of the cells inside the spheroid, will contribute to the deeper understanding of mechanistic properties of cancer treatments [116].

Unfortunately, 3D cell culture operating techniques can be time consuming and the reproducibility of some techniques are questionable [117]. Furthermore, if the size of the spheroid is greater than 150 μm, necrosis of the core can occur due to inadequate nutrient and oxygen diffusion [118], although this is greatly dependent on the type of spheroid system being used [108]. Drug penetration can also be restricted by the size of the spheroid, diffusion through the ECM and cell-to-cell and cell-matrix adhesion, influencing the effect of chemotherapy [114].

3.2.3.1. 3D multicellular models mediating MDR:

One practical application of a 3D multicellular model consisted of two NSCLC cell lines, isolated from primary lung cancer pleural effusions in patients who have not received any chemotherapy. The two cell lines INER-37 (well-differentiated adenocarcinoma) and INER-51 (weak-differentiated adenocarcinoma) each had a differing histological appearance, with the former cell line being a smaller aggregate (550 ± 25 μm), whilst the latter appeared to be a much larger aggregate (1314 ± 30 μm). These two cell lines were cultured separately as multicellular spheroid aggregates as well as monolayer models, while differentiation and changes in drug sensitivity were monitored. The ultimate goal was to disprove that drug resistance in these multicellular models was primarily caused by lower surface exposure of drugs to cells, due to lower diffusion of drugs into the larger spheroids, as had been previously stated [119]. Upon gene analysis using real-time polymerase chain reaction (RT-PCR) it was found that INER-37 induced gene expression of both *mdr1* and *mrp1* in multicellular spheroids, while this was not the case in the 2D cell culture model. The *mdr1* gene is ultimately responsible for the expression of the major MDR surface efflux transporter, P-gp [120]. INER-51 showed no changes to *mdr1* expression between culture conditions, and *mrp1* expression was absent in both models. Other changes were also induced, such as larger cells going into a quiescent state thus allowing less DNA damage and faster DNA repair capabilities. Several anticancer compounds (including etoposide, doxorubicin and camptothecin) were used in this study to compare the chemo sensitivity of cells grown in multicellular tumour models (3D models) and those in conventional 2D models. Ultimately, it was found that the 3D models conferred MDR to cells through numerous gene expression changes, most notably the induction of MDR transporters e.g. P-gp [107].

3.2.3.2. Hollow fibre-based spheroid cultures:

Biocompatible polyvinylidine fluoride (PVDF) hollow fibres were introduced into this model as an inducing agent of tumour cells, to accumulate into solid masses resembling spheroids [121]. Large concentrations of tumour cells are injected into the hollow lumen of the fibres, and thereafter they are periodically sealed and left in Petri dishes to proliferate into solid spheroid masses. The fibres, which acted as bioreactors, are then separated and transferred into cell culture plate wells, containing the test drug. This model produces versatile drug resistant 3D cancer spheroids, but the growth of these cells are severely limited by the fibre walls. This approach needs consideration of maximum cell growth and the time that is paired with it, to prevent a decline in cell numbers. Migration studies are also not permitted in these models, since the hollow fibres serve as a barrier to large molecules, limiting these studies to only toxicity screening [122-124]. Nevertheless, this model allows the quantification of multiple metabolic precursors and has immense potential in MDR studies, but to our knowledge, this model has not featured immensely in efflux-based resistance studies.

3.2.3.3. Cell printing:

Cell printing is based on bio-additive technology with the ability to build 3D spheroid constructs in a carrier medium [125]. This technology entails implanting the cells layer by layer, and assembles a heterogeneous

tumour model with a relative high density capacity. Biological 3D printing can be modified to produce high-throughput *in vivo*-like tumour models with ECM-cell interactions on a large scale. It allows controlled cell distribution by the researchers, thus creating a biomimetic design that resembles the *in vivo* situation, as well as allowing alterations to the tumour morphology. These alterations can be used to test specific tumour properties, accommodate certain assays and to investigate tumour behaviour [125, 126]. The ability to adjust cell morphology gives researchers the opportunity to create models with specific MDR properties, e.g. increased MDR1 gene expression.

4. *IN VIVO* MODELS FOR MULTIDRUG RESISTANT CANCER TREATMENT SCREENING

Although *in vitro* cell culture-based models are fundamental building-blocks in preclinical cancer research, *in vivo* animal models are still used as the decisive factor in the preclinical anticancer drug screening process before further clinical trials in humans may be conducted [127-128]. *In vivo* animal models resemble in part the *in vivo* responses and physiological conditions in humans [129], providing predictive means to study patient responses to anticancer drugs. Mice are the most commonly used animal model in cancer treatment research as their genome is well-characterised, making the manipulation of their genes much easier with the established genetic engineering techniques already available. Furthermore, their small size and short development period alleviates excessive breeding and maintenance costs in comparison to larger animals [130-132]. The most frequently used mouse cancer models involve xenografts, where human cell-derived tumours or human tumour explants (patient-derived) are implanted into immunocompromised mice, and genetically engineered mouse models (GEMMs) that are genetically

manipulated to form spontaneous tumours. As both these models have been applied in cancer research to study efflux transporter-related MDR, they will be discussed in the following sections. However, it is important to mention that other rodent models also exist, but have not necessarily been commonly utilised for efflux-related MDR cancer studies.

Mouse allografts involve the injection of immunocompetent mice with murine cancer cells or tumours. This model eliminates the bias of xenografts, which use only immunocompromised mice [133-134]. However, the interspecies differences between human and mouse cells result in poor translation between the drug efficacy in allograft models compared to humans, as comprehensively reviewed by Rangarajan and Weinberg [131]. Despite such discrepancies, mouse allografts have been used to generate orthoallobanks of NSCLC for preclinical drug screening [135], as well as basal cell carcinoma models for carcinogenesis and drug pathway studies [136].

By exposing rodents to carcinogens such as DMBA (dimethylbenz(a)anthracene), MNU (N-methyl-N-nitrosourea) and NNK (4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone), spontaneous carcinogen-induced tumours can develop [137-138]. However, the locality of tumour formation is not always constant or controllable. In addition, there is the unpredictable tumour formation rate and the adverse effects (cancer-unrelated) of such chemical substances on overall tissues [139].

4.1. *In vivo* hollow fibre assay models

In 1995, the NCI of the USA developed the hollow fibre assay (HFA) for the preliminary *in vivo* screening of anticancer drugs as a means to bridge the gap between *in vitro* cell-based assays and *in vivo* xenograft models [140]. This assay entails the culturing of 12 human cancer cell lines (of 6 different histologies) in highly permeable biocompatible PVDF hollow fibres, followed by implanting them into nude mice at both subcutaneous and intraperitoneal locations. After anticancer drug exposure for 4-6 days, the fibres are removed and cytotoxicity effects detected with colorimetric tetrazolium-based dye (i.e. MTT) [140-141]. The majority of HFA-based data have been used in prioritising anticancer drugs with a high probability of antitumour activity in follow-up *in vivo* xenograft models. By implementing a few modifications to the original NCI protocol such as extending the drug exposure period, using immunocompetent mice or conducting other end-point measurements, several studies have proven the potential of HFA to study antitumour vasculature drugs and tumour angiogenesis [142-143], DNA damaging agents [144], proteasome-inhibiting drugs [145] and the antitumour effects of active plant constituents [146-147]. The antitumour activity determined with the HFA has been found to positively correlate with that of xenograft models [148-149].

4.2. Xenograft cancer models

Xenograft cancer models are based on the implantation of a tumour(s) into immunocompromised mice, which ensures that no rejection of the induced tumour occurs [150]. Such immunocompromised mice can either be athymic nude mice, with a mutation in the *Foxn1* gene [151], or severely compromised immunodeficient (SCID) mice, with a nucleotide polymorphism in the *Prkdc* gene causing abnormalities to or complete absence of T and B lymphocytes [152-153]. The tumour implantations can either be done orthotopically or heterotopically [154-155]. Orthotopic models are formed when the tumour is directly implanted into its organ of origin, whereas heterotopic models are placed directly underneath the skin (subcutaneous). As subcutaneous heterotopic xenografts can be observed with the naked eye in hairless (nude) mice and the procedure is much easier and less invasive than orthotopic surgeries, this model is frequently used for rapid cytotoxic or cytostatic anticancer drug screening [156-157]. In contrast, orthotopic tumours provide a more appropriate measure to study the tumour at its primary site of origin and to evaluate possible metastasis and invasion [156]. However, orthotopic models have to be excised from sacrificed mice to measure the tumour volume, and therefore only end-point measurements can be taken. Xenograft cancer models can be classified as either cell-derived or patient-derived, as discussed in the following sections, with the focus on efflux transporter-based models used for anticancer drug screening applications.

4.2.1. Cell-derived xenografts:

The transplantation of *in vitro* cultured human cancer cell lines into mice with compromised immune systems is the traditional way to generate a xenograft. The specific type of induced cancer, the exact amount of cells required to form a solid tumour and the time it takes for tumour formation, all depend on the specific cancer cell line used [151, 154, 158]. Given that a considerable number of established cell lines are available for transplantation, a wide range of human cancer types can be studied. Nonetheless, the continuous passaging of the *in vitro* cell lines can lead to drifted cells with different genetic characteristics than their primary cancer tissue of origin [159-160]. In addition, the homogeneity of an injected cell mass does not recapitulate the complexity of a tumour with evolved cells in symbiotic growth [161]. The implantation of tumour cells in different sites than where they normally occur as a disease in humans can also contribute to discrepancies in translation [162]. As previously mentioned, the use of immunocompromised mice can also provide misleading results as not all cancers arise in immunodeficient patients [133, 163]. Even though cell-derived xenografts (CDXs) have several limitations, this *in vivo* model is still frequently used for preclinical anticancer treatment screening.

Overall, for the screening of anticancer drugs against MDR tumours associated with efflux transporter up-regulation, human cancer cells showing efflux transporter over-expression are injected either subcutaneously (used predominantly) or orthotopically into immunocompromised mice generating a CDX. After the allocated drug treatment period, the cell-based tumour growth is evaluated for subsequent changes to the tumour size and volume. This is followed by immunohistochemical (IHC) analysis or Western Blot and quantitative reverse transcription polymerase chain reaction (RT-qPCR) experiments to identify the transporter protein expression levels. By using these end-point measurements, CDXs can provide valuable

information regarding the efflux transporter proteins responsible for drug resistance in specific cancers [164-166], the modulation effect of drug combinations on efflux-related MDR in cancer [167-169] and the potential of herbal medicine to re-sensitise tumours towards anticancer drugs [170].

By using *in vivo* CDXs in conjunction with *in vitro* cytotoxicity assays, several studies have proposed the combinational therapy of drugs as an effective regiment to overcome cancer MDR. Table **2** provides a broad overview of a range of CDXs used for efflux transporter-based anticancer drug screening. Argov *et al*. [169] studied the modulation effect of fluoxetine (Prozac) on the moderate drug resistance of doxorubicin in subcutaneous human colon cancer (HCT-15) CDXs expressing only P-gp. Fluoxetine was found to reverse moderate MDR *in vitro* by inhibiting the P-gp efflux pump and improving doxorubicin intracellular accumulation. Tumour progression was drastically delayed when fluoxetine was combined with doxorubicin *in vivo*, which proved the combination therapy to be as efficient as bevacizumab treatment. Chakravarty *et al*. [168] used the aggressive MDR breast cancer cell line, MCF-7/Dox, in orthotopic xenografts to investigate the anticancer efficacy of the clinically approved antiretroviral drug, nelfinavir. Multiple exposures to nelfinavir *in vitro* enhanced intracellular doxorubicin levels and inhibited P-gp expression and efflux. The co-exposure to nelfinavir and doxorubicin resulted in a 65-80% reduction of *in vivo* tumour growth, in comparison to each drug alone. As this breast cancer model over-expressed P-gp, nelfinavir was shown to counteract MDR by inhibiting P-gp activity. Another study utilised human embryonic kidney cells (HEK293) transfected with the ABCG2 transporter, also known as BCRP, to generate ABCG2-expressing xenografts [170]. These MDR xenografts were implemented to assess the potential of MBLI-87, a non-toxic ABCG2-specific inhibitor, to increase irinotecan accumulation by modulating ABCG2-related drug resistance. The MBLI-87 inhibitor was able to re-sensitise the resistant tumours to irinotecan therapy, although it had no significant effect when treatments were discontinued for 14 days and reconstituted thereafter. This incident was similar to clinical findings also stating premature discontinuation to be a cause of survival failure [171]. Cell-derived xenografts have also been used to evaluate the anticancer efficacy of drug-loaded nanoparticles against efflux-related cancer MDR. Patil *et al*. [172] used poly(D,L-lactide-*co*-glycolide) (PLGA) and polyethyleneimine (PEI) to induce gene silencing in P-gp over-expressing xenograft models (JC cells used). By using nanoparticles encapsulating PLGA-PEI and paclitaxel and with the surface functionalised with biotin for specific tumour targeting, the efficacy of paclitaxel to inhibit *in vivo* tumour growth was significantly higher compared to treatments without gene silencing. Therefore, the use of dual-agent nanoparticles focusing on gene silencing and cytotoxic drug encapsulation shows potential to defeat cancer MDR, especially when the particle surface is coated with specific ligands commonly recognised by cancer cell receptors, such as biotin [172].

4.2.2. Patient-derived xenografts:

In an attempt to improve on the shortcomings of CDXs, the direct transplantation of a patient's primary tumour material as fresh explants into immunocompromised mice has been developed [153, 173-174]. These models, known as patient-derived xenografts (PDXs) or tumour grafts, more closely recapitulate the parental tumour behaviour, histology, genetic diversity and metastatic patterns [175-177]. Bearing in mind that the tumour is directly transplanted into the murine host, the *in vitro* manipulation of the tumour is completely eliminated, ensuring the preservation of the original tumour heterogeneity. With the genomic analysis of 1075 established PDXs and corresponding cell lines, Gao *et al*. [178] demonstrated that several signalling pathways were over- or under-represented in cell lines, whereas matching PDXs showed accurate representation thereof. Moreover, only minimum genetic drift occurred between the passages of a particular PDX, in contrast to the loss of certain developmental pathways in the cell lines upon continuous *in vitro* culturing. Such similarities between PDXs and the original tumours were also observed for human breast cancer models [175]. This explains the deferral of the *in vitro* NCI-60 cell panels, used for more than 25 years as the golden standard for anticancer drug screening, in favour of PDX collections that closely mimic the human tumour drug response [179]. The concept of using PDXs as mouse surrogates (newly referred to as "avatars") in chemotherapeutic research has evolved into a promising practice to improve patient treatment regimens. A patient's tumour is used to generate a number of PDXs in various murine hosts and these xenografts are then studied concurrently or ahead of the actual patient treatment program [180]. This "avatar" approach can help guide the therapeutic management of the clinical trial and introduce key features necessary for the development of personalised medicine [181]. Patient-derived xenografts have also been shown to accurately portray the biodistribution of anticancer drugs when coupled to radiolabelled tracers and examined with micropositron emission tomography (microPET) imaging [182].

Patient-derived xenografts are more commonly implanted subcutaneously, but orthotopic and mammary fat pad implantations have also been done before [174-175]. The development of validated PDX models is a long-term process with high costs. Just the average tumour latency, from tumour implantation to graft appearance, takes up to 12 months. Sadly, patients with rapidly growing tumours will most probably not benefit from their "avatar" models, established for their personalised therapy, as these model development programs are too time-consuming. Furthermore, the engraftment into an immunocompromised host has the potential to select for a more aggressive phenotype than originally present in the human patient [175]. The lack of an intact immune system in the immunocompromised murine host hinders the ability to study cancer-related immune responses in PDXs [183-184]. However, this limitation has been addressed with bioengineering approaches using GEMMs (discussed in the following section), immune cells and fibroblasts to establish an immunocompetent mouse model [185].

Irrespective of the abovementioned disadvantages of PDXs, these models can provide predictive preclinical means to evaluate patient drug responses, especially those related to efflux transporters involved in cancer MDR (Table **2**). Earlier research conducted by Arvelo *et al*. [186] investigated the potential of the P-gp inhibitor, verapamil, to counter MDR in SCLC PDXs. By combining verapamil with CyCAV, a multifaceted chemotherapeutic dosing consisting of cyclophosphamide, cisplatin, doxorubicin (adriamycin) and etoposide, the drug efficacy was improved against the resistant tumours. Therefore, verapamil inhibited the P-gp efflux transporter responsible for MDR in SCLCs. A recent study investigated the role of the transporter ABCB5 in the drug resistance of neuroendocrine skin cancer or Merkel cell carcinoma (MCC) [187]. After the treatment of intraperitoneal MCC PDXs with carboplatin or etoposide, the expression level of ABCB5 was 8-fold higher than the control PDXs. But, when these anticancer drugs were combined with an ABCB5 blocking antibody, tumour growth was significantly reduced and tumour apoptosis drastically enhanced. These results clearly showed the potential of ABCB5-targeted drug resistance reversal therapies to enhance the success of MCC treatment in the clinic.

4.3. Genetically engineered mouse models

Genetically engineered mouse models (GEMMs) are the most advanced *in vivo* models used for human cancer research. These models involve the genetic manipulation of cancer-related genes for drug efficacy screening, studying the interactions between chemotherapeutic agents as well as research on the role of these target genes in tumour biology and tumorigenesis [133]. For the purpose of this review, GEMMs can be categorised as either transgenic or endogenous [163, 188]. In essence, transgenic GEMMs are generated through the microinjection of cDNA with specific promoters into the nuclei of fertilised oocytes, which induces the expression of targeted oncogenes [189-190]. However, the expression level of such oncogenes may not always be predictable or accurately controlled, as apoptosis or senescence may also result from such manipulations [191]. Endogenous GEMMs, on the other hand, can either lose the ability to express oncogenes through the disruption or inactivation of the target allele (through knockout techniques) or express modified regions of the target allele by the insertion of the desired mutation into the target allele (knock-in approaches) [189]. Yet, several of these allele disruption approaches can cause embryonic lethality or developmental defects as these mutations are present in the whole mouse [192]. Through the use of conditional alleles requiring site-specific recombinases to induce mutations, the locality of mutations can be restricted to specific tissues [133].

The use of GEMMs in cancer research harbours a number of improvements on other model pitfalls. As GEMMs are generated in the presence of an intact immune system [193], the inadequacy of xenografts to study cancer-related immune responses is overcome [134]. Furthermore, the histopathological and molecular analyses of GEMMs have shown the accurate recapitulation of essential characteristics of human tumours, which is not always the case for CDXs. Liu *et al*. [194] developed a mouse model for human BRCA1-associated breast cancer through the simultaneous deletion of the *Brca1* and *p53* alleles. These tumours were found to closely resemble human basal-like breast cancers based on expansive growth, the expression of basal epithelial biomarkers and genomic instability. A year later, Rottenberg and colleagues used the same breast cancer GEMMs (*K14cre;Brca1*$^{F/F}$*;p53*$^{F/F}$) to investigate the mechanism involved in the acquired resistance of the PARP inhibitor, AZD2281 [195]. The over-expression of the P-gp efflux transporter was the observed mechanism of acquired resistance as the P-gp inhibitor, tariquidar, successfully reversed the resistance. By breeding mice carrying *Abcg2*-deleted alleles with the *K14cre;Brca1*$^{F/F}$*;p53*$^{F/F}$ GEMM, Zander *et al.* [196] confirmed that the ABCG2 transporter confers resistance to the topoisomerase I inhibitor, topotecan, *in vivo*. Therefore, this specific model proves to be a valuable predictor of human *BRCA1*-mutated breast cancers in preclinical trials.

Knockout GEMMs have been used to investigate the role of efflux transporters in anticancer drug failure and cancer MDR. Table **2** provides evidence of several knockout models. An *Mrp*4 knockout mouse model showed that MRP4 restricts topotecan drug distribution and its over-expression caused resistance to topotecan [197]. Marchetti *et al*. [198] used *Mdr1a/1b*$^{-/-}$ (P-gp) knockout mice to study the pharmacokinetics of the C-4 methyl carbonate paclitaxel analogue, BMS-275,183, in the presence of the proton pump inhibitor, pantoprazole. After an exposure period of 6 h, the apparent oral bioavailability of *Mdr1a/1b*$^{-/-}$ mice in comparison to wild-type mice was 97% and 63%, respectively. Significant increases were also observed in the absolute brain concentrations of BMS-275,183 in knockout mice, compared to control groups. The *in vivo* double-knockout GEMMs confirmed the affinity of BMS-275,183 for the P-gp efflux transporter. Not only knockout approaches are used; knock-in models can also provide information on efflux transporters. Gu *et al*. [199] created a mouse model by inserting a firefly luciferase (*fLUC*) gene into the murine *mdr*1a genetic locus. This model proved to faithfully show basal *mdr*1a expression, as well as induced expression after xenobiotic treatment. Changes in luminescence intensities were observed after treatment with paclitaxel, docetaxel and pregnenolone-16α-carbonitrile. These correlated to the statistically significant induction of *mdr*1a.*fLUC* expression in drug-exposed mice compared to controls, demonstrating the feasibility of linking the conditional knock-in system with a luminescent reporter gene for potential *in vivo* tumour bio-imaging.

5. IMPROVEMENTS FOR PRECLINICAL MDR TREATMENT SCREENING

For the past 30 to 40 years, the NCI made significant contributions to the list of standard drugs available for chemotherapy. The majority of these drugs were tested on the NCI-60 cell panel, even though today researchers are sceptic about using conventional 2D flat cultures for anticancer efficacy screening. Such scepticism can be substantiated by the proven discrepancies in conventional 2D cell culture growth patterns, when compared to human tumour tissues. Calitz *et al*. [200] and Wrzesinski *et al*.

[65] clearly note the main differences occurring between cells cultured in 2D and 3D. Conventional 2D cultures require synthetic growth surfaces with artificial nutrient sources, while regular sub-culturing (e.g. trypsinisation) of the cell population is necessary to ensure the availability of sufficient growth space and nutrients. Therefore, 2D cell cultures are in a constant state of proliferation and active cell growth to recover from sub-culturing. In contrast, dynamic 3D spheroids have reached a state of "dynamic equilibrium" after about 18 days' post-trypsinisation [201]. During this stage, these spheroids start to physiologically mature and form functional structures and characteristics of the original tumour tissue. Given that the dynamic spheroids are allowed to completely recover from laboratory practices (e.g. trypsinisation), the formation of an ECM is an important feature of such 3D cultures. An ECM ensures proper cell-to-cell communication, which is important in true human tumours. This explains why the NCI recently shifted its preclinical screening focus to the use of other models, such as PDXs [179].

Considering that each *in vitro* and *in vivo* model discussed in this paper has its own advantages and limitations, no single model can comprehensively elucidate on the entire efflux transporter-facilitated drug resistance mechanism of cancer as yet. In fact, a combination of both *in vitro* and *in vivo* models would enhance the preclinical decision-making of a chosen drug for further human clinical trials, where the advantages of a particular model overshadow the shortcomings of the other model. For example, preliminary screening of various drug targets at a wide range of concentrations for anticancer efficacy using conventional 2D flat cell cultures, followed by further in depth screening of the most potent compounds (as determined by 2D screening) using advanced 3D cancer spheroids. From this, only the most promising drug targets with the least detrimental side-effects are tested in PDXs, after which one drug is selected for human trials. This approach can greatly decrease the number of animals needed for research purposes, potentially decreasing drug development costs and addressing the ethical issues involved with animal-based research. It may also increase the success rate of clinical trials, since more accurate pre-screening was possible. It is also suggested that clinicians and academic researchers collaborate continuously, to ensure early communication and advice on relevant findings.

Keeping in mind that human tumours form complex structures, interacting with other normal tissues and no tumour exists as the single cell types found in monoculture models. Therefore, several future directions to overcome cancer MDR should be aimed at developing techniques to produce co-cultures containing different cancer cell types. High-throughput hanging drop technology has been used to successfully produce a 3D co-culture of DLD1 colon cancer cells with mouse NIH3T3 fibroblasts, for the validation of RNAi therapy [202]. Another study co-cultured NSCLC cells with T-lymphocytes, using scaffolds, to develop a 3D model for the exploration of tumour-immune responses [203]. Therefore, the use of *in vitro* 3D co-cultures in general can provide a more realistic portrayal of the tumour-related responses associated with anticancer drug treatments.

Adding to the heterogeneity and complexity of a human tumour is the fact that tumours can contain their own sub-population of stem cells responsible for cellular "plasticity". These cancer stem cells (CSCs) can shift as a response to external/internal stimuli from a quiescent state to actively proliferating; from chemosensitive to chemoresistant (by overexpressing ABC-efflux transporters) and from an epithelial to a mesenchymal phenotype (see Fiori *et al*. [204] and Zakaria *et al*. [205] for a comprehensive discussion on CSC biology). The hypothesis is that these CSCs hold the key to cancer recurrence (after remission has been medically confirmed) or the emergence of the same type of cancer in various organs. Therefore, by targeting CSCs specifically, improved anticancer treatment strategies can evolve for clinical applications.

3D cell culture models have proven to be suitable for use in CSC research. Stankevicius *et al*. [206] used scaffold-attached laminin rich ECM and scaffold-free multicellular spheroid models (liquid-overlay technique) to investigate CSC marker expression, associated with self-renewal and epithelial to mesenchymal transition, of human colorectal carcinoma (CRC) cells (DLD1 and HT29). In comparison to 2D culture conditions, the CRC cells showed higher expression of the CSC-associated genes when cultured in 3D. This is in accordance with the findings of Reynolds *et al*. [207], who found that CSC content was the highest in 3D embedded spheroid models compared to 2D cultures.

To this end, complex 3D cell culture-based models continuously demonstrate truthful resemblances to the *in vivo* or human-like environment in which tumours can arise. Seeing as tumour complexity and stem cell characteristics can add to the long list of tumour properties portrayed in 3D models, this approach can contribute to this approach becoming the gold standard in efflux-based MDR screening of anticancer drugs.

6. CONCLUSIONS

The causative effect of drug efflux transporters, especially those of the ABC-superfamily, to the occurrence of MDR in cancer is widely recognised. A collection of therapeutic strategies exist to combat efflux-based MDR mechanisms in cancer including the co-administration of efflux transporter inhibitors/modulators, RNAi therapy, metronomic chemotherapy combined with immunotherapy, to name but a few. The preclinical screening of such therapeutic strategies form a fundamental part of the drug development process and both *in vitro* and *in vivo* models are used for this purpose; each with their own strengths and limitations. *In vitro* models applied can range from easily maintained conventional 2D flat cultures (having poor translatability towards *in vivo* tissues) to more intricate 3D culture systems that closely imitate the true *in vivo* microenvironment. *In vivo* animal models, predominantly used for efflux-based MDR treatment screening, involve the grafting of either cell-derived or patient-derived tumours into immunodeficient rodents to generate xenografts. Although predictive patient drug responses can be obtained from xenografting, the model development is extremely time-consuming and the

absence of a fully functioning immune system in the murine host restricts studies on cancer immune responses. By genetically manipulating cancer-associated genes in mouse models, accurate representations of human tumour properties can be achieved while using models with intact immune systems.

Given that cancer research to overcoming efflux transporter-driven MDR is a multifaceted approach, no particular model should be used solitary. In fact, the utilisation of a complete "package" of preclinical screening models would provide a better understanding and refinement of which therapeutic strategies to explore in further effective clinical trials.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest, financial or otherwise.

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Table 1. ***In vitro*** **efflux-based models used for anticancer drug screening.**

Model	Drug target	Transporter studied	Outcome	Reference
2D flat cultures				
MCF-7 (MCF-7/ADR) cells (Doxorubicin-resistant human breast cancer cells)	• Resveratrol • Doxorubicin	MDR1 (P-gp) • MRP1/MRP2 • BCRP	• Doxorubicin-induced cell death is potentiated by resveratrol. • Enhanced intracellular doxorubicin accumulation is facilitated by resveratrol. • MDR1 and MRP1 mRNA-expression is down regulated. • Resveratrol acts as a P-gp inhibitor. • No effect was witnessed on MRP2 and BCRP.	[208]
LoVo and LoVoDX cells (colon cancer cells resistant to doxorubicin)	• Melatonin • Doxorubicin	P-gp	• In some concentrations melatonin intensified the cytotoxicity effect of doxorubicin in the LoVoDX cells.	[209]
H460/MX20; HEK293/pcDNA3.1; HEK/R482T cells (Large cell lung cancer cells; human embryonic kidney cells)	• CCTA-1523 (ABCG2 inhibitor) • Mitoxantrone • Doxorubicin • SN-38 • Cisplatin	ABCG2	• CCTA-1523 is a potent, selective & reversible modulator of ABCG2. • CCTA-1523 enhances the cytotoxicity of mitoxantrone & SN-38.	[210]
SW620/Ad300 cells (human colon cancer cells)	• Regorafenib (multikinase inhibitor) • Paclitaxel • Doxorubicin • Vincristine • Cisplatin	ABCB1	• Regorafenib reversed the ABCB1-mediated MDR & increased the accumulation of [3H]-paclitaxel in ABCB1-overexpressing cells, by suppressing efflux activity of ABCB1, but not altering expression level & localisation of ABCB1. • Regorafenib & paclitaxel synergistically shrink resistant colorectal tumours. • Regorafenib inhibits the efflux activity of ABCB1 transporter.	[211]
K562 and K562/ADM cells (human chronic myeloid leukaemia (CML) cells)	• Imatinib • Etoposide	MRP1 MDR1 / P-gp	• Use of imatinib may be preferable over the use of etoposide in the treatment of CML.	[212]

BxPC3, Cfpac-1, and HPAC cells (human pancreatic cancer cells)	• CG200745 (HDAC inhibitor) • Erlotinib • Gemcitabine	MRP3 MRP4	• A synergistic inhibitory & apoptotic effect with CG200745 was witnessed when combined with gemcitabine/erlotinib in pancreatic cancer cells. • Decreased expression of MRP mRNA.	[213]
KB_{v200} (drug resistant) and HEK293/ABCB1 stable transfected cells (human embryonic kidney cells)	• Pristimerin	P-gp / ABCB1 / MDR1	• Pristimerin inhibited cell proliferation & induced apoptosis in both cell lines. • Decreased P-gp activity in a dose-dependent manner was independent of mRNA levels but primarily owing to its protein stability. • Disturbed the subcellular distribution of P-gp with decreased location in the plasma membrane.	[214]
		Micelles developed		
MCF-7 / ADR cells (human drug resistant breast cancer cells)	• Doxorubicin • Hyaluronic acid-g-poly(l-histidine) (HA-PHis) and d-α-tocopheryl polyethylene glycol 2000 (TPGS2k) copolymers	P-gp	• Incorporation of doxorubicin into a nanoparticle limited distribution (thus reducing cardio toxicity). • The doxorubicin-loaded mixed micelles (HPHM/TPGS2k) caused increased MDR sensitisation due to reversal of transporter efflux. • Blank HPHM/TPGS2k inhibited P-gp activity by reducing mitochondrial membrane potential & depletion of ATP but without inhibition of P-gp expression.	[215]
MCF-7 and MCF-7/ADR cells (human breast cancer cells and their multidrug resistant phenotype)	• Paclitaxel • d-α-tocopheryl polyethylene glycol 1000 succinate (TPGS) and the mPEG-SS-PTX conjugate	P-gp	• TPGS is a P-gp inhibitor that can block the cancer cell action of pumping drugs out of cells. • Mixed micelles can effectively improve the accumulation of paclitaxel in multidrug resistant MCF-7 cells.	[216]
SKOV-3TR and A2780-Adr cells (Ovarian carcinoma cells)	• Paclitaxel • Tariquidar (P-gp inhibitor) • Transferrin (Tf)-modified polyethylene glycol-phosphatidyl ethanolamine-based micellar delivery system	P-gp	• Tariquidar increased the intracellular paclitaxel levels & its cytotoxicity. • Tf-targeting of these micellar nanoformulations can further enhance their efficacy by targeting deeper layers of tumours.	[217]

		Nanoparticles developed		
MCF-7/T cells (Taxol-resistant breast cancer cells)	• Selenium / Ruthenium (nanoparticles)	P-gp	• The delivery of siRNA by means of Se/Ru- Metal-Organic Framework nanoparticles, decreased MDR genes in MCF-7/T.	[218]
HCT 116 cells (human colon cancer cells)	• Paclitaxel combined with rod-shaped gadolinium arsenite nanoparticles	P-gp	• The hybrid paclitaxel-loaded gadolinium arsenite nanoparticle is used to decrease MDR of paclitaxel.	[219]
MCF-7 and MCF-7/ADR cells (Doxorubicin resistant breast cancer cells)	• Doxorubicin in combination with siMDR1	P-gp	• The liposome decreased doxorubicin resistance.	[220]
T24 and TCC‑SUP cells (Bladder cancer cells & acquired resistant cells towards gemcitabine and vinblastine)	• Nanoparticle albumin‑bound (nab) paclitaxel	ABCB1 (P-gp)	• Hyperexpressive ABCB1 cell lines showed a comparable resistance pattern towards both paclitaxel & nab paclitaxel.	[221]
MCF-7/ADR cells (Doxorubicin resistant breast cancer cells)	• Doxorubicin combined with microRNAs	P-gp	• The higher miR-129-5p expression led to P-gp inhibition & decreased cellular efflux of doxorubicin.	[55]
Advanced complex cell-cultures				
HEK cells cultured as scaffold-free, 3D spheroids formed in nonadhesive micro-molds (human embryonic kidney cells)	• Ko143 (inhibitor) • Gefitinib • Elacridar	ABCG2	• This model gave a better idea of pharmacokinetic characteristics of transporter inhibitors. • Drug uptake and penetration was modelled more effectively. • Overall effectiveness of inhibitors was lower than in 2D models due to diffusion barriers in spheroids. • Elacridar showed effectiveness 5 hours after removal, thus showing differentiation abilities of the model between static and dynamic inhibitors.	[222]
NHLF, SAEC and HMVEC-L co-cultured cells dispensed on poly-HEMA-coated plates and centrifuged to form 3D aggregates.	• Recombinant human Wnt5a • Cisplatin • LiCl (chemical activator of Wnt5a) • IWR-1 (pathway inhibitor)	ABCB1 ABCG2	• Transporter expression was induced in primary healthy lung tissue 3D aggregates, by adding precursors responsible for transporter expression. • Chemical manipulation of Wnt5a pathway induced or reduced transporter expression.	[223]

MCF-7 and DOX resistant MCF-7/ADR cells entrapped in alginate-oligochitosan microcapsules to form 3D aggregates.	• Doxorubicin derivatives (Palm-N2H-DOX, N-Palm-DOX, DOX-5FU, DOX-TPP and DOX-AMG)	BCRP P-gp	• Determining the most effective derivative (Palm-N2H-DOX) in the MDR 3D cancer model. • This model had increased resistance towards drugs in comparison to flat culture models.	[224]
Adriamycin-resistant cells, MCF-7R, and parental control cell line, MCF 7, seeded in silk-collagen scaffolds to form 3D aggregate models.	• Doxorubicin • Carboplatin • Paclitaxel	P-gp MRP2	• 3D aggregates had distinct cell proliferation, cellular aggregate formation and expression of drug resistance-related genes/proteins compared to that of 2D models. • The distinctive characteristics made the model more comparable to the *in vivo* environment. • Drug resistance of the 3D model increased due to alterations in the cell cycle distribution and increased presence of breast cancer stem cells.	[225]
HER2-positive breast cancer cell lines cultured as 3D models using the poly-HEMA method.	• Neratinib (HER2 targeted therapy) • Docetaxel (chemotherapy)	HER2	• This 3D model indicated a higher innate resistance due to alterations in cell survival precursors such as the receptor proteins, drug transporters and metabolic enzymes when compared to the 2D cells. • An overall increased expression of drug targets, cell survival proteins and drug transporters was also reported when compared to the 2D counterpart. • An overall increased cellular viability was reported when 3D cells were dosed with toxic concentrations in 2D.	[226]

Table 2. ***In vivo*** **efflux-based models used for anticancer drug screening.**

Model	Drug target	Transporter studied	Outcome	Reference
Cell-derived xenografts				
		Subcutaneous implantations		
ABCG2-HEK293 cells (human embryonic kidney cells)	• MBLI-87 (ABCG2 inhibitor) • Irinotecan	ABCG2 / BCRP	• Significant sensitisation to irinotecan. • Increased irinotecan's effect. • MBLI-87 prevents drug efflux by ABCG2 inhibition.	[170]
ABCB1-KB-C2; ABCG2-H460/MX-20 & ABCC10-HEK/MRP7 cells (human non-small cell lung cancer cells, human embryonic kidney cells)	• Nilotinib	ABCB1; ABCG2; ABCC10	• Nilotinib potentiates the anticancer effect of paclitaxel & doxorubicin in MDR xenograft models.	[227]
SKOV-3 cells (human ovarian cancer cell line)	• NSC23925 (P-gp inhibitor) • Paclitaxel	P-gp / MDR1	• Combination of paclitaxel & NSC23925 showed inhibition of tumour growth. • NSC23925 prevented development of paclitaxel resistance *in vivo*.	[164]
MCF-7 / ADR cells (human breast cancer cells resistant to adriamycin)	• Psi-Pgp-tGC nanoparticles • Doxorubicin	P-gp	• Psi-Pgp-tGC nanoparticles down-regulated P-gp expression. • Nanoparticles potentiated doxorubicin-mediated inhibition of tumour growth & showed lower tumour volume.	[228]
KB_{V200} cells (human keratin-forming HeLa-derivative, ABCB1-overexpressing cells)	• Trametinib • Vincristine • Doxorubicin	ABCB1	• Trametinib in combination with vincristine inhibited the tumour growth of ABCB1-overexpressing xenografts. • Trametinib used in combination therapy has the potential to combat efflux pump related MDR.	[229]

MDA-MB-231 and BT-474 cells transfected with shRNA targeting ABCC1 or ABCC3 (human breast cancer cells)	• Doxorubicin • Mitoxantrone • 5-fluorouracil	ABCC1 & ABCC3	• Like ABCC1, ABCC3 is also overexpressed in primary breast cancers. • ABCC3 was responsible for drug resistance, whereas ABCC3 knockdown reversed resistance.	[230]
C4-2B, TaxR and DU145-DTXR cells (Docetaxel resistant human prostate cancer cells)	• Bicalutamide • Enzalutamide • Docetaxel	ABCB1	• Combination of bicalutamide & docetaxel overcomes resistance. • Bicalutamide and enzalutamide inhibit ABCB1 transporter activity.	[231]
HCT-15 cells (human colorectal adenocarcinoma)	• Fluoxetine (Prozac) • Doxorubicin • Bevacizumab (avastin)	P-gp; MRP & BCRP – tested, but not detected	• Combination of doxorubicin & fluoxetine showed significant slow-down of tumour progression, comparable to aggressive treatment with bevacizumab. • Fluoxetine improved doxorubicin intracellular accumulation & uptake. • Ability of fluoxetine to modulate resistance *in vivo*.	[169]
JFCR013-2 cells (human non-small cell lung cancer cells resistant to ceritinib)	• Ceritinib • Crizotinib • Alectinib	P-gp	• P-gp overexpression is the major cause of ceritinib & crizotinib resistance in NSCLC cells. • Ceritinib & crizotinib are P-gp substrates.	[166]
T24 cells (human bladder cancer cells)	• Emodin • Cisplatin	MRP1	• By downregulating the MRP1 expression, emodin improves the chemosensitivity to cisplatin.	[167]
H460 cells (human large cell lung carcinoma cells)	• Sulindac • Doxorubicin	MRP1	• Sulindac reduced doxorubicin resistance by inhibiting MRP1 activity.	[232]
JC cells (mouse primary mammary gland adenocarcinoma cells)	• Paclitaxel • Poly(D,L-lactide-co-glycolide) nanoparticles	P-gp	• Nanoparticles, containing both paclitaxel and P-gp targeted siRNA, showed effective MDR1 gene silencing & the improved accumulation of paclitaxel in drug-resistant cells.	[172]

		Orthotopic implantations		
MCF-7/DOX cells (human breast cancer cells)	• Nelfinavir (inhibitor) • Doxorubicin	P-gp	• Co-exposure to the anti-HIV-drug nelfinavir enhanced the antitumor efficacy of doxorubicin.	[168]
MDA-MB-231 cells (human breast cancer cells)	• Curcumin • Paclitaxel • Cisplatin • Doxorubicin	ABCG2 ABCC1	• Curcumin downregulated ABCG2 and ABCC1 expression. • Curcumin enhanced the sensitivity of the cancer to paclitaxel, cisplatin, doxorubicin & mitomycin C.	[233]
Patient-derived xenografts				
		Subcutaneous implantations		
Small cell lung cancer	• Cyclophos-phamide • Cisplatin • Doxorubicin • Etoposide • Verapamil (inhibitor)	P-gp / MDR1	• The co-administration of verapamil enhanced the antitumor efficacy of CyCav treatment regimen.	[186]
Human colorectal tumours	• PF-309 (inhibitor)	P-gp / ABCB1 gene	• PF-309 efficacy is affected by the expression of P-gp in tumours.	[234]
Merkel cell carcinoma (MCC)	• Carboplatin • Etoposide	ABCB5	• MCC cancers showing carboplatin and etoposide resistance, had high ABCB5 expression levels. • Tumour growth was reduced by the reversal of drug resistance, through the administration of ABCB5 blockade.	[187]
Non-small cell lung cancer	• Etoposide • Carboplatin • Gemcitabine • Paclitaxel • Erlotinib	BCRP MDR1 MRP1	• A low correlation between BCRP expression & PDX response to etoposide was found. • No other efflux transporter expression levels were correlated to any drug responses *in vivo*.	[235]
Retinoblastoma (eye cancer)	• Topotecan • Melphalan	ABCB1 ABCC1 ABCG2	• The single exposure to melphalan or topotecan did not influence the expression levels of the efflux transporters in the PDXs.	[236]

Genetically engineered mouse models				
		Knock-in mice		
*mdr*1a.*fLUC* knock-in mice	• Paclitaxel • Docetaxel • Pregnenolone-16α-carbonitrile (PCN)	MDR1	• By inserting a firefly luciferase (*fLUC*) gene into the *mdr*1a locus of the murine host, luminescence intensities could be accurately detected. • *mdr*1a.*fLUC* expression was shown to change with paclitaxel, docetaxel or PCN treatments.	[199]
		Knockout mice		
Mdr1a/1b$^{-/-}$ double knockout mice	• BMS-275,183 (analogue of paclitaxel) • Pantoprazole (proton pump inhibitor)	P-gp / ABCB1 MRP2 / ABCC2 BCRP / ABCG2	• By genetically deleting P-gp, it was shown that P-gp plays a role in the pharmacokinetics & brain distribution of BMS-275,183.	[198]
Mrp4$^{-/-}$ knockout mice	• Topotecan	MRP4	• The distribution of the anticancer drug, topotecan, is restricted by the Mrp4 transporter. • The overexpression of Mrp4 causes topotecan resistance.	[197]
Mdr1a/b/Mrp2-/- triple knockout mice	• Irinotecan • Methotrexate • Doxorubicin	P-gp / MDR1 MRP2	• Methotrexate is a good substrate for *Mrp2*, whereas irinotecan is poorly effluxed.	[237]
Doxorubicin-sensitive & doxorubicin-resistant *Brca1$^{-/-}$;p53$^{-/-}$* tumours in *K14cre;Brca1$^{F/F}$;p53$^{F/F}$* mice	• Doxorubicin • Tariquidar	MDR1	• Doxorubicin resistance is caused by increased expression of the *Mdr1* gene encoding P-gp. • The third-generation P-gp inhibitor, tariquidar, can reverse this resistance.	[238]
Brca1$^{-/-}$;p53$^{-/-}$ tumours in *K14cre;Brca1$^{F/F}$;p53$^{F/F}$* mice	• Doxorubicin • AZD2281 (inhibitor) • Tariquidar (inhibitor)	P-gp / *Abcb1a* and *Abcb1b*	• The up-regulation of P-gp induced acquired resistance to AZD2281. • The addition of the P-gp inhibitor, tariquidar, reversed this resistance.	[195]

Abcg2-deleted alleles bred with *K14*cre;*Brca1*$^{F/F}$;*p53*$^{F/F}$ mice	• Topotecan	BCRP / ABCG2	• ABCG2 contributes to topotecan resistance *in vivo*.	[196]
Slco1a/1b$^{-/-}$;*1A2*^{tg}; *Slco1a/1b*$^{-/-}$;*1B1*^{tg}; *Slco1a/1b*$^{-/-}$;*1B3*^{tg} mice	• Paclitaxel • Methotrexate	OATP1B1 OATP1B3 OATP1A2	• Methotrexate is a substrate of all three human OATP1A/B transporters. • Paclitaxel is transported by OATP1B3 and OATP1A2, but not OATP1B1.	[239]

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Chapter 4
Preliminary anticancer efficacy screening

4.1. Introduction

A decreased therapeutic response towards chemotherapy in cancer management is frequently caused by multidrug resistance (MDR). In overcoming MDR, the general therapeutic response in human malignancies can be increased (Mbaveng *et al.*, 2017; Stark & Assaraf, 2017). Many people, especially in Africa, rely on plant remedies for treating diseases such as cancer (Mbele *et al.*, 2017). The anticancer efficacy of these plant extracts can be evaluated in cultured mammalian cells through cell inhibition assays, and an IC_{50} value (the concentration of the extracts which result in a 50% reduction in cancer cell viability) can be calculated (Casuga *et al.*, 2016). Several assays are available for cytotoxicity testing, but the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay is largely considered the pillar of cytotoxicity assays and is frequently used for high-throughput screening of medicinal plant extracts (Van Tonder *et al.*, 2015).

The principle of the MTT assay lies in the enzymes, such as dehydrogenases and mitochondrial nicotinamide adenine dinucleotide phosphate (NADP) hydrogen (H)-dependent oxidoreductases, present in metabolically active cells. These enzymes can reduce yellow tetrazolium salts to purple formazan crystals, which enables detection of metabolically active cells (Mosmann, 1983; Berridge *et al.*, 2005). The MTT assay is known to be a rapid and simplistic method with high levels of effectiveness (Bahuguna *et al.*, 2017; Präbst *et al.*, 2017). However, the conversion of the salt to formazan crystals depends on the metabolic rate of the cells and the number of mitochondria present, and it is possible that the plasma membrane may be injured due to the needle shaped formazan crystals (Lü *et al.*, 2012; Van Tonder *et al.*, 2015). The reduction of the MTT salt has been shown to differ between several cell lines, and the amount of MTT being reduced has been shown to decrease as the cell culture ages (Vistica *et al.*, 1991). Furthermore, the phytochemicals in plant extracts may interact with the MTT compound through antioxidant activity, which can cause false positive results (Karakaş *et al.*, 2017).

Despite these limitations, the MTT assay is still widely used in cancer research and for the screening of phytochemicals. The cytotoxic effects of fascaplysin (a natural product with anticancer activity) was determined to be between 134 and 1 740 nM against a panel of non-small cell lung cancer (NSCLC), small cell lung cancer (SCLC) and human embryonic kidney cells (HEK293) (Hamilton, 2014). The combination of paclitaxel and curcumin in a multifunctional nanomedicine-based drug delivery system was also examined in a breast cancer cell line expressing P-glycoprotein (P-gp)

(MCF-7/ADR) using MTT (Baek & Cho, 2017). Recently, the anticancer properties of several Indian medicinal plant extracts were also determined with the MTT assay (Nile *et al.*, 2017).

In this study, the MTT assay was used as a cost effective screening method to determine the range of anticancer efficacy of three selected traditional medicinal plant, namely *Aloe vera* (gel material and polysaccharides fraction), *Sutherlandia frutescens* and *Xysmalobium undulatum*. From this investigation IC_{50} values were determined statistically for use in subsequent more advanced evaluations, presented in Chapter 5.

4.2. Materials and methods

4.2.1. Experimental approach

The anticancer potential of the selected plant material were evaluated with the MTT assay after exposure to a concentration range of each selected crude plant extract for 96 h. The relative cell inhibition values were then used to calculate IC_{50} ranges for each crude extract, in each cell line. The mammalian cell lines included in this study consisted of LLC-PK1 porcine kidney cells as a non-cancerous control, and three human SCLC cell lines – H69V (chemosensitive), H69AR (this cell line is considered multidrug resistant due to the overexpression of the multidrug resistance-associated protein 1 (MRP1)) and NCI-H69/LX4 (this cell line is also multidrug resistant, given that it is P-gp hyperexpressive).

4.2.2. Materials and reagents

Raw powdered plant material of *S. frutescens* (SFFW) and *X. undulatum* (XU174) were purchased from Afrinatural holdings (Prestige Laboratory Supplies CC, KwaZulu-Natal, South Africa). The *A. vera* gel material (Daltonmax 700® #7004Q11PK01), was supplied by Improve, USA, Inc (Texas, USA). The polysaccharides of *A. vera* used in this study were previously precipitated for the Daltonmax 700® gel material (Beneke *et al.*, 2013). The European Collection of Authenticated Cell Cultures (ECACC) was the authorised supplier for both the H69V (ECACC; #91091803) and NCI-H69/LX4 (ECACC; #96042329) cell lines. The American Tissue Culture Collection (ATCC) supplied the H69AR (ATCC®; #CRL-11351™) and LLC-PK1 (ATCC®; #CL-101™) cell lines. The culture medium used included Roswell Park Memorial Institute (RPMI) 1640 (Gibco, #A10491-01 and #11835-063; Thermo Fisher Scientific, Johannesburg, South Africa) and Dulbecco's Modified Eagle's medium (DMEM) (HyClone, #SH30243.01; Separations, Johannesburg, South Africa).

Culture medium supplements included foetal bovine serum (FBS) (Gibco, #10270-106; Thermo Fisher Scientific, Johannesburg, South Africa), L-glutamine (Lonza, 200 mM, #BE17-605E; Whitehead Scientific, Cape Town, South Africa), non-essential amino acids (NEAA) (Lonza, 100X, #BE13-114E; Whitehead Scientific, Cape Town, South Africa), penicillin/streptomycin (Pen/Strep) (Lonza, 10 000 Penicillin U/ml/ 10 000 Streptomycin U/ml, #DE17-602E; Whitehead Scientific, Cape

Town, South Africa) and doxorubicin hydrochloride (DOX) (#D1515; Sigma-Aldrich (Pty) Ltd, Johannesburg, South Africa). Other reagents used during sub-culturing were purchased from Whitehead Scientific (Cape Town, South Africa) and included trypsin- ethylenediaminetetraacetic acid (EDTA) (Lonza, #BE17-161F), and phosphate-buffered saline (PBS) (HyClone, 1X, #SH30256.01). Trypan blue (#T8154) was obtained from Sigma-Aldrich (Johannesburg, South Africa). The adherent cell lines were seeded in flat bottom 96-well plates (Costar®, #3596, Corning Incorporated, New York, United States of America), whereas V-bottom 96-well plates (Costar®, #3894, Corning Incorporated, New York, USA) were used to seed the suspension cell line. Thiazolyl blue tetrazolium bromide powder (#M5655), Triton X-100 (#SLBB1491V) and dimethyl sulfoxide (DMSO) (#SHBJ4602) were purchased from Sigma-Aldrich, Johannesburg, South Africa.

4.3. Crude plant extract preparation

Traditionally, aqueous decoctions of *S. frutescens* and *X. undulatum* are prepared and used as a hot beverage for the treatment of several illnesses, including cancer (Van Wyk *et al.*, 2009; Vorster *et al.*, 2012). To investigate the anticancer efficacy of the plants selected for this study it was, therefore, decided to prepare a crude aqueous extract to mimic these traditional ethno-botanical decoctions more closely.

4.3.1. *Aloe vera* gel material and precipitated polysaccharides

The *A. vera* gel material was supplied in a powder form, and no additional preparation techniques was done on the gel fraction. The polysaccharides were previously precipitated from *A. vera* Dalton Max700® by means of centrifugal separation at 4000 rpm for 10 min, followed by washing with 80% ethanol (*v/v*), and finally lyophilised (Beneke *et al.*, 2013). The amount of aloveros, a known marker molecule was quantified in 2018 (Table 4.2).

4.3.2. *Sutherlandia frutescens*

An aqueous plant suspension of *S. frutescens* was prepared at a ratio of 1:10 (product:water). For each 10 g raw powder, 100 ml distilled water was added and the suspension was sonicated at 45ºC for 45 min in a digital Eumax® ultrasonic cleaner (Labotec, Midrand, South Africa). Following sonication, the suspension was centrifuged at 5 000 *x g* for 10 min in a Sigma 3-16 KL Laborzentrifugen (Germany). The supernatant was collected and the pellet was re-suspended in water, followed by the same sonication and centrifugation conditions as above. The combined supernatants were centrifuged at 1 218 *x g* for 5 min, followed by filtration of the supernatant. The filtration was performed through Whatman™ filtration paper with a pore diameter of 125 mm (Whatman™, #1114125, Sigma, Johannesburg, South Africa). The collected filtrate was frozen overnight in a -86ºC freezer (Forma Scientific, Marietta OHIO, USA), and following lyophilisation in a Virtis freeze dryer (SP Scientific, Gardiner, New York, USA) it was powdered with a mortar and pestle and stored in a desiccator until further use.

4.3.3. *Xysmalobium undulatum*

An aqueous suspension of *X. undulatum* was prepared in a 1:10 product to water ratio in distilled water. The suspension was sonicated at 45ºC for 45 min before centrifugation for 10 min at 5 000 *x g*. The supernatant was collected and the pellet was re-suspended in water, and the sonication and centrifugation repeated. The combined supernatants were then filtered through 125 mm filtration paper (Whatman™, #1114125, Sigma, South Africa) and frozen overnight at -86ºC. The frozen plant extract was lyophilised, crushed with a mortar and pestle to powder and kept in a desiccator for further experiments.

4.4. Chemical fingerprinting of the crude aqueous extracts

All crude extracts prepared from plant material need to be chemically characterised. This enables the identification, comparison and detection of the main active phytochemicals in the prepared crude aqueous extracts. The ultra-performance liquid chromatography (UPLC) analyses were performed by Prof. A Viljoen (Tshwane University of Technology, South Africa), while nuclear magnetic resonance H-NMR spectroscopy of the *A. vera* gel was done previously (Beneke *et al.,* 2012). The H-NMR spectroscopy of the *A. vera* polysaccharide was done by Spectral service (Cologne, Germany).

4.4.1. *Aloe vera* (gel material and precipitated polysaccharides)

The NMR fingerprinting of the *A. vera* gel material included identification of several marker molecules, including aloverose (referred to as either acemannan or partly acetylated polymannose), malic acid, glucose and citric acid. The quantified marker molecules are listed in Table 4.1.

Table 4.1: The chemical fingerprint of *A. vera* gel material by means of H-NMR spectroscopy as adapted from (Beneke *et al.*, 2012)

Concentration [%] of selected constituents in the *A. vera* gel material (Daltonmax700®)			
Aloverose	Glucose	Malic acid	Citric acid
15.200	9.800	20.700	2.000

The chemical fingerprinting of the precipitated polysaccharides detected the following marker molecules; aloverose, glucose, malic acid, citric acid and calcium. The quantification of these marker molecules are presented in Table 4.2.

Table 4.2: Quantification of selected components following chemical fingerprinting of the *A. vera* precipitated polysaccharide fraction with H-MNR spectroscopy

Concentration [%] of selected constituents in the precipitated polysaccharide fraction of the *A. vera* gel material (Daltonmax700®)				
Aloverose	Glucose	Malic acid	Citric acid	Calcium
47.1	0.5	3.5	3.5	4.7

4.4.2. *Sutherlandia frutescens*

Prior to the UPLC analysis a sample of the *S. frutescens* extract was prepared (2 ml of methanol and 2 mg of freeze-dried powder). The sample was sonicated for 10 min and syringed filtered (0.2 µm filter). Through UPLC a cycloartane-like triterpene glycoside (SU1) marker molecule was identified. The UPLC analysis of *S. frutescens* aqueous extract was performed on a Waters Acquity UPLC system, equipped with a photodiode array (PDA) detector (Waters, Milford, MA, USA) and an Acquity UPLC BEH C18 column (150 mm × 2.1 mm, i.d., 1.7 µm particle size, Waters) maintained at 40ºC. The mobile phase consisted of (A) 0.1% formic acid in water and (B) acetonitrile, at a flow rate of 0.35 ml/min and an injection volume of 5.0 µl (full-loop injection). A gradient elution was used at 80% (A): 20% (B) for the first 0.5 min, changing to 40% (A): 60% (B) in 13 min, adjusting to 100% (B) in 2.5 min, holding for 1 min and back to the initial ratio in 1 min. Mass spectrometry was operated in negative ion electrospray mode. The desolvation gas used was N_2, at a temperature of 350ºC, a flow rate of 500 l/h and the source temperature was set to 100ºC. The capillary and cone voltages were set to 2 500 and 45 V, respectively. Scans were collected throughout the run between 100 and 1 500 m/z.

4.4.3. *Xysmalobium undulatum*

The UPLC analysis of *X. undulatum* aqueous extract was performed on a Waters Acquity UPLC system with a PDA detector as described for *S. frutescens,* with the following variations. The sample prepared for the UPLS consisted of 2 mg of *X. undulatum* freeze-dried material and 2 ml of methanol which was sonicated for 10 min and syringe filtered (0.2 µm). The mobile phase consisted of 0.1% formic acid in water (solvent A) and acetonitrile (solvent B) at a flow rate of 0.3 ml/min. The gradient elution was as follows: 85% A: 15% B to 65% A: 35% B in 10 min, changed to 50% A: 50% B in 0.5 min, keeping for 1 min and back to initial ratio in 0.5 min. The running time was 12 min. The samples were injected in the mobile phase at an injection volume of 2.0 µl (full-loop injection). Mass spectrometry was operated in positive ion electrospray mode, and the desolvation temperature was set to 250ºC at a flow rate of 600 l/h. The capillary and cone voltages were set to 3 000 and 40 V, respectively. Data were collected between 100 and 1 000 *m/z*.

4.5. Mammalian cell culture models

4.5.1. Culturing and sub-culturing procedures

4.5.1.1. Culturing of the H69V adherent cell line

The H69V cell line was cultured in RPMI 1640 (#11835-063) culture medium, supplemented with 10% FBS, 2 mM L-glutamine, 1% NEAA and 1% Pen/Strep. Cells were incubated at 37ºC in 5% CO_2 and 95% humidified air in an ESCO CelCulture CO_2 incubator (ESCO Micro Pte Ltd, Changi, Singapore). The culture medium was replaced every third day, and an inverted light microscope (Nikon Eclipse TS100/TS100F, Nikon Instruments, Tokyo, Japan) was used to observe the cell confluence. Cells were sub-cultured at approximately 80% confluence, following trypsinisation. The spent culture medium was removed, followed by rinsing with 10 ml PBS, twice. The cells were trypsinised in 3 ml trypsin at 37ºC, for 4 min. Cells were agitated to aid detachment, and 1 ml FBS together with 5 ml of culture medium was added. The cell suspension was transferred to a 15 ml centrifuge tube and centrifuged for 5 min at 140 *x g*. The supernatant was decanted and 5 ml culture medium was used to re-suspend the cell pellet. The cell suspension was subcultured into new flasks, usually at a ratio between 1:4 and 1:10, and culture media was added to a final volume of 15 ml per flask. The H69V cells used for experimental work were between passages 13 and 18.

4.5.1.2. Culturing of the H69AR adherent cell line

The multidrug resistant H69AR cell line was cultured in RPMI 1640 (#A10491-01) culture medium, supplemented with 1% NEAA, 20% FBS, 1% Pen/Strep and 2 mM L-glutamine. Cells were incubated with 5% CO_2 and 95% humidified air at 37˚C. Culture medium was exchanged every second day. The cells were sub-cultured at approximately 80% confluence, following trypsinisation as described for the H69V cells. The H69AR cells used for experiments were between passages 5 and 9.

4.5.1.3. Culturing of the NCI-H69/LX4 suspension cell line

The P-gp hyperexpressive NCI-H69/LX4 cell line was cultured in RPMI 1640 (#11835-063) culture medium with additional 10% FBS, 1% NEAA, 1% Pen/Strep, 2 mM L-glutamine and DOX (0.4 µg/ml). The culture medium was replaced every second day, and cells were incubated at 37ºC, with 5% CO_2 and 95% humidified air. Since this cell line forms aggregates in suspension, the cells were centrifuged before replacing the culture medium. A serological pipette was used to transfer the whole cell suspension from the cell culture flask to a 15 ml centrifuge tube, and centrifuged (Hettich, Tuttlingen, Germany) for 5 min at 200 *x g*. If the cells started to adhere to the flask surface, a cell scraper was used to loosen them before transfer. The supernatant was decanted and 5 ml of culture medium was used to re-suspend the cells, before transferring them to a new flask. An additional 10 ml of culture medium was added to the flask.

At approximately 80% cell confluence, the suspension cell line was sub-cultured after the culture flask was scraped. The cell suspension was transferred to a 15 ml centrifuge tube and centrifuged for 5 min at 200 *x g*. The supernatant was decanted and the pellet was re-suspended in 5 ml culture medium. The cell suspension was divided according to the desired sub-culturing ratio (never more than 1:3) and the culture medium adjusted accordingly. NCI-H69/LX4 cells were used for experimental work between passages 6 and 18.

4.5.1.4. Culturing of the LLC-PK1 adherent cell line

LLC-PK1 cells were cultured in DMEM (high glucose) culture medium with the following additives: 1% NEAA, 10% FBS, 1% Pen/Strep and 2 mM L-glutamine. The culture medium was replaced every second day, and the culturing conditions consisted of a humidity of 95%, with 5% CO_2 at 37ºC. Cells were sub-cultured at approximately 80% confluence following trypsinisation as described for the other cell lines, although the flask was incubated for 9 - 10 min after addition of the trypsin. LLC-PK1 cells were used for experimental work between passages 13 and 16.

4.6. Seeding of cells in 96-well plates

The SCLC cell line is known to have a short doubling time and a rapid growth rate, allowing SCLC cells to act as an aggressive cancer (Tang *et al.*, 2016). The cell viability reducing properties of the plant extracts were to be evaluated through comparison with normal physiological cellular growth over a time period of 96 h. The 96 h time frame is comparable to literature, including a study conducted by Mirza *et al.* (2013) where the cytotoxicity of various natural compounds on breast cancer cells was tested by means of MTT after 96 h exposure. The normal physiological growth pattern of each of the selected cell lines was first evaluated, as well as the optimal seeding density to obtain an 80% confluence at 96 h, before seeding the 96-well plates.

Prior to seeding cell suspensions were prepared as described above, followed by cell counting. The adherent cell lines were counted with a haemocytometer (Marienfeld-Superior, 0.0025 mm^2, Germany), by loading 10 µl of cell counting solution (prepared with 25 µl trypan blue, 15 µl PBS and 10 µl cells) in each counting chamber. The final seeding concentrations were prepared and 200 µl cell suspension pipetted per well into flat bottom 96-well plates. The outer wells of each plate were filled with 200 µl PBS to reduce the edge effect, and the plates were incubated at 37ºC, 5% CO_2 and 95% humidified air.

The suspension cell line was counted with an automated Millipore Scepter™ 2.0 (Millipore Corporation, Billerica, USA) with 60 µm sensors (#PHCC60050, Sensors, Merck, USA). A 1:10 dilution of the cell suspension was prepared with PBS, and the cell count/ml determined with the Scepter™ 2.0, followed by adjusting for the dilution factor. Cells were subsequently seeded in V-bottom 96-well plates.

Cells were seeded in 96-well plates at the following cell densities: H69V at 7 000 cells/well, H69AR at 16 000 cells/well, LLC-PK1 at 7 000 cells/well and NCI-H69/LX4 at 30 000 cells/well.

4.7. Preparation of plant solutions for cytotoxicity assays

The medicinal plant solutions were freshly prepared daily and preheated to 37ºC before adding to the cells at experimentally selected concentrations.

4.7.1. *Aloe vera* (gel material and precipitated polysaccharides)

A 1 mg/ml *A. vera* gel material and polysaccharide stock solution was prepared in the various cell culture mediums, using a volumetric flask. The solutions were vortexed using a Lasec vortex mixer (Benchmark Scientific Inc, Edison, New Jersey, USA) and placed in a water bath (Grant Instruments Ltd, Cambridge, UK) to form a homogeneous solution. The 1 mg/ml stock solutions of both the gel material and polysaccharides were pasteurised before preparing the final concentrations in the relevant culture mediums, and these concentrations ranged between 0.008 and 1 mg/ml. The pasteurisation was done by heating the stock solution for 15 sec. at 73ºC, followed by immediate cooling to 9ºC in an ice bath (monitoring the temperature with a Digitron thermometer (Digitron, 2088T, Sifam Instruments Limited Torquay, England)).

4.7.2. *Sutherlandia frutescens*

S. frutescens stock solutions of 8 mg/ml was prepared with the various cell culture mediums in volumetric flasks. The final stock solution was vortexed and filter sterilised using a 0.22 µm syringe filter. The final concentrations were prepared with the various culture mediums, and ranged between 0.05 - 8 mg/ml.

4.7.3. *Xysmalobium undulatum*

The stock solutions of 3.5 mg/ml *X. undulatum* were prepared in the various growth mediums in volumetric flasks. The stock solution was vortexed, followed by filtration through a 0.22 µm syringe filter. The final concentrations were prepared with the various culture mediums, and ranged between 0.008 - 3.5 mg/ml.

4.8. MTT cytotoxicity assay

The MTT assay method to determine the relative percentage cell viability was adapted from Wentzel *et al.* (2017). After seeding in the 96-well plates, a 24 h incubation period ensured the full recovery of the cells before initiation of the treatments. The 3 adherent cell lines were treated by removing the culture medium from the plates, and then adding the selected crude extract concentrations to each treatment well as 200 µl aliquots (0 h). Culture medium containing the various treatments were replaced daily (24, 48 and 72 h). The untreated control wells (cells not treated with extracts) received fresh supplemented culture medium at the abovementioned time points. The DMSO background

control wells (cells treated only with DMSO to eliminate the background interference) and the dead cell control (cells treated with Triton X-100 after 96 h culture) also received fresh supplemented culture medium at the abovementioned time points. A culture medium background control (non-additive media only) and highest plant concentration control (highest plant extract concentration dissolved in non-additive media) were also included to screen for interference of the extracts with the MTT assay.

After the 96 h period, the dead cell control wells were treated for 15 min with 200 µl of 0.2% Triton X-100 (dissolved in PBS), and then carefully rinsing the wells twice with 100 µl PBS. This was followed by the addition of 180 µl non-additive medium (specific for each line). The rest of the wells containing cells (excluding the DMSO background controls) were rinsed twice with 100 µl PBS, followed by the addition of non-additive medium. Fresh MTT stock solution (5 mg/ml) was prepared in PBS and covered with aluminium foil to prevent light exposure. A volume of 20 µl of MTT stock solution was added to each well (final concentration of 0.5 mg/ml), excluding the DMSO background control wells (Shandiz *et al.*, 2017). The plate was wrapped in aluminium foil and placed onto a compact rocker for 5 min, followed by further incubation at 37ºC for 4 h. The medium of each well (except the culture medium background controls and the highest plant concentration controls) was replaced with 200 µl DMSO, followed by shaking for 1 h on the compact rocker. Absorbance was read at 560 and 630 nm using a SpectraMax® plate reader (Paradigm® Multi-Mode Detection Platform, Molecular Devices®, Separations, Gauteng, South Africa).

The suspension line NCI-H69/LX4 in the V-bottom plates were centrifuged at 250 *x g* for 5 min at 23ºC, before replacing the contents of each well as stated above. All the treatment and control wells received DOX-free culture medium to ensure that no herb-drug interactions occurred. Furthermore, after the 96 h exposure period, the plate was centrifuged at 250 *x g* for 5 min at 23ºC. The dead cell control was treated with Triton X-100 for 15 min. All the remaining wells, except the DMSO background control, was rinsed with 100 µl non-additive RPMI 1640 medium, followed by centrifugation at the same conditions as stated above. Subsequently, the 100 µl in each well (excluding the DMSO background control) was replaced with 90 µl non-additive RPMI 1640 medium and 10 µl MTT stock suspension (5 mg/ml). The same incubation period as used for the adherent cell lines was applied. After the 4 h incubation period, the plate was centrifuged at 1 218 *x g* for 5 min at 23ºC, followed by removal of 150 µl of the media in the DMSO background control wells with 150 µl DMSO. In all the remaining wells containing the MTT solution, 50 µl/well was replaced with 150 µl DMSO, and each well was agitated by pipetting. The plate was covered in aluminium foil and incubated for 1 h on a compact rocker. Absorbance was measured at 560 and 630 nm, as described previously.

4.9. Sample quantification

The SpectraMax® plate reader was set to measure the absorbance of the 96-well plates at a wavelength of 560 nm for the sample values, and 630 nm for the background of each well. Each extract's highest concentration was tested to eliminate the possible interference of the plant material with the measurements of the MTT assay. The cell viability of the untreated control was expected to be 100%, and each sample was expressed as a percentage relative to the untreated control. The DMSO control was used as the blank. Cells treated with Triton X-100 represented a dead control. The percentage cell viability was determined using Equation 1,

$$Cell\ viabillity\ \% = \frac{(\Delta Sample - \Delta Blank)}{(\Delta Control - \Delta Blank)} \times 100 \qquad \textbf{Equation 1}$$

where $\Delta Sample$ refers to the absorbance of the treated cells at 560 nm with the absorbance of treated cells as 630 nm subtracted. $\Delta Blank$ represents the absorbance of the DMSO background control at 560 nm with the absorbance of the DMSO background control at 630 nm subtracted. $\Delta Control$ refers to the absorbance of the untreated control at 560 nm after subtraction of the absorbance of the untreated control at 630 nm. The percentage cell inhibition was subsequently calculated with Equation 2.

$$Cell\ inhibition\ \% = 100 - cell\ viability\ \% \qquad \textbf{Equation 2}$$

4.10. Statistical analysis

All experiments were performed in triplicate. SPSS statistical analysis software (IBM Analytics, Version 25), in conjunction with the Probit Analysis Method, were used to generate IC_{50} values and 95% confidence limit ranges for each plant extract on the various cell lines, using the data from the MTT analyses.

4.11. Results and discussion

4.11.1. Chemical characterisation of the plant extracts

4.11.1.1. *Aloe vera* (gel material and precipitated polysaccharides)

The H-NMR spectra of *A. vera* gel material is presented in Figure 4.1. As indicated, several marker molecules were detected and identified in the Dalton Max 700® *A. vera* gel in varying quantities. A quantitative H-NMR spectroscopy analysis of the *A. vera* polysaccharides detected 47.1% Aloverose (known polysaccharide) in the sample (Figure 4.2).

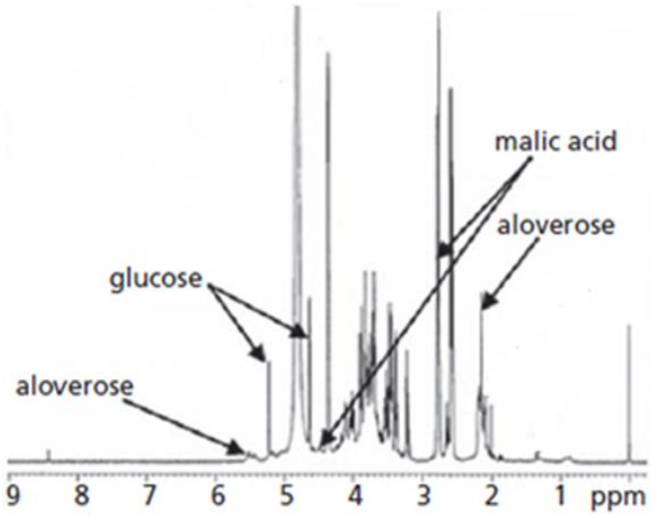


Figure 4.1: The H-NMR spectra of *Aloe vera* gel material indicating the presence of several marker molecules according to marker molecule peaks identified by Jiao *et al.*, 2010.

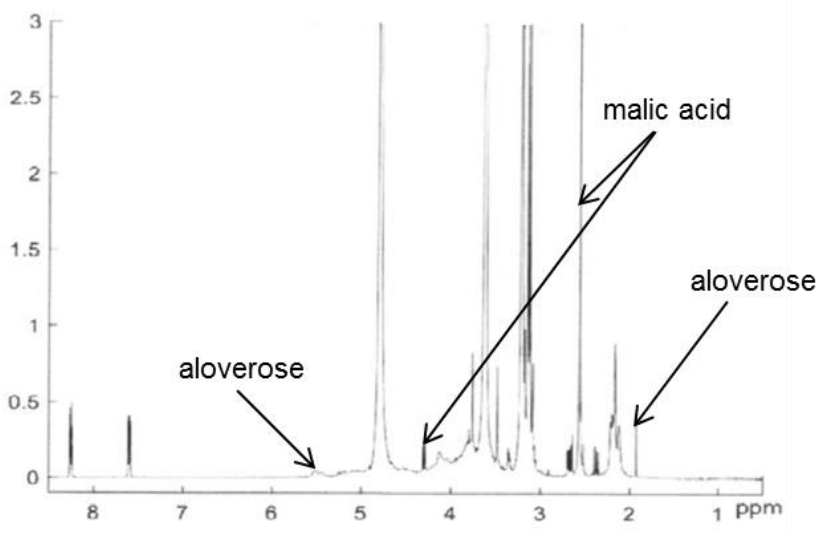


Figure 4.2: The H-NMR spectra of *Aloe vera* precipitated polysaccharides.

4.11.1.2. *Sutherlandia frutescens*

The liquid chromatography-mass spectrometry (LC-MS) chromatogram of the *S. frutescens* crude aqueous extract is shown in Figure 4.3. The presence of the known marker molecule SU1 was confirmed and quantified as 10.1 µg/mg (n = 2).

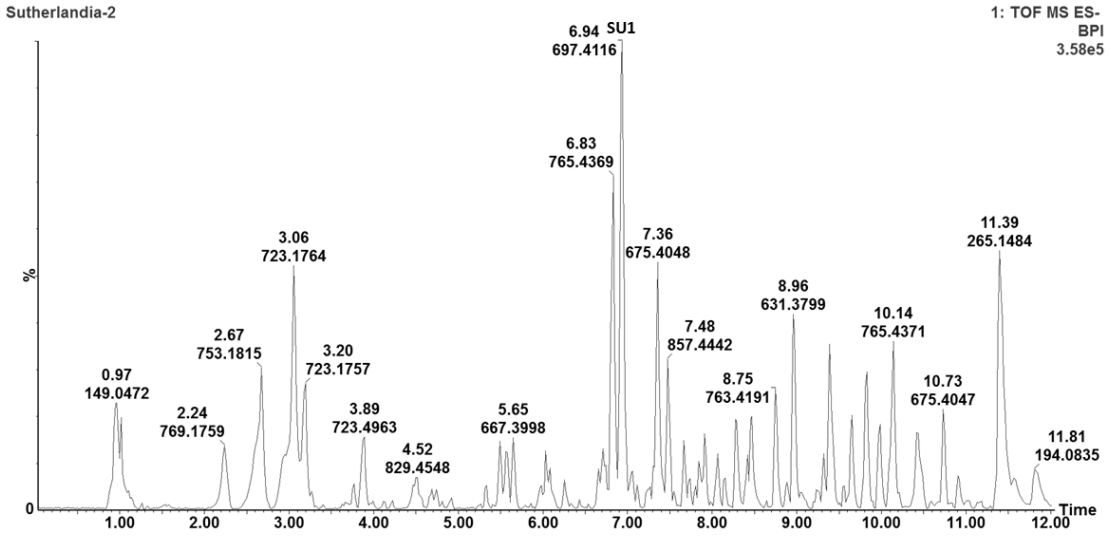


Figure 4.3: The LC-MS chromatogram of the *Sutherlandia frutescens* crude aqueous extract.

4.11.1.3. *Xysmalobium undulatum*

The chemical characterisation of the *X. undulatum* crude aqueous extract via UPLC analysis is presented in Figure 4.4. The marker molecule, Uzarin, was also identified to be present in the extract.

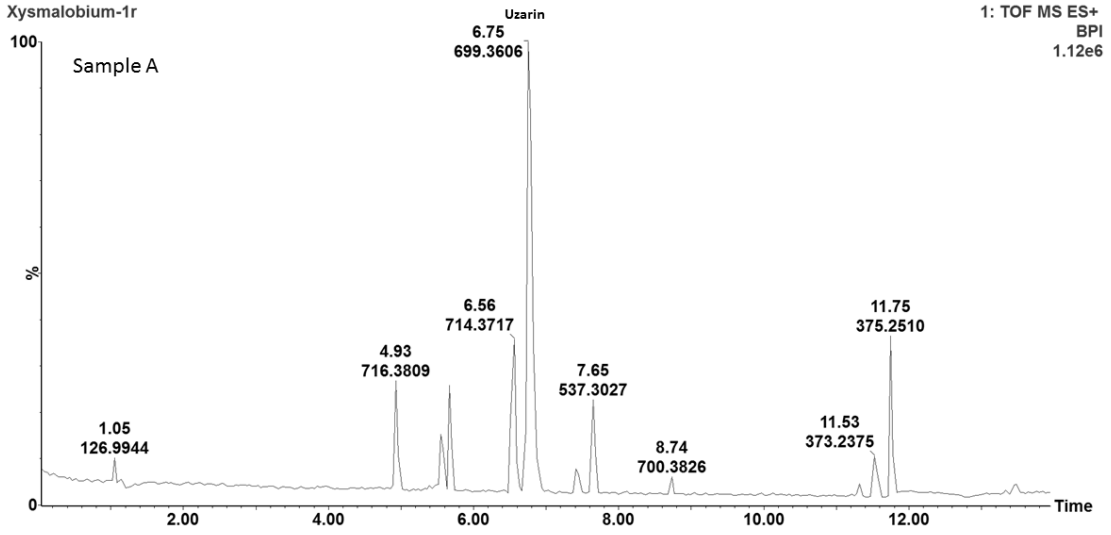


Figure 4.4: The UPLC chromatogram of the *Xysmalobium undulatum* crude aqueous extract.

4.11.2. Cytotoxicity evaluation

4.11.2.1. *Aloe vera* gel material

4.11.2.1.1. LLC-PK1 - non-cancerous cell line

The relative inhibition of cell viability compared to an untreated control, following treatment of the LLC-PK1 pig embryonic kidney cell line with a concentration series of *A. vera* gel material, is shown in Figure 4.5.

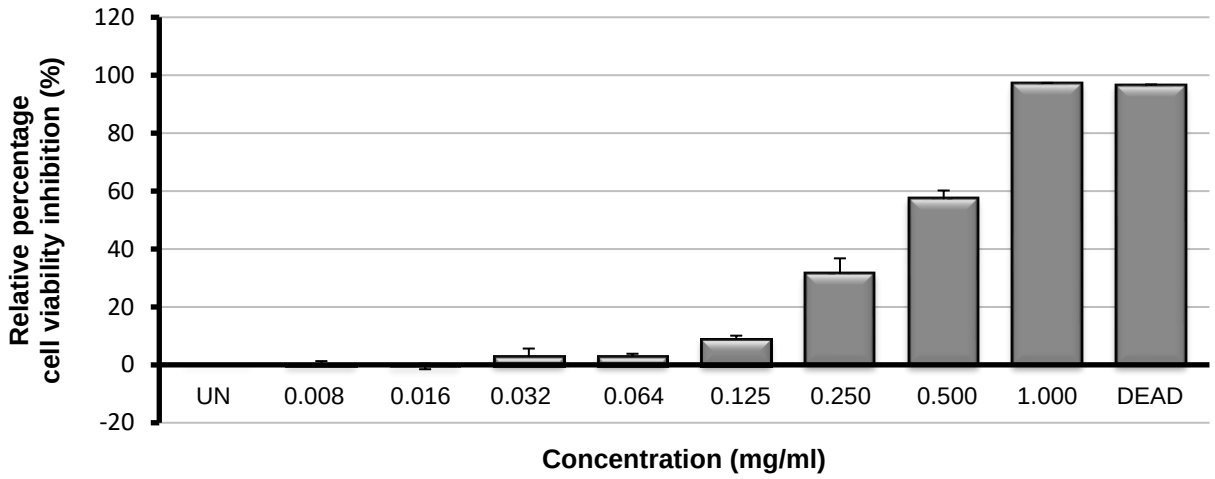


Figure 4.5: Inhibition of cell viability relative to an untreated control, following treatment of the LLC-PK1 cell line with a concentration series of *A. vera* gel material for 96 h (*n* = 3; error bars = standard deviation). The positive control consisted of cells treated with Triton X-100 (DEAD), while the untreated cells are indicated as UN.

An increase in the viability inhibition of the embryonic kidney cell line, relative to the untreated control, could be observed following treatment with *A. vera* gel for 96 h in a concentration dependent manner. Treatment with 1.000 mg/ml resulted in complete reduction of viability, comparable to the dead cell control, with almost no viable cells left after 96 h. From results displayed in Figure 4.5, an IC_{50} value between 0.250 and 0.500 mg/ml was estimated. The MTT results suggested that the maximum non-cytotoxic concentration of *A. vera* gel against the non-cancerous cells was 0.125 mg/ml (approximately 10% reduction in viability).

It is important, however, to note that the *A. vera* gel material was administered as a suspension which could have contributed to the increased reduction of cell viability due to an occlusive effect. However, this will need to be clarified through further studies.

4.11.2.1.2. H69V - chemosensitive SCLC cell line

The relative cell viability inhibition of the chemosensitive H69V SCLC cell line following treatment with *A. vera* gel for 96 h is shown in Figure 4.6. Treatment of this chemosensitive cancerous line

with *A. vera* gel indicated that concentrations as low as 0.008 mg/ml had a marked inhibitory effect of approximately 25% on cell viability, while complete viability reduction could be observed above 0.500 mg/ml (>95%).

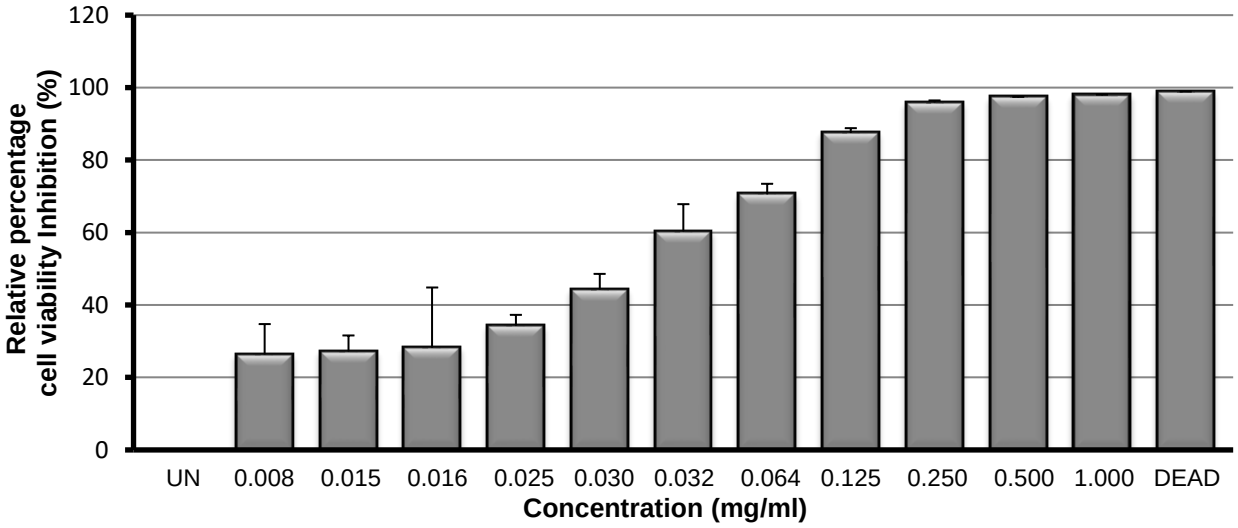


Figure 4.6: Inhibition of cell viability relative to an untreated control, following treatment of the H69V cell line with a concentration series of *A. vera* gel material for 96 h (*n* = 3; error bars = standard deviation). The positive control consisted of cells treated with Triton X-100 (DEAD), while the untreated cells are indicated as UN.

The cell viability inhibitory effect of *A. vera* gel material on the H69V cell line appears to follow a typical sigmoidal trend, with the tumoricidal outcome between 0.008 and 0.016 mg/ml forming a plateau. This is followed by a linear, dose-dependent viability reduction (between 0.016 and 0.250 mg/ml) and, finally, another plateau of maximum cell viability reduction beyond 0.250 mg/ml. Comparing the viability inhibition results obtained in this chemosensitive SCLC cell line with the values measured in the non-cancerous LLC-PK1 line, it appears that *A. vera* gel not only has anticancer potential, but that this potential can possibly be obtained at concentrations which do not appear to have cytotoxic effects towards non-cancerous cells. A study by Im *et al.* (2016) showed a suppressive effect of *A. vera* gel towards colitis-associated colon carcinogenesis, which supports the tumoricidal effects of *A. vera* gel material observed in this study.

4.11.2.1.3. H69AR - multidrug resistant SCLC cell line (MRP1 hyperexpressive)

Aloe vera gel reduced cell viability in the drug resistant H69AR SCLC cell line in a concentration-dependent manner, as determined with the MTT assay (Figure 4.7). A relative viability inhibition of approximately 15% could be observed at a concentration of 0.030 mg/ml, reaching more than 90% after exposure to 1 mg/ml *A. vera* gel for 96 h.

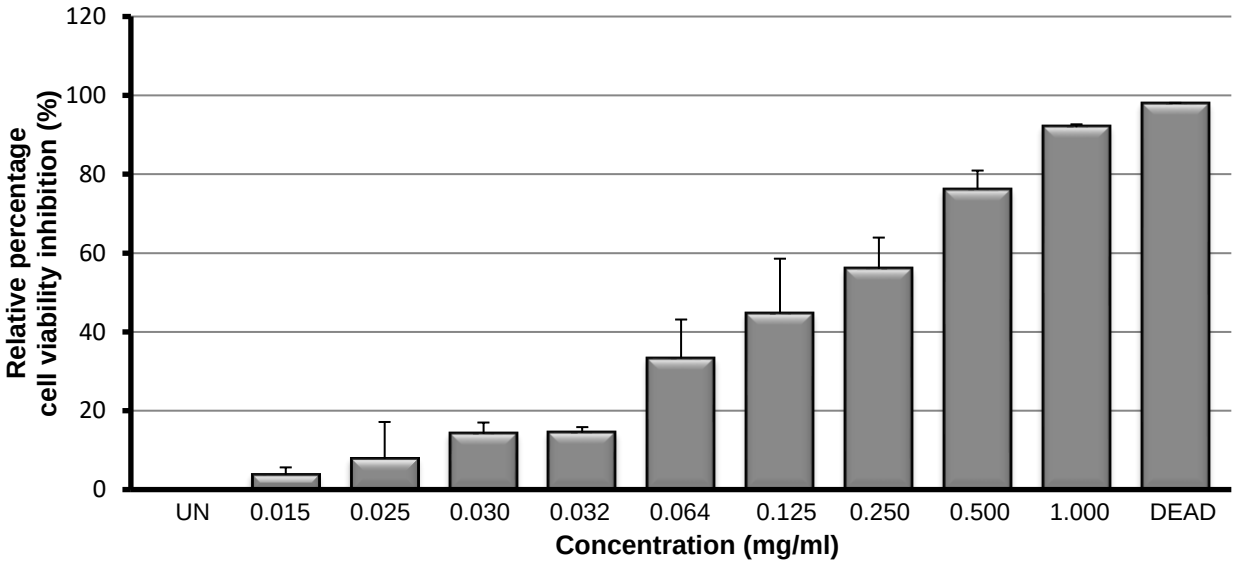


Figure 4.7: Inhibition of cell viability relative to an untreated control, following treatment of the H69AR cell line with a concentration series of *A. vera* gel material for 96 h (*n* = 3; error bars = standard deviation). The positive control consisted of cells treated with Triton X-100 (DEAD), while the untreated cells are indicated as UN.

The IC_{50} value for *A. vera* gel in the H69AR cell line was estimated between 0.125 and 0.250 mg/ml. These values suggest that although there is still anticancer efficacy in comparison to the LLC-PK1 cells, these effects were considerably lower in the MRP1 hyperexpressive H69AR line than in the chemosensitive cell line. As shown in Figure 4.7, at 0.125 mg/ml the *A. vera* gel resulted in 44.71% relative inhibition in the H69AR cells, compared to 87.48% in the H69V line.

Firstly, this highlights the resistant nature of this cell line compared to the chemosensitive line. Secondly, it could signify that the constituents in the *A. vera* gel resulting in the anticancer effects may potentially be MRP1 substrates, since the hyperexpression of the MRP1 transporters decreased the anticancer effects of the *A. vera* gel.

4.11.2.1.4. NCI-H69/LX4 - multidrug resistant SCLC cell line (P-gp hyperexpressive)

Following treatment of the NCI-H69/LX4 cell line for 96 h with a concentration series of *A. vera* gel material, a decrease in relative cell viability could once again be observed (Figure 4.8). The anticancer potential of *A. vera* gel in the NCI-H69/LX4 cell line was achieved in a similar dose-dependent manner as in the H69V and the H69AR cell lines. The estimated IC_{50} value in the NCI-H69/LX4 cell line was between 0.125 - 0.250 mg/ml, similar to that of the MRP1 hyperexpressive cell line. This suggests that some of the active constituents of the *A. vera* gel may also be P-gp substrates. The *A. vera* gel fraction has previously been shown to alter the P-gp mediated efflux of cimetidine, suggesting possible P-gp inhibition (Beneke *et al.*, 2013). This may, therefore, have been a result of competitive inhibition, but further studies are needed to evaluate this. The NCI-H69/LX4 cell model shows high standard deviations, which may be attributed to the suspended

growth characteristic of the cell line. The cells may cluster in different densities, resulting in inconsistent drug exposure and large standard deviations.

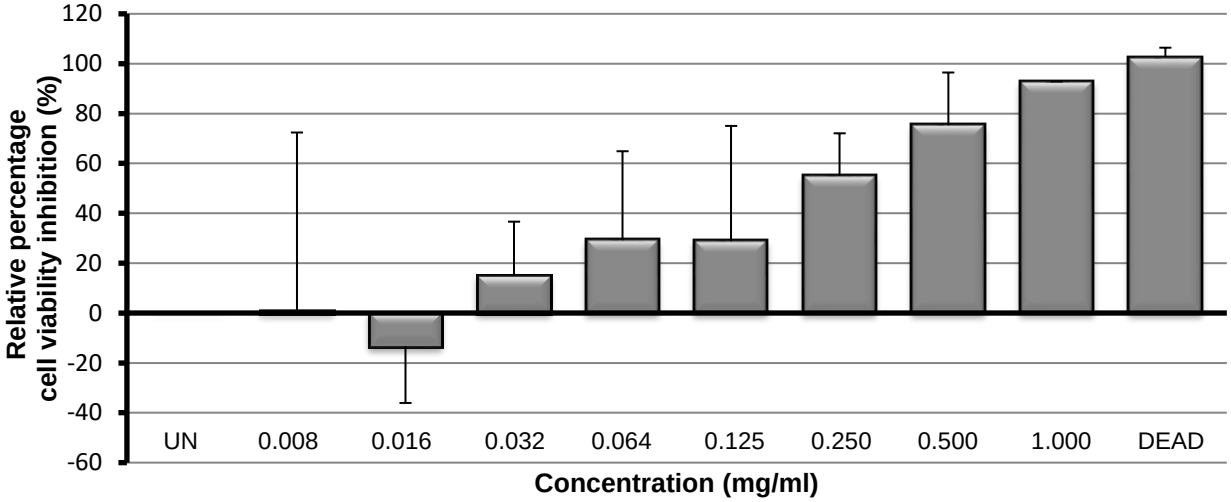


Figure 4.8: Inhibition of cell viability relative to an untreated control, following treatment of the NCI-H69/LX4 cell line with a concentration series of *A. vera* gel material for 96 h (*n* = 3; error bars = standard deviation). The positive control consisted of cells treated with Triton X-100 (DEAD), while the untreated cells are indicated as UN.

4.11.2.1.5. Summary of the *A. vera* gel material MTT results

The MTT data indicated concentration dependent anticancer efficacy and cytotoxicity of *A. vera* gel material in the cell lines used for this study. The *A. vera* gel material demonstrated moderate cytotoxicity in the pig embryonic kidney cell line between 0.025 – 0.500 mg/ml, and therefore limits the use of *A. vera* against cancer above these concentrations. The resistance of the multidrug resistant efflux transporter hyperexpressive cancer cells towards *A. vera* was observed through increased IC_{50} ranges, in comparison to that of the chemosensitive cell line. It is evident that the active phytochemicals in the *A. vera* gel are probable MRP1 and P-gp substrates.

4.11.2.2. *Aloe vera* precipitated polysaccharides

4.11.2.2.1. LLC-PK1 - non-cancerous cell line

The polysaccharide fraction of *A. vera* gel material at concentrations between 0.008 and 0.500 mg/ml showed no notable cytotoxic effect with the MTT assay after 96 h on the LLC-PK1 cell line (Figure 4.9). A moderate cell viability reduction of 33.15% did occur at 1.000 mg/ml, however, future studies can be considered to investigate the possibility of an occlusive effect on the cells. The MTT data illustrated in Figure 4.9 did not result in significant cell viability reduction at the tested concentrations for the *A. vera* polysaccharides on the non-cancerous cell line. Although, statistically, an IC_{50} range between 1.113 and 1.351 mg/ml was predicted (Table 4.3, to be discussed in Section 4.14).

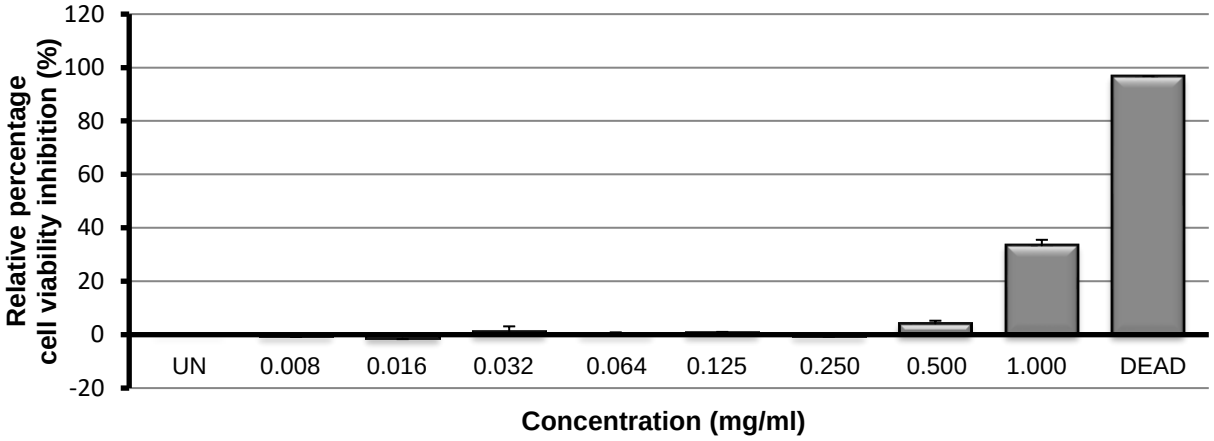


Figure 4.9: Inhibition of cell viability relative to an untreated control, following treatment of the LLC-PK1 cell line with a concentration series of *A. vera* precipitated polysaccharides for 96 h (*n* = 3; error bars = standard deviation). The positive control consisted of cells treated with Triton X-100 (DEAD), while the untreated cells are indicated as UN.

4.11.2.2.2. H69V - chemosensitive SCLC cell line

The relative cell viability of the H69V line decreased in a concentration dependent manner following treatment with *A. vera* polysaccharides (between 0.008 and 1.000 mg/ml) after 96 h (Figure 4.10). The anticancer efficacy of the *A. vera* polysaccharides against the chemosensitive SCLC cell line presented as a typical sigmoidal curve, suggesting an IC_{50} value around 0.200 mg/ml. By comparing the efficacy of *A. vera* polysaccharides at concentrations between 0.008 and 0.500 mg/ml on both the LLC-PK1 and the H69V cell lines, the *A. vera* polysaccharides can be suggested to be tumoricidal.

The *A. vera* polysaccharides at 0.500 mg/ml reduced the cell viability of the H69V cell line up to 81.5%, yet, only 3.0% cell viability reduction occurred at the same concentration in the LLC-PK1 cell line. This supports the possible use of *A. vera* polysaccharides for cancer management. Acemannan, a polysaccharide previously isolated from *A. vera,* was reviewed and found to have been used for the treatment of fibrosarcoma in domesticated animals (Sierra-Garcia *et al.*, 2014).

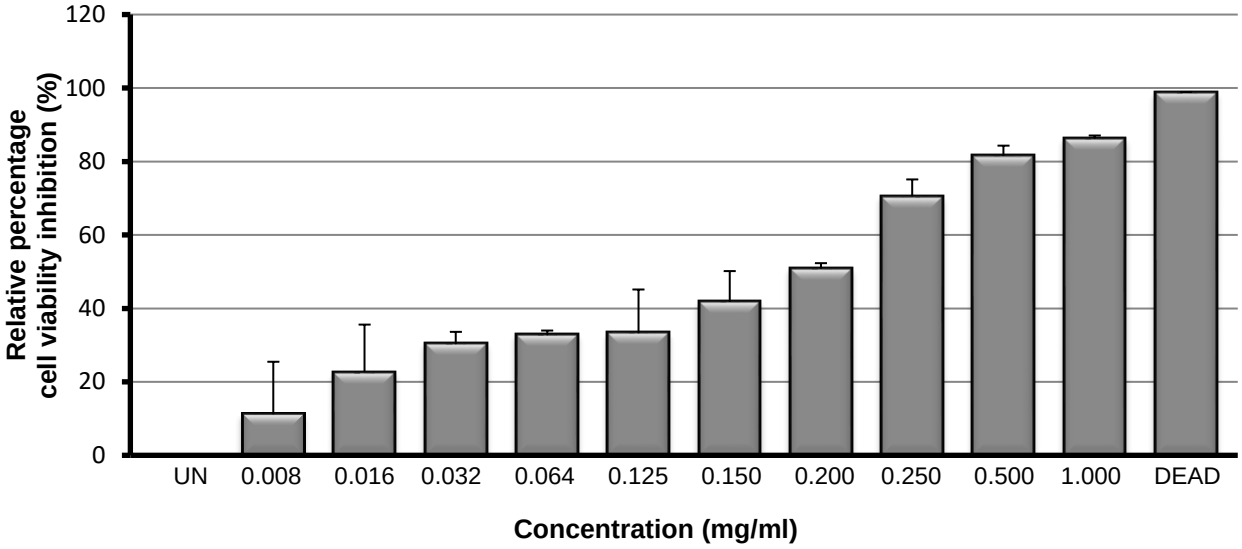


Figure 4.10: Inhibition of cell viability relative to an untreated control, following treatment of the H69V cell line with a concentration series of *A. vera* precipitated polysaccharides for 96 h (*n* = 3; error bars = standard deviation). The positive control consisted of cells treated with Triton X-100 (DEAD), while the untreated cells are indicated as UN.

4.11.2.2.3. H69AR - multidrug resistant SCLC cell line (MRP1 hyperexpressive)

The hyperexpression of MRP1 in the H69AR cell line altered the anticancer potency of the *A. vera* polysaccharide fraction. Nevertheless, the anticancer effects observed occurred in a dose-dependent manner (Figure 4.11). The IC_{50} value was estimated between 0.500 – 1.000 mg/ml, however, as previously indicated the likelihood of an occlusive effect at concentrations above

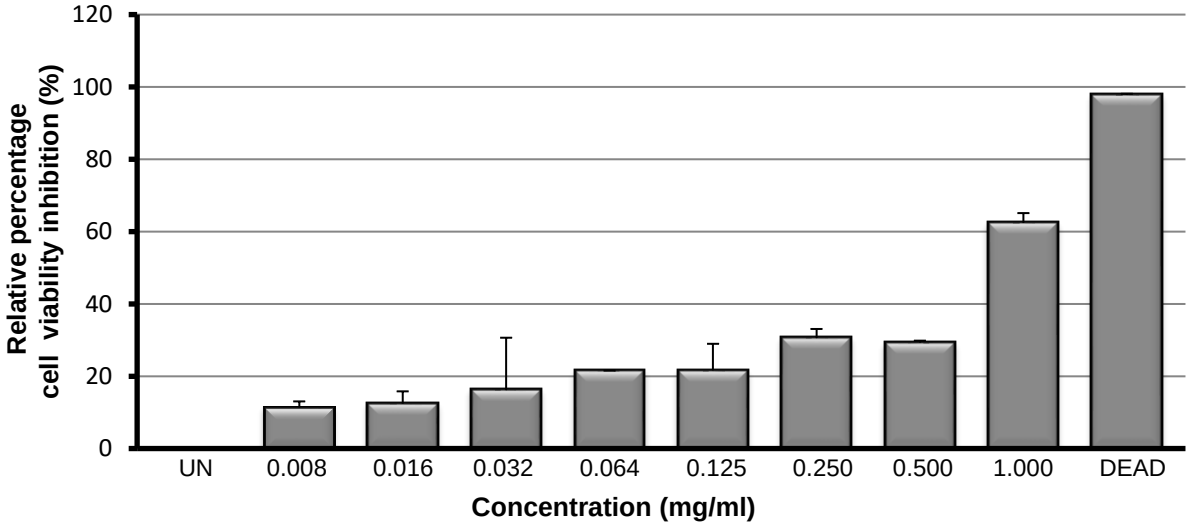


Figure 4.11: Inhibition of cell viability relative to an untreated control, following treatment of the H69AR cell line with a concentration series of *A. vera* precipitated polysaccharides for 96 h (*n* = 3; error bars = standard deviation). The positive control consisted of cells treated with Triton X-100 (DEAD), while the untreated cells are indicated as UN.

0.5 mg/ml in the LLC-PK1 cell line can be studied in the future. The *A. vera* precipitated polysaccharides showed anticancer efficacy against MRP1 hyperexpressive cells at concentrations not cytotoxic when tested on the non-cancerous cell line. It is, however, evident that the polysaccharide fraction from *A. vera* may be MRP1 substrates when comparing the cell viability reduction at 0.500 mg/ml in both the chemosensitive and the MRP1 hyperexpressive cell lines.

4.11.2.2.4. NCI-H69/LX4 - multidrug resistant SCLC cell line (P-gp hyperexpressive)

A non-linear proliferation effect was measured between 0.008 and 1.000 mg/ml after treatment of the NCI-H69/LX4 cells with *A. vera* polysaccharides for 96 h (Figure 4.12). The MTT data purported that *A. vera* polysaccharides do not have any anticancer effects towards P-gp hyperexpressive lung cancer cells at concentrations between 0.008 - 1.000 mg/ml. The proliferation effect of the polysaccharides decreased in a concentration dependent manner between 0.008 and 0.064 mg/ml. It is suggested that the P-gp efflux pumps may be saturated at concentrations higher than 0.064 mg/ml, due to the moderate plateau of the proliferation effect observed in Figure 4.12. The proliferation effect of *A. vera* polysaccharides occurred to a larger extent in the NCI-H69/LX4 cell line than in the LLC-PK1 cells, where a 0.34% cell proliferation at 0.008 mg/ml was detected. Thus, the hyperexpression of P-gp transporters may influence the intracellular concentration of the proliferative polysaccharides.

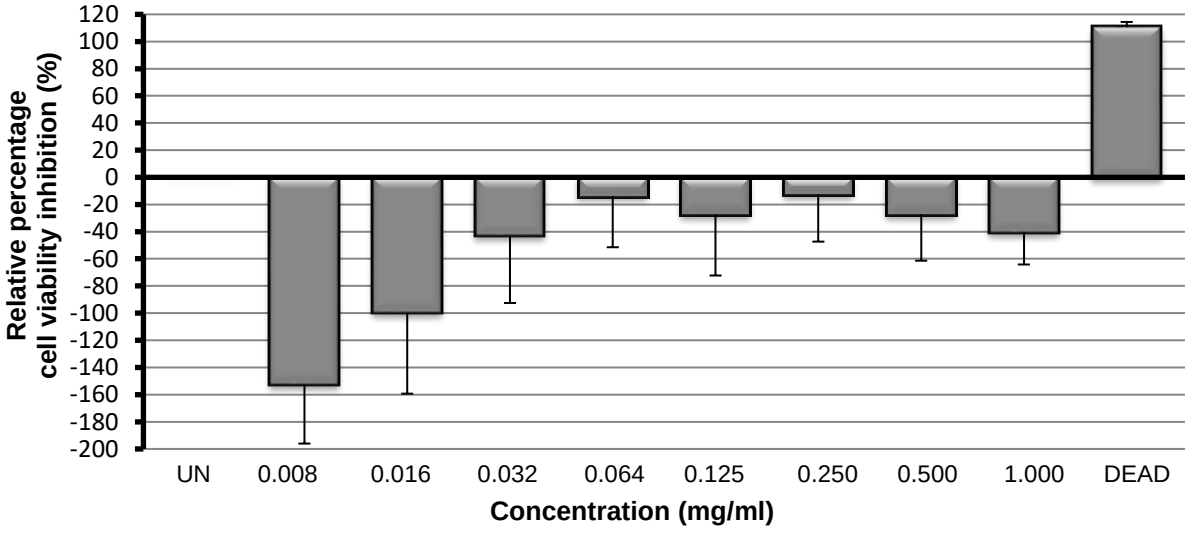


Figure 4.12: Inhibition of cell viability relative to an untreated control, following treatment of the NCI-H69/LX4 cell line with a concentration series of *A. vera* precipitated polysaccharides for 96 h (*n* = 3; error bars = standard deviation). The positive control consisted of cells treated with Triton X-100 (DEAD), while the untreated cells are indicated as UN.

The efflux of cimetidine, a substrate for P-gp transporter, was previously shown to be inhibited by the co-administration of *A. vera* polysaccharides (Beneke *et al.*, 2013). By comparing the relative cell viability reduction of the MRP1 hyperexpressive H69AR cells to the cell proliferation seen in the

P-gp hyperexpressive cell line, the anticancer compounds in *A. vera* polysaccharides are suggested to be substrates for P-gp. In theory, if the *A. vera* polysaccharide active components are P-gp substrates, the possibility arises for its co-administration with known cancer drugs to increase the intracellular concentration of the cancer drug, possibly resulting in increased cell viability reduction. Especially if the cancer drug is susceptible to P-gp efflux.

4.11.2.2.5. Summary of the *A. vera* precipitated polysaccharide MTT results

It is purported that the polysaccharide fraction of *A. vera* gel material can cause cytotoxicity at concentrations higher than 0.500 mg/ml, although concentrations between 0.008 and 1.000 mg/ml did not inhibit viability significantly in either the LLC-PK1 or the NCI-H69/LX4 cell line. This suggests both a low level of cytotoxicity and insufficient anticancer efficacy towards P-gp hyperexpressive cancer cells. Nevertheless, cell viability reduction was detected in both the chemosensitive and MRP1 hyperexpressive cell lines. The *A. vera* polysaccharides are, therefore, suggested to be P-gp substrates rather than MRP1 substrates.

4.11.2.3. *Sutherlandia frutescens*

4.11.2.3.1. LLC-PK1 - non-cancerous cell line

The cytotoxic potential of the *S. frutescens* crude aqueous extract was evaluated after a 96 h period of exposure to a concentration range of 0.200 – 8.000 mg/ml in the LLC-PK1 cell line (Figure 4.13). The relative cell inhibition graph displays a non-cytotoxic plateau effect between 0.200 and 6.000 mg/ml, indicating no cellular viability inhibition. However, an IC_{50} value was estimated at concentrations greater than 8.000 mg/ml. The cytotoxic effects of *S. frutescens* occurred in a dose dependent manner at concentrations above 6.000 mg/ml. These findings of the MTT data are in accordance with a study conducted by Stander *et al.* (2009), where a 10.000 mg/ml aqueous *S. frutescens* extract (obtained from the Karoo regain in South Africa) inhibited 54% cell growth of a normal mammary gland cell line (MCF-12A). The difference in concentrations resulting in a 50% cell viability reduction is most probably a result of the different target tissue, or demographical differences in the collection sites of the plant material. These differences are highlighted by another study, where the cell viability of the MCF-12A cell line was estimated at 93.22% after 48 h of treatment with a 1 mg/ml aqueous *S. frutescens* extract (Vorster *et al.*, 2012).

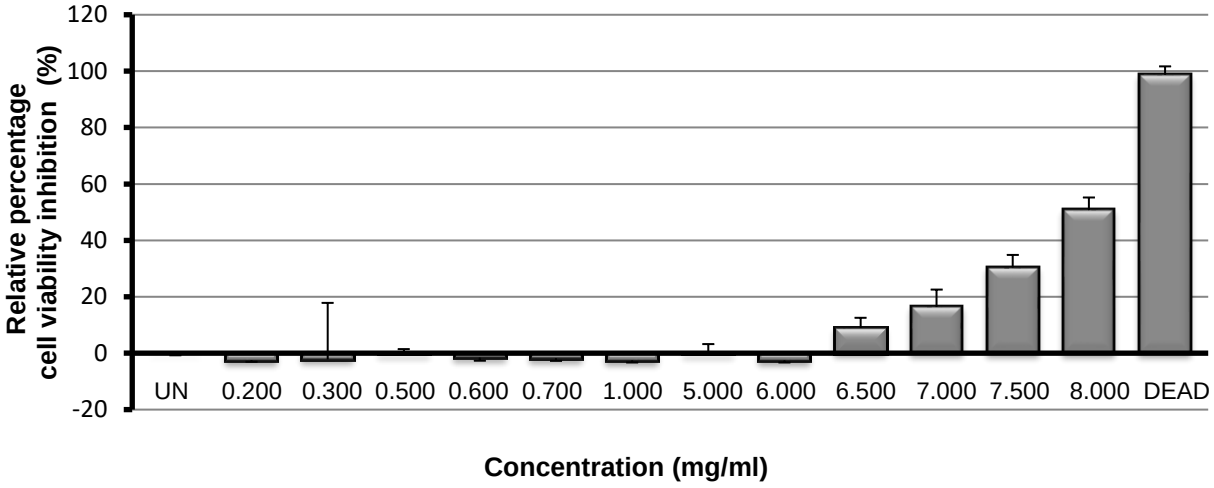


Figure 4.13: Inhibition of cell viability relative to an untreated control, following treatment of the LLC-PK1 cell line with a concentration series of *S. frutescens* for 96 h (*n* = 3; Error bars = standard deviation). The positive control consisted of cells treated with TritonX (DEAD), while the untreated cells are indicated as UN.

4.11.2.3.2. H69V - chemosensitive SCLC cell line

The anticancer potential of *S. frutescens* extract was screened in the chemosensitive cell line at concentrations between 0.100 – 5.000 mg/ml (Figure 4.14). *S. frutescens* demonstrated anticancer efficacy in a concentration dependent manner between 0.250 and 3.500 mg/ml, although, a plateau effect was observed at concentrations higher than 2.500 mg/ml. The chemosensitive cell line appeared much more susceptible towards *S. frutescens* in comparison with the LLC-PK1 cell line, since concentrations between 0.250 and 3.500 mg/ml did not decrease the cell viability of the LLC-PK1 cell line in a considerable manner. In a study conducted by Vorster *et al.* (2012), a 1.000 mg/ml aqueous *S. frutescens* extract resulted in 51.9% cell growth inhibition in a breast cancer cell line (MCF-7) after 48 h. In the current study, 1.000 mg/ml extract resulted in approximately 80% cell viability reduction in the chemosensitive cell line after 96 h treatment. It is suggested that the anticancer efficacy of *S. frutescens* may therefore be determined by both the exposure time of the extract to the cells, and the tumour type.

4.11.2.3.3. H69AR - multidrug resistant SCLC cell line (MRP1 hyperexpressive)

A concentration dependent anticancer effect of *S. frutescens* extract was observed between 0.250 and 5.000 mg/ml after 96 h treatment in the H69AR cell line, as illustrated in Figure 4.15. In this sigmoidal shaped graph, a plateau effect was observed from 3.500 mg/ml at the maximum level of cell viability reduction as established in the dead cell control. The concentrations screened against the H69AR cell line indicated no significant cytotoxicity in the non-cancerous cell line, while demonstrated a higher level of resistance towards *S. frutescens* than the H69V cell line. The increased IC_{50} range in comparison to the H69V cell line, suggests the active phytochemicals in the

crude *S. frutescens* extract to be MRP1 substrates. A triterpenoid glycoside namely Sutherlandioside B, present in the *S. frutescens* plant, was previously also found to possibly be both a P-gp and MRP substrate (Madgula *et al.*, 2008).

4.11.2.3.4. NCI-H69/LX4 - multidrug resistant SCLC cell line (P-gp hyperexpressive)

The anticancer potential of *S. frutescens* crude aqueous extract was screened in the NCI-H69/LX4 cell line between 0.050 and 5.000 mg/ml after 96 h treatment, as shown in Figure 4.16. The MTT data suggest that the anticancer effects of *S. frutescens* occur in a concentration dependent manner against the P-gp hyperexpressive cell line. The influence of P-gp hyperexpression on the *S. frutescens* extract efficacy is demonstrated by the increased IC_{50} range in the NCI-H69/LX4 cell line, compared to a lower IC_{50} range detected in the chemosensitive cell line. The differences between detected IC_{50} ranges of the H69AR and NCI-H69/LX4 lines also suggest the active phytochemicals in the *S. frutescens* extract to have a lower affinity for the P-gp efflux transporter. However, *S. frutescens* was previously shown to inhibit intestinal P-gp efflux (Ondieki *et al.*, 2017). The overall findings indicated the NCI-H69/LX4 cell line to be less resistant to *S. frutescens* than the H69AR cell line. The NCI-H69/LX4 cell model showed moderate variation, with high standard deviations between 0.050 and 0.090 mg/ml, which may be a result of the non-uniform cell clusters.

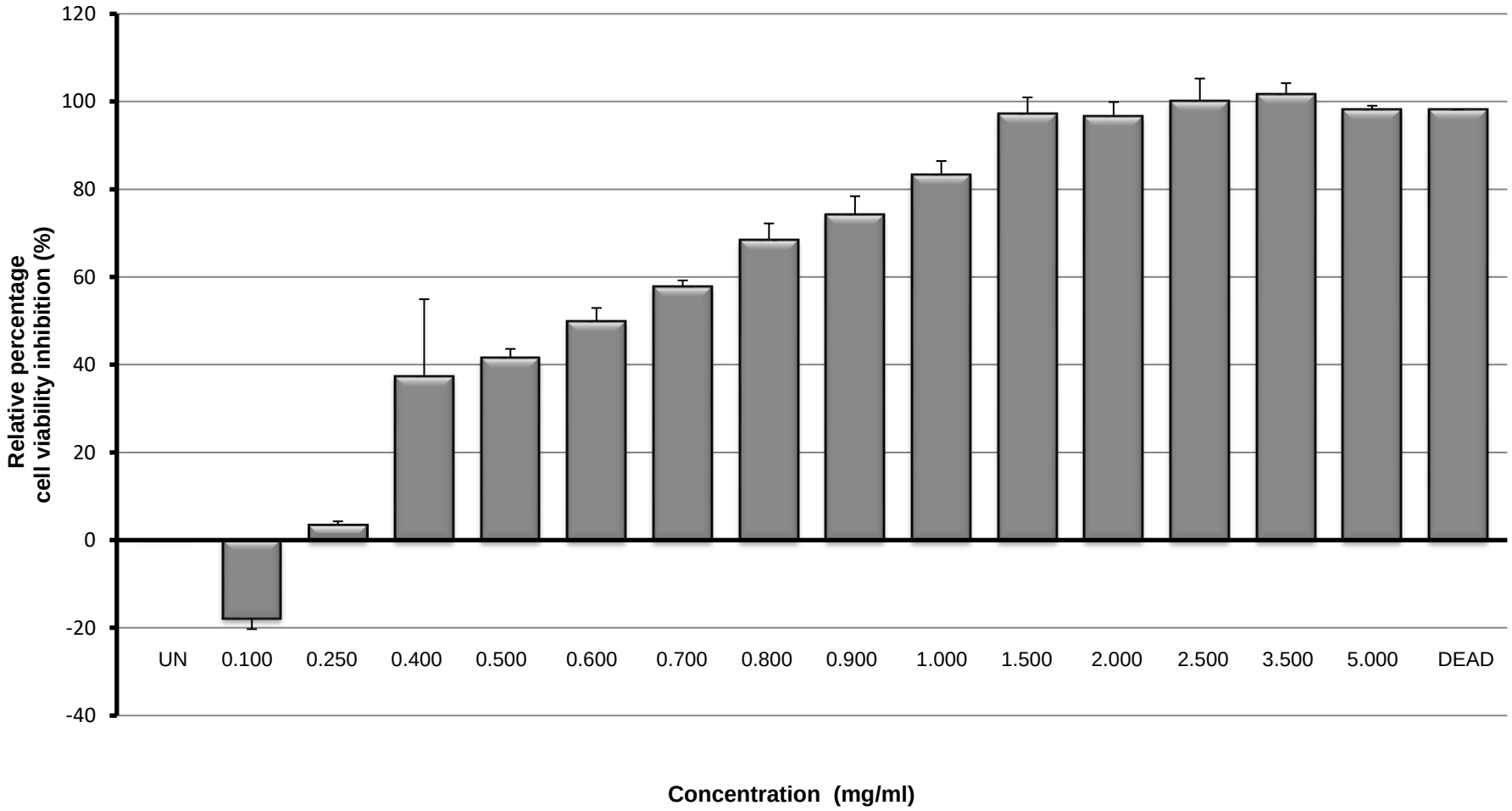


Figure 4.14: Inhibition of cell viability relative to an untreated control, following treatment of the H69V cell line with a concentration series of *S. frutescens* for 96 h (*n* = 3; Error bars = standard deviation). The positive control consisted of cells treated with Triton X (DEAD), while the untreated cells are indicated as UN.

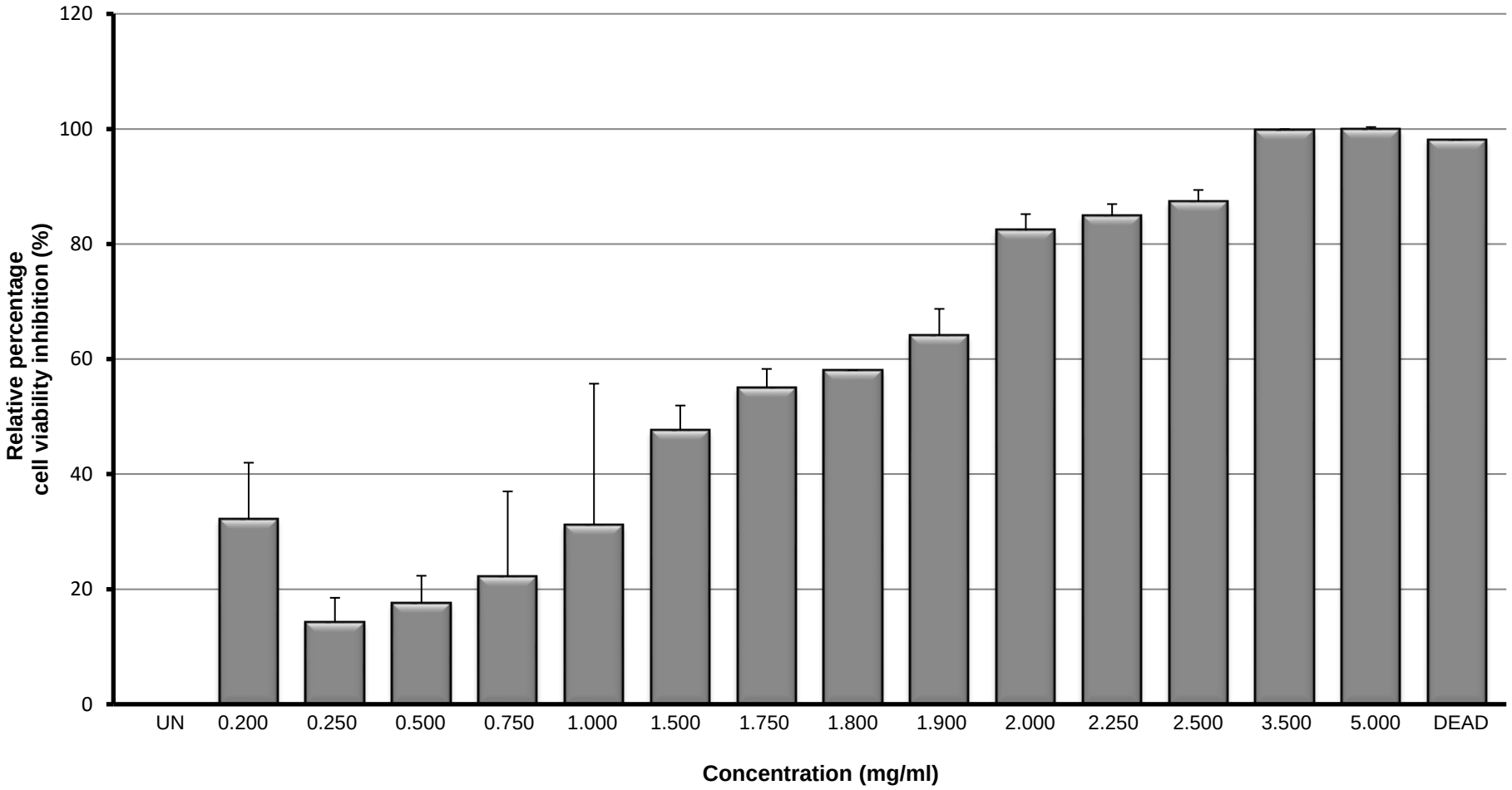


Figure 4.15: Inhibition of cell viability relative to an untreated control, following treatment of the H69AR cell line with a concentration series of *S. frutescens* for 96 h (*n* = 3; Error bars = standard deviation). The positive control consisted of cells being treated with Triton X (DEAD), while the untreated cells are indicated as UN.

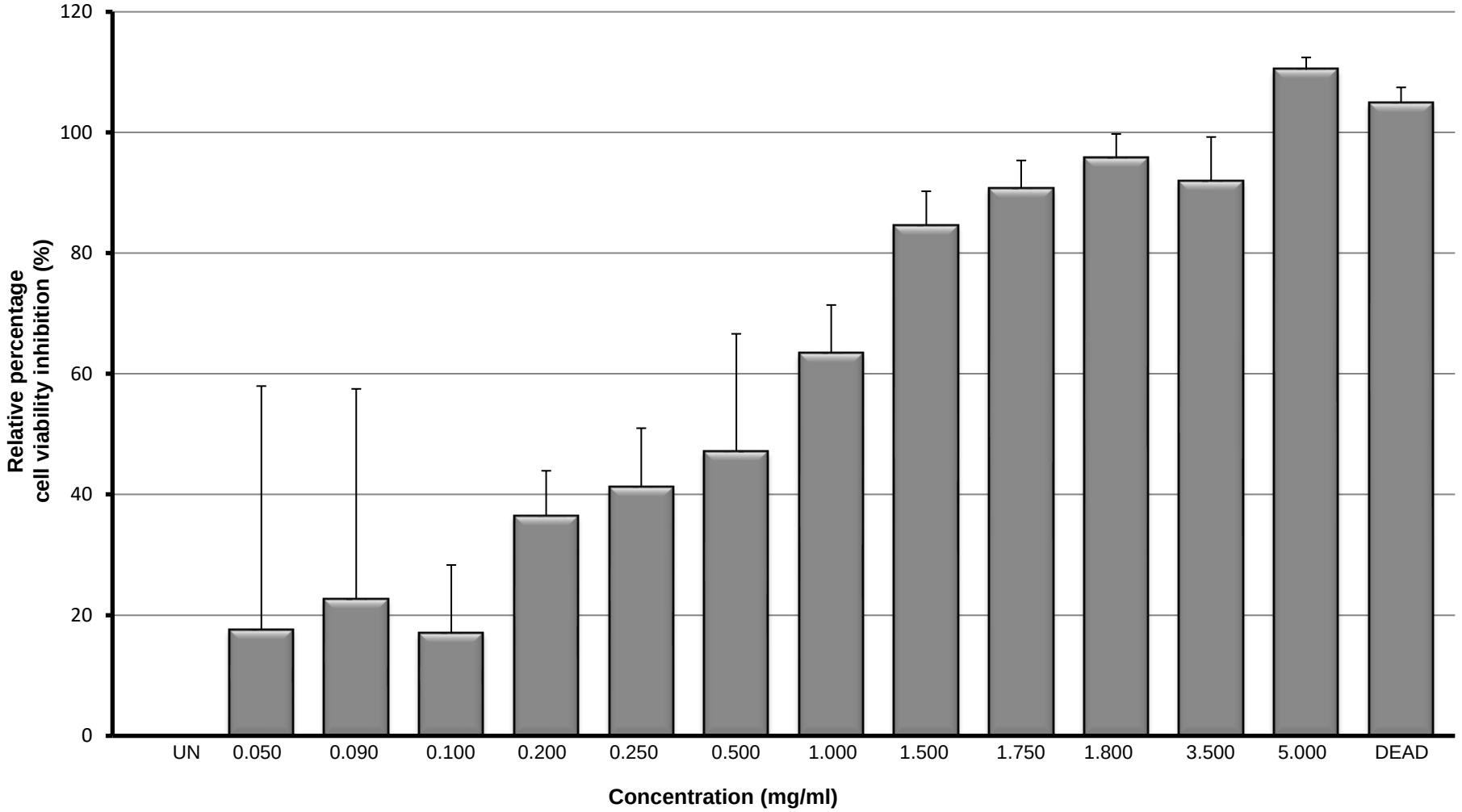


Figure 4.16: Inhibition of cell viability relative to an untreated control, following treatment of the NCI-H69/LX4 cell line with a concentration series of *S. frutescens* for 96 hours (*n* = 3; Error bars = standard deviation). The positive control consisted of cells treated with Triton X (DEAD), while the untreated cells are indicated as UN.

4.11.2.3.5. Summary of the *S. frutescens* MTT results

The non-cancerous cell line demonstrated reduced cell viability at *S. frutescens* concentrations greater than 6.000 mg/ml. This potential anticancer efficacy of *S. frutescens* crude aqueous extract followed a typical sigmoidal trend when screened in the three SCLC cell lines. The anticancer efficacy of the crude *S. frutescens* extract is suggested to be influenced by the exposure period and the cancer tissue characteristics. The phytochemicals in the aqueous *S. frutescens* extract are proposed to be MRP1 substrates, rather than P-gp substrates.

4.11.2.4. *Xysmalobium undulatum*

4.11.2.4.1. LLC-PK1 – non-cancerous cell line

The cytotoxic potential of the *X. undulatum* crude aqueous extract after 96 h exposure was investigated in the non-cancerous control cell line, as indicated in Figure 4.17. The decreased relative cell viability observed was dose dependent between 0.006 – 0.500 mg/ml. A plateau at maximum cytotoxic effect was observed above 0.300 mg/ml *X. undulatum*. An IC_{50} value was estimated to be approximately 0.008 mg/ml.

4.11.2.4.2. H69V – chemosensitive SCLC cell line

In Figure 4.18, the anticancer effect of *X. undulatum* on the chemosensitive SCLC cell line was illustrated in a dose dependent manner between 0.0004 and 0.1000 mg/ml after 96 h exposure. The graph shows a plateau of maximum cell viability reduction at concentrations above 0.0500 mg/ml. The anticancer effect of *X. undulatum* occurred at concentrations suggested to be cytotoxic for the non-cancerous LLC-PK1 cell line. Yet, a lower IC_{50} range between 0.004 - 0.007 mg/ml was estimated for the H69V cell line in comparison to the IC_{50} range of the LLC-PK1 cell line. Thus, the chemosensitive SCLC cell line was more susceptible to *X. undulatum* toxicity than the LLC-PK1 cell line.

4.11.2.4.3. H69AR - multidrug resistant SCLC cell line (MRP1 hyperexpressive)

The ability of *X. undulatum* to reduce the relative cell viability of the H69AR cell line was evaluated at concentrations between 0.008 – 0.500 mg/ml over 96 h (Figure 4.19). The reduction in cell viability of this MRP1 hyperexpressive cell line was concentration dependent, with a plateau effect approaching maximal inhibition at concentrations higher than 0.090 mg/ml. The MTT data indicated the IC_{50} value as approximately 0.020 mg/ml, which demonstrates the resistance of the H69AR cell line towards *X. undulatum.* By comparing the IC_{50} values of the H69AR and LLC-PK1 cell lines, it is evident that this concentration range may cause moderate cytotoxicity. Furthermore, the inhibitory effect of the *X. undulatum* extract occur at higher concentrations in the H69AR cell line than the H69V cell line, demonstrating the possibility of the active *X. undulatum* phytochemicals to be MRP1 substrates.

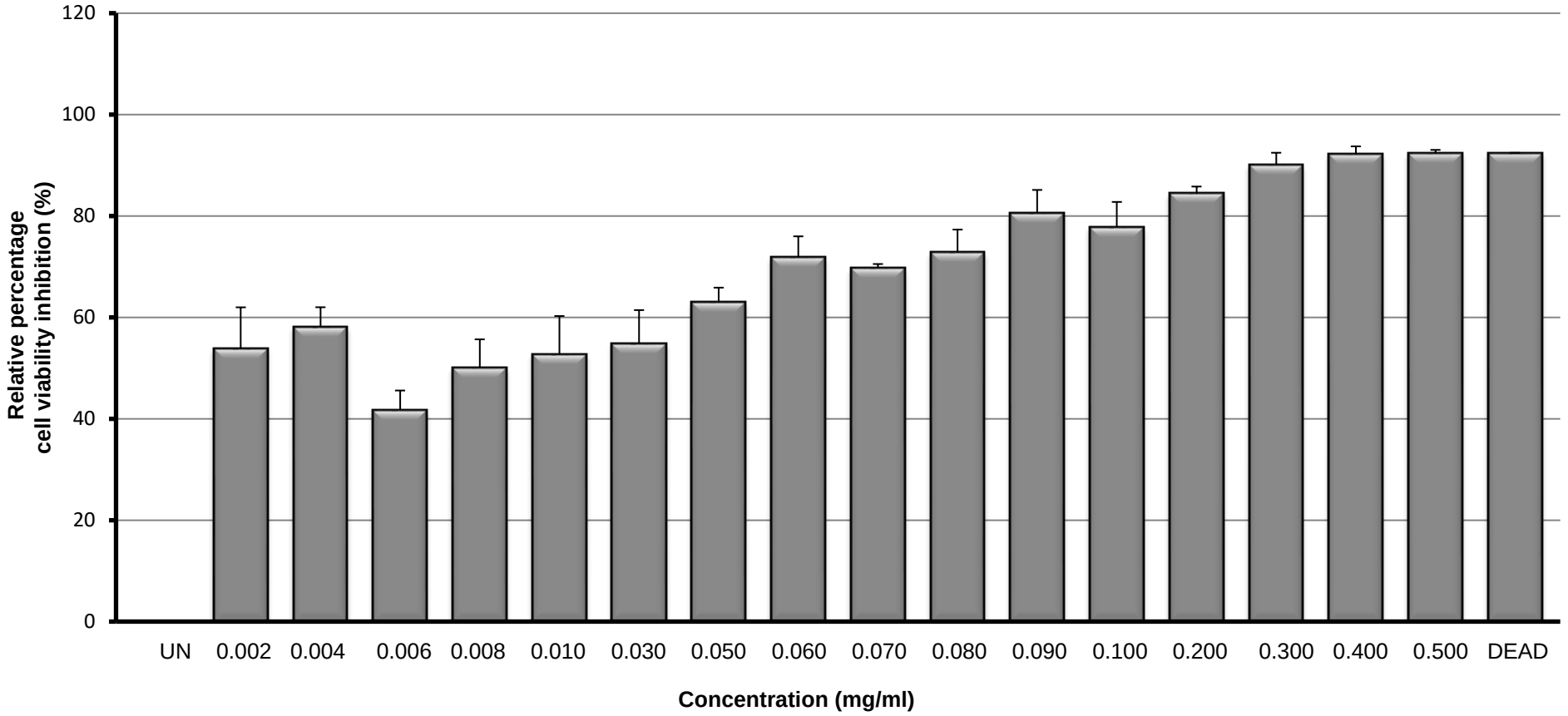


Figure 4.17: Inhibition of cell viability relative to the untreated control, following treatment of the LLC-KP1cell line with a concentration series of *X. undulatum* for 96 h (*n* = 3; Error bars = standard deviation). The positive control consisted of cells treated with Triton X (DEAD), while the untreated cells are indicated as UN.

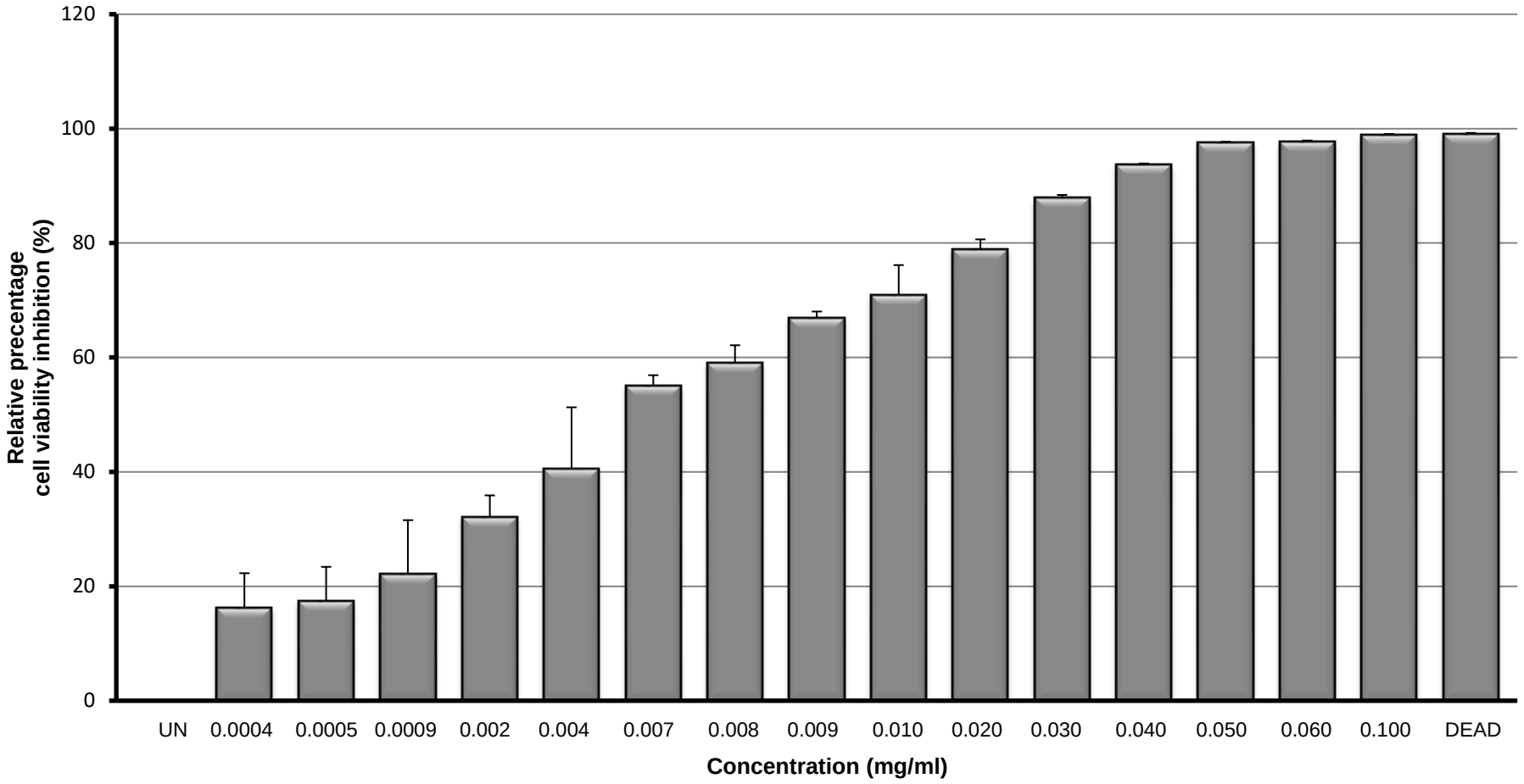


Figure 4.18: Inhibition of cell viability relative to an untreated control, following treatment of the H69V cell line with a concentration series of *X. undulatum* for 96 h (*n* = 3; Error bars = standard deviation). The positive control consisted of cells treated with Triton X (DEAD), while the untreated cells are indicated as UN.

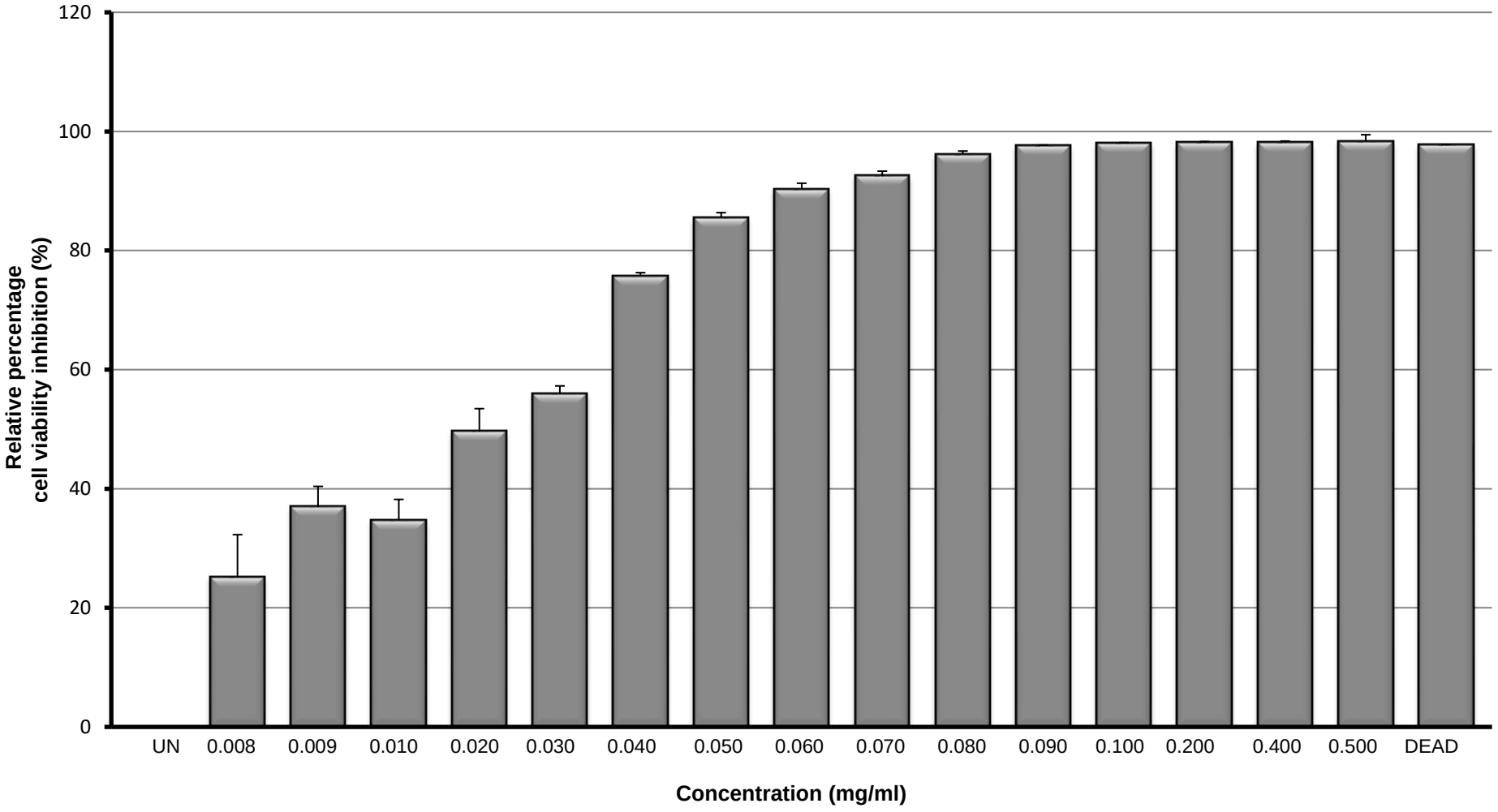


Figure 4.19: Inhibition of cell viability relative to an untreated control, following treatment of the H69AR cell line with a concentration series of *X. undulatum* for 96 h (*n* = 3; Error bars = standard deviation). The positive control consisted of cells treated with Triton X (DEAD), while the untreated cells are indicated as UN.

4.11.2.4.4. NCI-H69/LX4 - multidrug resistant SCLC cell line (P-gp hyperexpressive)

The anticancer properties of *X. undulatum* were screened between 0.010 and 0.080 mg/ml in the NCI-H69/LX4 cell line (Figure 4.20). The relative cell viability of the P-gp hyperexpressive cell line was reduced in a dose dependent manner by the crude aqueous *X. undulatum* extract, indicating the possible use in MDR cancer treatment. However, the IC_{50} value between 0.015 - 0.018 mg/ml observed for the NCI-H69/LX4 cell line have been shown to be cytotoxic in the LLC-PK1 cell line. Further consideration of the IC_{50} value revealed the possibility of the active *X. undulatum* phytochemicals to be P-gp substrates when compared to the H69V IC_{50} value. Thus, the transport of *X. undulatum* (containing Uzarin, a cardenolide) can potentially be influenced by P-gp efflux.

Cardenolides in general have been suggested to modify MDR in cancer by inhibiting P-gp (Krishna *et al.*, 2015). Further studies are needed to determine whether this specific plant containing cardenolides can cause P-gp inhibition in the NCI-H69/LX4 cell line.

4.11.2.4.5. Summary of the *X. undulatum* MTT results

The *X. undulatum* crude aqueous extract showed *in vitro* anticancer potential in both the chemosensitive and multidrug resistant SCLC cell lines. The MTT data suggest the possible use of *X. undulatum* for treatment of chemosensitive SCLC, given that the estimated IC_{50} value of the H69V cell line was lower than the IC_{50} value for the LLC-PK1 cell line. However, the cytotoxic effects of *X. undulatum* at IC_{50} concentrations needed for both the multidrug resistant SCLC lines negates its use in MDR cancer management. A higher concentration is needed to inhibit 50% of the H69AR cells compared to the chemosensitive cell line, demonstrating the possibility of the active phytochemicals in the *X. undulatum* extract to be MRP1 substrates, while the IC_{50} values determined for the P-gp hyperexpressive cells suggest the intracellular accumulation of the active components to be influenced to a greater extent by the MRP1 transporters than the P-gp transporters.

4.12. Statistical modelling of experimental MTT data

Preliminary screening of the anticancer potential of three selected medicinal plants, namely *A. vera* (gel material and polysaccharides), *S. frutescens* and *X. undulatum,* was done by means of the MTT assay. The influence of the plant material on the relative cell viability of the various selected cell lines was defined through an estimated IC_{50} value. The MTT data was, however, also analysed through statistical modelling.

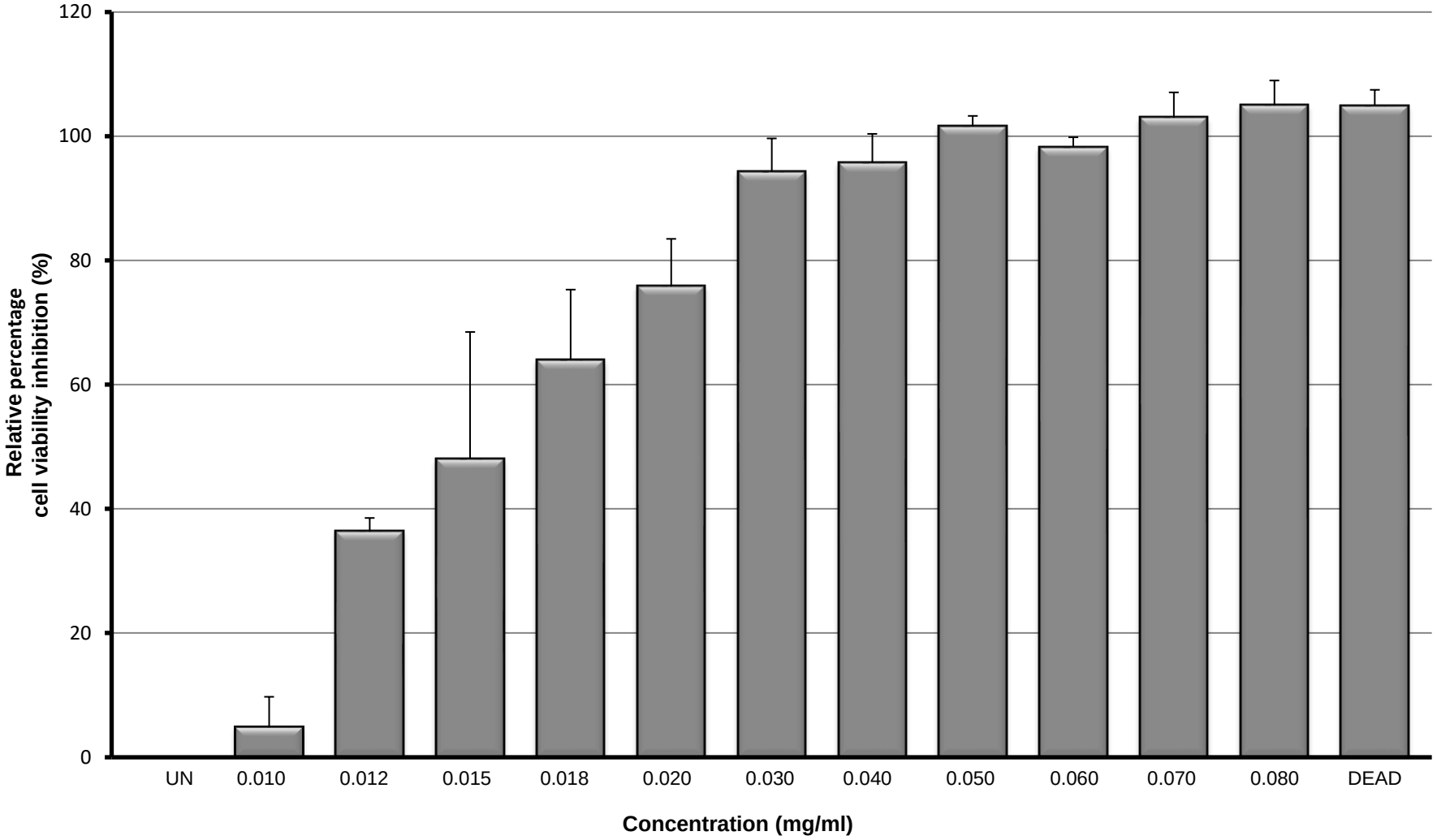


Figure 4.20: Inhibition of cell viability relative to an untreated control, following treatment of the NCI-H69/LX4 cell line with a concentration series of *X. undulatum* for 96 h (*n* = 3; Error bars = standard deviation). The positive control consisted of cells treated with Triton X (DEAD), while the untreated cells are indicated as UN.

The MTT data were analysed by means of a Probit Analysis Method, using SPSS statistical analysis software. The statistically determined IC_{50} values for the various plant extracts in the selected mammalian cell lines are summarised in Table 4.3, and correlates well with the experimental MTT findings. The impact of the hyperexpressed efflux transporters on the efficacy of the different plant extracts was further evaluated by calculating the cancer selectivity and resistance ratios. To indicate the level of resistance, the efficacy in the hyperexpressive cell lines are compared to the efficacy in the chemosensitive cell line (Hall *et al.*, 2009). A resistance ratio greater than 1.000 indicates a reduced efficacy of the extract towards the hyperexpressive cell line, whereas a value lower than 1.000 signifies higher anticancer efficacy against the hyperexpressive cell line. The *S. frutescens* (resistance ratio: 2.613) were the most effective anticancer agent against the MRP1 hyperexpressive SCLC cell line. The P-gp hyperexpressive cell line was found to be more susceptible to *S. frutescens* (known for traditional cancer management) as indicated by a resistance ratio of 0.918. Furthermore, *S. frutescens* showed the highest cancer selectivity (14.901) characteristics, when comparing efficacy against the chemosensitive SCLC cells to the non-cancerous cell line.

The *A. vera* gel material displayed both cytotoxic and anticancer effects, however, the cytotoxicity only occurred at higher concentrations than needed to achieve IC_{50} values in the three SCLC cell lines. The *A. vera* gel did inhibit the cell viability of the MRP1 and P-gp hyperexpressive cell lines. The cytotoxicity of the precipitated polysaccharides occurred at 1.201 mg/ml compared to the gel fragment resulting in cytotoxicity at 0.374 mg/ml, hence, the gel fragment has demonstrated cytotoxicity at a much lower dosage than the polysaccharides.

The precipitated polysaccharides, compared to the gel material, showed lower anticancer efficacy when tested on the chemosensitive cells, which is shown by the polysaccharide IC_{50} value of 0.179 mg/ml compared to 0.027 mg/ml for the gel. Thus, the possibility occurs of additional bioactive phytochemical present in the gel fraction, which are not in the polysaccharide fraction. The increased IC_{50} concentrations for the MDR SCLC cell lines, for both the gel and polysaccharide fractions, suggest the phytochemicals in these extracts to be substrates for both MRP1 and P-gp transporters.

The *S. frutescens* extract is purported to be both cytotoxic and tumoricidal. A 50% cell viability reduction of the LLC-PK1 cell line was detected at 8.017 mg/ml, while anticancer activities occurred at lower concentrations (such as 0.494 and 1.406 mg/ml). The cytotoxic effects observed for the *S. frutescens* extract occurred at concentrations greater than needed for possible anticancer efficacy. The phytochemicals in the *S. frutescens* extract can be considered possible P-gp (0.494 mg/ml), and to a higher extent MRP1 substrates due to the increased IC_{50} of 1.406 mg/ml detected with the MRP1 hyperexpressive cell line.

Table 4.3: Summary of the statistically determined cell viability reduction, cancer selectivity and resistance ratios for the various plant extracts

		Cell models			
Medicinal plant	**Parameters**	LLC-PK1	H69V	H69AR	NCI-H69/LX4
A. vera (gel)	IC_{50} (mg/ml)	0.374	0.027	0.227	0.201
	IC_{50} range (mg/ml)[$]	0.349-0.400	0.023-0.031	0.203-0.253	0.166-0.237
	Cancer Selectivity*	–	13.830	1.649	1.860
	Resistance Ratio[#]	–	–	8.387	7.435
A. vera (polysaccharides)	IC_{50} (mg/ml)	1.201	0.179	0.719	1.346
	IC_{50} range (mg/ml)[$]	1.113 -1.351	0.152-0.208	0.640-0.807	0.961-2.218
	Cancer Selectivity*	–	6.702	1.670	0.893
	Resistance Ratio[#]	–	–	4.017	6.702
S. frutescens	IC_{50} (mg/ml)	8.017	0.538	1.406	0.494
	IC_{50} range (mg/ml)[$]	7.90-8.168	0.486-0.588	1.318-1.485	0.418-0.567
	Cancer Selectivity*	–	14.901	5.704	16.235
	Resistance Ratio[#]	–	–	2.613	0.918
X. undulatum	IC_{50} (mg/ml)	0.010	0.004	0.0199	0.015
	IC_{50} range (mg/ml)[$]	0.006-0.014	0.003-0.005	0.017-0.023	0.012-0.016
	Cancer Selectivity*	–	2.322	0.483	0.660
	Resistance Ratio[#]	–	–	4.810	3.518

*Cancer selectivity was determined as the ratio of the IC_{50} of the LLC-PK1 cells divided by the IC_{50} of the SCLC cell line.
[#]The resistance ratio of the cell lines was determined by the ratio of the IC_{50} of the hyperexpressive cell lines divided by the IC_{50} of the H69V chemosensitive cell line.
[$]The IC_{50} ranges are indicative of a 95% confidence interval (n = 3).

The anticancer efficacy of the *X. undulatum* extract was screened at lower concentrations compared to the *A. vera* (gel and polysaccharides) and *S. frutescens* extracts. However, it is evident that the cytotoxicity potential of *X. undulatum* negates its use in cancer management. The phytochemical constituents of *X. undulatum* are considered to be substrates for P-gp and MRP1.

The overall observation indicated the four medicinal plant extracts used in this study to reduce the cell viability of the tested SCLC cell lines, and their phytochemical components, are possible substrates for MRP1 and P-gp transport proteins.

4.13. Conclusion

In Chapter 4 the preliminary anticancer screening of selected medicinal plants resulted in the estimation of *in vitro* IC_{50} values. The observations made in Chapter 4 were, subsequently, further investigated by conducting an additional evaluation of the intracellular adenosine triphosphate (ATP) and extracellular adenylate kinase (AK) levels of the studied cell lines following treatment with the statistically determined IC_{50} values (Chapter 5).

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Chapter 5

Advanced anticancer efficacy evaluation

Chapter 5 consists of a research article prepared for submission to *the Journal of Ethnopharmacology*. The manuscript was prepared according to the specific author guidelines as shown in Appendix B.

In vitro screening of selected medicinal plants as treatment for drug resistant small cell lung cancer

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Abstract

Ethnopharmacological relevance

Traditional medicinal plants such as Aloe vera, Sutherlandia frutescens and Xysmalobium undulatum have been used to treat several illnesses, including cancer. It is important to gain further information on the efficacy of these traditional medicinal plants against specific cancers, including chemosensitive and chemoresistant small cell lung cancer (SCLC) cells.

Aim of the study

SCLC is a high grade neuroendocrine malignancy, which is responsible for high mortality rates worldwide. The hyperexpression of several adenosine triphosphates (ATP)-binding cassette (ABC) efflux transporters in SCLC tissues is often responsible for occurrence of multidrug resistance (MDR) in these tumours. MDR reduces the chances of a positive outcome of SCLC chemotherapeutic treatment. The aim of this study is to investigate an ethno-medicinal approach to combat cancer through evaluation of the selected traditionally used medicinal plants against chemosensitive and chemoresistant SCLC cell models.

Materials and methods

The anticancer effects of crude aqueous extracts of Sutherlandia frutescens and Xysmalobium undulatum, as well as Aloe vera gel material and precipitated polysaccharides were studied on different SCLC cell lines. The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay was used as a preliminary screening method to determine the relative reduction in cell viability (IC) of each selected SCLC cell line, after a 96 h exposure to the various plant materials. Subsequently, the effect of the selected plant materials on intracellular ATP and extracellular adenylate kinase (AK) levels of the different SCLC and LLC-PK1 cells were measured to establish their anticancer efficacy and cytotoxic potential more accurately.

Results

All the selected plant materials investigated in this study resulted in a statistically significant reduction in cell viability for all of the SCLC cell lines ($p<0.05$), although a measure of resistance was observed in the chemoresistant cell lines. The anticancer phytochemicals in the crude extracts are therefore most probably subjected to MRP1 and P-gp related efflux. The aqueous S. frutescens extract was found to possibly induce necrosis in the multidrug resistance-associated protein 1 (MRP1) hyperexpressive SCLC cell line at 1.406 mg/ml, while the MTT data suggested that S. frutescens extract had the highest cancer selectivity ratio in P-glycoprotein (P-gp) hyperexpressive SCLC management of all the materials investigated. The cytotoxic effect of the X. undulatum extract on non-cancerous cells (LLC-PK1 cell line) contradicts its use in SCLC management.

Conclusions

The selected medicinal plant materials significantly altered both the intracellular ATP and extracellular AK levels of the chemosensitive and chemoresistant SCLC cell lines, indicating anticancer effects against SCLC cells. However, indications of cytotoxicity to some extent were also detected in a non-cancerous LLC-PK1 cell line for all of the plant materials.

Keywords: *Aloe vera,* crude aqueous extracts, multidrug resistance, Small cell lung carcinoma, *Sutherlandia frutescens*, *Xysmalobium undulatum*.

1. Introduction

Lung cancer is one of the leading causes of cancer deaths worldwide, with 2.09 million deaths reported in 2018 (World Health Organization, 2018). Small cell lung carcinoma (SCLC), a high grade neuroendocrine malignancy, accounts for an estimated 15% of all lung cancer diagnoses (Tanaka et al., 2018). The systemic treatment option for managing SCLC has remained relatively unchanged for the last 40 years, as platinum-based chemotherapy in combination with etoposide is still commonly used without long-term success. Inconsistent chemotherapeutic outcomes are caused by obstacles such as platinum resistance and cross-resistance against several anticancer drugs (Mollaoglu et al., 2017; Peng et al., 2018). The term multidrug resistance (MDR) is used to define cancer cells with an insensitivity or resistance to a variety of structurally unrelated anticancer drugs (Sivak et al., 2017). Up-regulation of several ABC efflux transporter proteins is a frequent contributing factor to the occurrence of MDR in cancer cells (Kathawala et al., 2015). The three major efflux transporters responsible for MDR in cancer cells are P-glycoprotein (P-gp; ABCB1), multidrug resistance-associated protein 1 (MRP1; ABCC1) and breast cancer resistance protein (BCRP; ABCG2) (Sharom, 2008). The possibility arise that by reducing the efflux of anticancer drugs

from the intra-to-extracellular environment, the impact of MDR in cancer cells can be reduced (Wu et al., 2014).

The ethno-botanical use of several medicinal plant extracts and phytochemicals have drawn considerable attention as an alternative treatment option in overcoming cancer MDR, potentially through modulation (i.e. inhibition) of xenobiotic efflux (Ferreira et al., 2014; Yuan & Li, 2014; Ochwang'i et al., 2018).

Aloe vera (*Aloe Barbadensis* Miller), grouped under the *Asphodelaceae* botanical family, is a succulent known for lance-spear shaped leaves with jagged edges (Gupta et al., 2018). The mucilage (gel-like consistence) within the leaves is considered to be beneficial and is traditionally harvested by means of a hand filleted method, using a sharp knife to expose the pulp from which the mucilage or gel can be extracted (Ramachandra & Rao, 2008). The pulp of the leaves is composed of various chemical constituents, including anthraquinones, carbohydrates, chromones, enzymes, inorganic compounds, non-essential and essential amino acids, saccharides, proteins and vitamins (Hamman, 2008). *A. vera,* commonly referred to as the first aid plant or burn plant, is purported to be beneficial in the treatment of several health conditions such as cardiovascular diseases, arthritis, diabetes and cancer, but also has antimicrobial and immunomodulatory efficacy (Nazir & Ahsan, 2017). It has been proposed that the active polysaccharides and glycoproteins in *A. vera* contribute to the chemo-preventive properties of this plant (Singh et al., 2000).

Sutherlandia frutescens (tribe *Galegeae*, family *Fabaceae*) is a shrub indigenous to the southern Africa region (including South Africa, southern Botswana, southern Namibia and Lesotho). This shrub, reaching heights of up to 2.5 m, has characteristic red boat-like keel petal flowers (Van Wyk & Albrecht, 2008). In South Africa, traditional healers prepare *S. frutescens*, also known as cancer bush, by making an infusion of the stem and leaves to treat cancer. The Khoi-San and Nama populations also treated fever and rinsed wounds with decoctions of *S. frutescens* (Aboyade et al., 2014). Pharmacologically, the cycloartane-type triterpenoids in *S. frutescens* have been found to act in a cancer chemo-preventive manner, supporting the traditional use of this medicinal plant in cancer treatment (Van Wyk et al., 2009). An aqueous extract was also found to alter the proliferation of an MCF-7 breast cancer cell line, further supporting the beneficial anticancer properties of this medicinal plant (Vorster et al., 2012).

Xysmalobium undulatum (family *Apocynaceae*), referred to as Uzara, milkwort or wild cotton, is widely distributed across the southern African landscape and is traditionally used for a variety of health conditions, including diarrhoea, afterbirth cramps and headaches (Balogun et al., 2016). The following phytochemicals of *X. undulatum* are all grouped as cardenolides, considered to have P-gp inhibitory activity that can potentially target cancer MDR: uzarin, urezin, ascleposide xysmalorin, xysmalobin, uzaroside and glucoascleposide (Krishna et al., 2015).

In this study, the anticancer efficacy of crude aqueous extracts and inner leaf materials from three selected medicinal plants frequently used in South Africa were investigated using *in vitro* cell culture models. These plants included *A. vera* (gel material and a precipitated polysaccharide fraction), *S. frutescens* and *X. undulatum* (aqueous extracts). A panel of three chemosensitive and chemoresistant SCLC cell lines and one non-cancerous cell line were used to determine the potential anticancer properties of the selected plant materials. Secondly, the potential of each plant to overcome MDR induced by hyperexpressed efflux transporters were investigated. This was done through pre-screening of a range of concentrations of each plant extract by means of the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay. Subsequently, the effect of selected concentrations of each plant extract on cell viability was further assessed with the intracellular adenosine triphosphate (ATP) and extracellular adenylate kinase (AK) assays.

2. Materials and methods

2.1. Study design

The anticancer efficacy and cytotoxicity of four plant materials, namely *A. vera* gel material, *A. vera* polysaccharides precipitated from the gel, aqueous extract of *S. frutescens* and aqueous extract of *X. undulatum* were determined on different SCLC cell lines and a non-cancerous pig embryonic kidney (LLC-PK1) cell line. The SCLC cell lines consisted of a chemosensitive (H69V), chemoresistant MRP1 hyperexpressive (H69AR) and a chemoresistant P-gp hyperexpressive (NCI-H69/LX4) cell line.

The preliminary anticancer potential of a series of plant material concentrations was evaluated by means of an MTT assay, following 96 h of exposure with daily application of fresh solutions. The data were subsequently statistically evaluated to determine the concentrations where 50% and 75% reduction in cell viability relative to an untreated control (IC_{50} and IC_{75}, respectively) occurred.

These values were then used to choose concentrations for further evaluation of the anticancer and cytotoxic potential of the plant materials. After seeding, the cells were allowed to recover from trypsinisation for 24 h before the various cell lines were treated daily with plant material solutions according to their wet biomass, to mimic *in vivo* dosing more closely. After a period of 96 h, intracellular ATP, indicative of cell viability, and extracellular AK, marker of cell death, levels were determined.

2.2. Plant materials

The raw powdered plant material of *S. frutescens* (#SFFW) and *X. undulatum* (#XU174) were purchased from Afrinatural holdings (Prestige Laboratory Supplies CC, KwaZulu-Natal, South Africa). The *A. vera* gel material was supplied by Improve, USA, Inc (Texas, USA), and

polysaccharides were precipitated from this gel material as previously described (Beneke et al., 2013).

2.3. Preparation of the crude aqueous extracts

Crude aqueous extracts of *S. frutescens* and *X. undulatum* were prepared as previously described (Calitz et al., 2018). Briefly, an aqueous suspension of the raw plant material was prepared in a 1:10 w/v ratio. The plant suspensions were sonicated using a Eumax® ultrasonic bath (Labotec, Midrand, South Africa) at 45ºC for 45 min, followed by centrifugation (Sigma 3-16 KL Laborzentrifugen, Germany) at 5000 x *g* for 10 min. The supernatant was transferred to a new tube and the pellet re-suspended in 10 ml water, followed by the same sonication and centrifugation conditions as above. The respective pooled supernatants collected, from both the *S. frutescens* and *X. undulatum* suspensions, were filtered through 125 mm Whatman filtration paper (Whatman™, China). The filtrates were frozen at -80°C and lyophilised with a Virtis freeze dryer (SP Scientific, Gardiner, New York). The lyophilised extract powders were pulverised with a mortar and pestle, and stored in a desiccator until further use. The *A. vera* polysaccharides were precipitated from an aqueous *A. vera* gel solution as described by Beneke et al. (2013), using 80% ethanol *v/v* in a 1:4 ratio. The precipitated polysaccharides were allowed to segregate under gravity to the bottom of the beaker and separated by centrifugation, after which it was frozen and lyophilised.

2.4. Chemical characterisation

2.4.1. *A. vera* gel material and precipitated polysaccharides

Both the Daltonmax 700® *A. vera* gel material and precipitated polysaccharide materials used in this study were characterised by means of quantitative nuclear magnetic resonance (^{1}H-NMR) analysis for marker molecules indicative of *A. vera* gel material. These marker molecules included aloverose (or acetylated polymannose), glucose, malic acid and citric acid (Jiao et al., 2010).

2.4.2. *Sutherlandia frutescens*

Samples were prepared for Ultra-high performance liquid chromatography mass spectrometry (UPLC-MS) by adding 2 ml methanol to 2 mg of *S. frutescens* dried plant material. The methanolic extract was sonicated for 10 min and then filtered (0.2 µm syringe filter). The marker molecule for *S. frutescens* was a cycloartane-like triterpene glycoside, named SU1.

UPLC analysis of *S. frutescens* was performed on a Waters Acquity UPLC system, equipped with a photodiode array (PDA) detector (Waters, Milford, MA, USA) and an Acquity UPLC BEH C_{18} column (150 mm × 2.1 mm, i.d., 1.7 µm particle size, Waters) maintained at 40°C. The mobile phase consisted of (A) 0.1% formic acid in water and (B) acetonitrile, at a flow rate of 0.35 ml/min and an injection volume of 5.0 µl (full-loop injection). A gradient elution was used at 80% (A): 20% (B) for the first 0.5 min, changing to 40% (A): 60% (B) in 13 min, adjusting to 100% (B) in

2.5 min, holding for 1 min and back to the initial ratio in 1 min. Mass spectrometry was operated in negative ion electrospray mode. The desolvation gas used was N_2, at a temperature of 350°C, a flow rate of 500 l/h and the source temperature was set to 100°C. The capillary and cone voltages were set to 2 500 and 45 V, respectively. Scans were collected throughout the run between 100 and 1 500 *m/z*.

2.4.3. *Xysmalobium undulatum*

The marker molecule used to characterize the *X. undulatum* extract was uzarin. Briefly, 2 mg of the *X. undulatum* dried plant material was dissolved in 2 ml methanol, and sonicated for 10 min. The methanolic extract was syringe filtered through a 0.2 µm filter, resulting in the sample used for the UPLC analysis performed on a Waters Acquity UPLC system, with a PDA detector as described for *S. frutescens*, with the following variations: The mobile phase consisted of 0.1% formic acid in water (solvent A) and acetonitrile (solvent B) at a flow rate of 0.3 ml/min. The gradient elution was as follows: 85% A: 15% B to 65% A: 35% B in 10 min, changed to 50% A: 50% B in 0.5 min, keeping for 1 min and back to initial ratio in 0.5 min. The running time was 12 min. The samples were injected in the mobile phase with an injection volume of 2.0 µl (full-loop injection). Mass spectrometry operated in positive ion electrospray mode, and the desolvation temperature was set to 250 °C at a flow rate of 600 l/h. The capillary and cone voltages were set to 3 000 and 40 V, respectively. Data were collected between 100 and 1 000 *m/z*.

2.5. Preparation of the test solutions

A 1.000 mg/ml stock solution was prepared for both the *A. vera* gel material and precipitated polysaccharides in the designated supplemented cell culture growth medium, and then pasteurised. The pasteurisation entailed heating of the solution to 73°C for 15 sec, and then cooling to 9°C in an ice bath. The temperature was monitored with a Digitron thermometer (Digitron, 2088T, Sifam Instruments Limited Torquay, England). After pasteurisation, the final *A. vera* gel material and precipitated polysaccharide suspensions, ranging between 0.008 and 1.000 mg/ml, were prepared by means of dilution in the various supplemented mediums.

The *S. frutescens* and *X. undulatum* stock solutions were prepared in the designated supplemented mediums, vortexed and filtered through a 0.22 µm syringe filter (Anatech Instruments (Pty) Ltd, Johannesburg, South Africa). The final test solutions were diluted in the various supplemented mediums to concentration ranges 0.050 - 8.000 mg/ml for *S. frutescens,* and 0.008 - 3.500 mg/ml for *X. undulatum.* The concentrations prepeared for each stock solution for all the plant material were determined experimentally.

2.6. Selected cell culture models

2.6.1. Culturing of the cell lines

The SCLC and non-tumorigenic cell lines used in this study were purchased from the European Collection of Authenticated Cell Cultures (ECACC) and the American Tissue Culture Collection (ATCC). These included the NCI-H69/LX4 (ECACC 96042329) cell line known to overexpress P-gp, a chemosensitive H69V (ECACC 91091803) cell line, H69AR (ATCC® CRL-11351™) cell line (hyperexpressing MRP1) and a porcine embryonic kidney LLC-PK1 (ATCC® CL-101™) cell line.

The NCI-H69/LX4 cell line was cultured in Roswell Park Memorial Institute (RPMI) 1640 growth medium (Gibco, Thermo Fisher Scientific, Johannesburg, South Africa), supplemented with 10% foetal bovine serum (FBS) (Gibco, #10270-106; Thermo Fisher Scientific), 1% non-essential amino acids (NEAA) (Lonza, 100X, Whitehead Scientific, Cape Town, South Africa), 1% of 10 000 Penicillin U/ml/10 000 Streptomycin U/ml (Pen/Strep) (Lonza, Whitehead Scientific), 2 mM L-glutamine (Lonza, Whitehead Scientific) and 0.4 µg/ml doxorubicin hydrochloride (DOX) (Sigma-Aldrich (Pty) Ltd, Gauteng, South Africa). This will be referred to as supplemented media.

The H69V cell line was also cultured in supplemented RPMI 1640 medium as prepared for the NCI-H69/LX4 line, but without the DOX. The H69AR cell line was cultured in RPMI 1640 (ATCC modification, Gibco) growth medium, supplemented with 20% FBS, 1% NEAA, 2 mM L-glutamine and 1% Pen/Strep. The LLC-PK1 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) high glucose (4.5 mg/l) growth medium (HyClone, Separations, Gauteng, South-Africa), with the following additives: 2 mM L-glutamine, 1% NEAA, 10% FBS and 1% Pen/Strep.

All cultures were maintained in an ESCO CelCulture CO_2 incubator (ESCO Micro Pte Ltd, Singapore) at 37˚C, in 5% CO_2 and 95% humidified air. Cells were sub-cultured at 80% confluence, and medium was exchanged every two days for the H69AR, NCI-H69/LX4, LLC-PK1 cell lines and every third day for the H69V cell line.

2.6.2. Seeding and treatment of cultured cells

All three adherent cell lines (H69V, H69AR and LLC-PK1) were trypsinised prior to seeding into 96-well plates. The non-adherent NCI-H69/LX4 cell line was prepared as a cell suspension. The optimal seeding density of each cell line was determined beforehand, which resulted in 80% confluence after 120 h. A recovery period (24 h) was allowed after seeding, before the treatment commenced. Thereafter, the cells were treated at 24 h time intervals (i.e. 0, 24, 48 and 72 h) with the experimental plant material solutions for a period of 96 h.

2.7. The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide assay

The metabolic activity of the cultured cells was determined with the MTT assay (Sigma-Aldrich). Briefly, the adherent cell lines were seeded in 96-well flat bottom plates (Costar®, Corning Incorporated, New York, United States of America) in a volume of 200 µl/well at the following concentrations: 7000 cells/well for the H69V cell line; 16 000 cells/well for the H69AR cell line and 7000 cells/well for the LLC-PK1 cell line. The NCI-H69/LX4 suspension cell line was seeded in 96-well V-bottom plates (Costar®, Corning Incorporated) at 200 µl/well containing 30 000 cells/well.

After the 24 h recovery period, the test solutions containing the plant materials were added. Each cell line was treated every 24 h for a duration of 96 h, with a concentration series of each plant material solution in supplemented growth media. The adherent cell lines were then rinsed twice with 100 µl/well PBS and incubated with a 0.5 mg/ml MTT in PBS solution for 4 h at 37˚C. The MTT solution was removed and 200 µl dimethyl sulfoxide (DMSO; Sigma-Aldrich) was added to each well. The plate was incubated for 1 h on a shaker at room temperature, and the absorbance was then measured at 560 nm and 630 nm with a SpectraMax® plate reader (Paradigm® Multi-Mode Detection Platform, Molecular Devices®, Separations). An untreated control group was included as an indicator of 100% cell viability. Wells containing DMSO were included as background controls, and cells treated with 200 µl of 0.2%Triton X-100 for 15 min generated a dead cell control.

The same method was followed with the non-adherent (or suspension) cell line (i.e. NCI-H69/LX4 cell line), but with an additional step. The 96-well V-bottom plates were centrifuged at 250 x *g* for 5 min at 23˚C during rinsing, before adding the test solutions or the MTT solution and the DMSO.

Following the MTT assays, the data were statistically analysed to determine the specific IC_{50} and IC_{75} concentrations of each crude aqueous extract and inner leaf material, for each cell line.

2.8. Advanced cell viability investigation

2.8.1. Treatment of the cell models

Each cell line was seeded in 96-well clear, flat bottom plates and 96-well clear bottomed black plates (Costar®, Corning Incorporated), as described above. Total soluble protein per well was determined every 24 h after seeding, which was used to determine the wet biomass in each well as an indicator of cell growth. It also enabled calculation of a treatment dose per mass of cells in each well, for the advanced cell viability study. This allowed similar dosing practices as those used with *in vivo* treatment regimens, where dosages are administered according to animal weight (mg/kg) (Fey & Wrzesinski, 2012).

For the ATP and AK studies, each cell line was treated with the respective H69V IC_{50} concentrations, and the specific IC_{75} concentrations of each selected plant material solution as determined for each cell line. All treatments were adjusted for wet biomass changes that occurred during cell growth.

LLC-PK1 cell line as a non-cancerous reference, while all the cell lines receiving no treatment acted as their own untreated controls. The H69V cell line served as a chemosensitive control for the chemoresistant cell lines.

2.8.2. Soluble protein quantification

The total soluble protein content per well was determined every 24 h in clear 96-well plates, using the Bradford protein assay as previously published (Calitz et al., 2018). Briefly, the supplemented medium was removed and 158 µl distilled water was added to each well, followed by the addition of 2 µl lysis buffer B (Wrzesinski & Fey, 2013) and 40 µl colour dye reagent (Bio-Rad Laboratories Ltd., Johannesburg, South Africa). The plate was incubated for 5 min at room temperature, and the absorbance was then measured at 595 nm. The soluble protein content was quantified relative to a bovine serum albumin standard (Bio-Rad Laboratories Ltd.).

2.8.3. Intracellular adenosine triphosphate (ATP)

The intracellular production of ATP is an indicator of cell viability, which was quantified using the Promega CellTiter-Glo assay (Whitehead Scientific), relative to an ATP standard (Sigma). After 96 h treatment in black 96-well plates, all supplemented medium was removed from the wells and replaced with 100 µl PBS. An equal volume of CellTiter-Glo reagent was added to each well and mixed thoroughly. The 96-well plate was covered and placed on a Bio-Rad compact rocker for 40 min before luminescence was measured, using a SpectraMax® plate reader. ATP was quantified relative to an ATP standard, and data were normalised relative to the untreated controls.

2.8.4. Extracellular adenylate kinase (AK)

Extracellular AK is a marker of cell death, and was detected through a non-destructive cytolysis and bioluminescent BioAssay Kit (ToxiLight™, Lonza). After the 96 h treatment of the cells, the spent medium (200 µl per well) of the 6 wells per tested concentration was removed (the spent medium of two well were pooled together, resulting in 400 µl spent medium per microcentrifuge tube), prior to the ATP assay. The spent medium samples were centrifuged at 140 *x g* for 5 min to remove any cells in suspension, followed by transfer of 350 µl supernatant to a new microcentrifuge tube. The second supernatant sample was centrifuged again for 15 min at 15 000 *x g*, and 20 µl of the subsequent supernatant was transferred into a black 96-well plate. The AK detection reagent (100 µl) was added to each well, and after a 20 min incubation period luminescence was detected with a SpectraMax® plate reader. The data was quantified relative to a known dead cell standard (H69V - 3.780×10^5 cells/ml, H69AR - 1.398×10^6 cells/ml, NCI-H69/LX4 - 3.274×10^6 cells/ml and LLC-PK1 - 1.654×10^5 cells/ml), and normalised to the untreated controls.

2.9. Statistical analysis and calculations

Following the MTT assays, the IC_{50} and IC_{75} values (with a 95% confidence interval) were determined for each crude aqueous extract in each cell model, using a Probit Analysis Method and SPSS statistical analysis software (IBM Analytics) (n = 3). The Probit Analysis calculates the confidence intervals of each dose-response, and further quantifies the respons as an IC-value. The cancer selectivity of each extract was determined by dividing the calculated IC_{50} for the non-cancerous cell line by the IC_{50} of the different SCLC cell lines. The resistance ratio was calculated by dividing the IC_{50} values of the hyperexpressive cell lines by the IC_{50} value of the chemosensitive cell line.

Statistical analyses of the advanced cell viability study data were conducted using STATISTICA 13.3 (Statsoft, Tulsa, OK, USA). A two-way analysis of variance (ANOVA) was used, followed by the Tukey's HSD and Kruskal-Wallis tests to determine statistically significant differences ($p < 0.05$) between each treatment and its untreated control, as well as between the treatment concentrations. The error bars represent the standard deviations of the mean, and all assays were conducted in six biological replicates.

3. Results and discussion

3.1. Chemical characterisation of the plant materials

The ^{1}H-NMR analysis of the Daltonmax 700® *A. vera* gel material confirmed the presence of aloverose (15.2%), glucose (9.8%), malic acid (20.7%) and citric acid (2%) (Figure 1A). The ^{1}H-NMR analysis of the *A. vera* precipitated polysaccharide fraction also indicated the presence of aloverose (47.1%), glucose (0.5%), malic acid (3.5%) and citric acid (3.5%) (Figure 1B).

The *S. frutescens* marker molecule, SU1, was quantified in the crude aqueous extract as 10.1 µg/mg dried plant material (Figure 1C), while the presence of Uzarin was confirmed in the *X. undulatum* extract (Figure 1D).

3.2. Preliminary efficacy and cytotoxicity screening

3.2.1. *Aloe vera* gel material

The reduction in cell viability, relative to the untreated controls, obtained with the MTT assay following treatment for 96 h, was expressed as IC_{50} values, which are shown in Table 1.

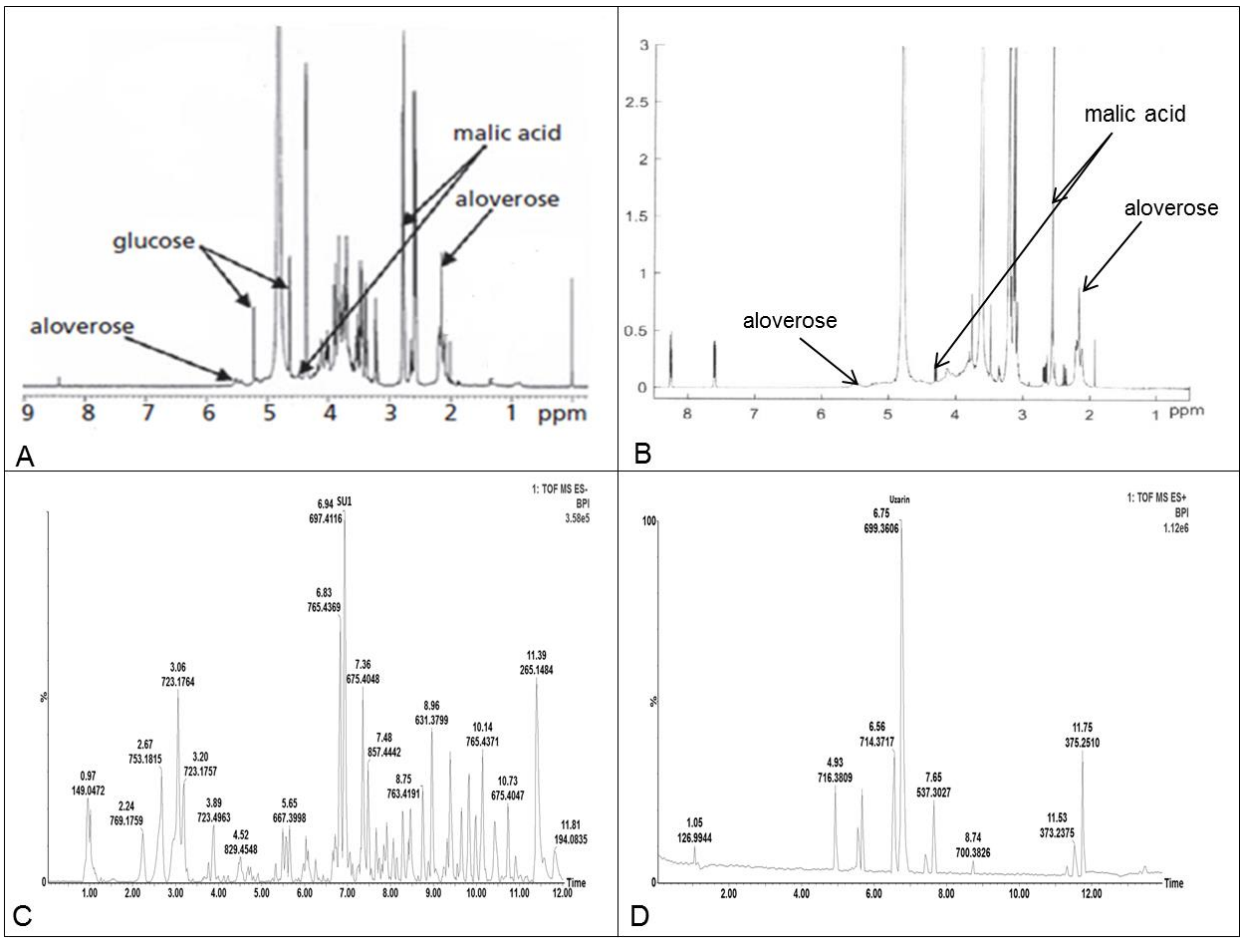


Figure 1: Chemical characterisation of the plant materials indicating the presence of marker molecules, with (A) *A. vera* gel material ^{1}H NMR spectrum, (B) *A. vera* precipitated polysaccharide ^{1}H NMR spectrum, (C) *S. frutescens* (LC-MS chromatogram) and (D) *X. undulatum* (UPLC chromatogram).

The *A. vera* gel material exhibited IC_{50} values of 0.027 mg/ml in the chemosensitive H69V cell line, 0.227 mg/ml in the MRP1 overexpressing H69AR cell line and 0.201 mg/ml in the NCI-H69/LX4 cell line (hyperexpressing P-gp). This indicated some anticancer efficacy of the *A. vera* gel material against the chemosensitive cell line, but it showed a lower effect against the chemoresistant cell lines. Furthermore, an IC_{50} value of 0.374 mg/ml in the non-cancerous LLC-PK1 cell line showed that the efficacy against the chemoresistant lines was mediocre. The IC_{50} values of *A. vera* gel material determined in B16F10 murine melanoma (skin cancer cell line) and NIH3T3 mouse embryonic fibroblast cell lines (non-cancerous cell line) were previously found to be 0.260 and 0.286 mg/ml, respectively (Çandöken et al., 2017). The anticancer efficacy of the *A. vera* gel material was, therefore, very similar in this study to the previous study, although the non-cancerous cell line (LLC-PK1) was affected slightly less.

3.2.2. *Aloe vera* precipitated polysaccharide fraction

The *A. vera* precipitated polysaccharide fraction demonstrated a IC_{50} value of 1.201 mg/ml in the non-cancerous LLC-PK1 cell line, after 96 h exposure. This is also similar to previous findings of IC_{50} values in different non-cancerous cell lines after a 72 h exposure period (1.232 mg/ml in embryo kidney cells and 1.782 mg/ml in ECV304 umbilicus vein endothelium cells) (Wang et al., 2004). The IC_{50} of the polysaccharides in the LLC-PK1 cell line was higher than that of the chemosensitive H69V cell line (0.179 mg/ml), demonstrating anticancer efficacy at potentially non-cytotoxic concentrations. This was also true for the MRP1 overexpressing, chemoresistant H69AR cell line (IC_{50} = 0.719 mg/ml), but it was not the case for the other chemoresistant cell line (NCI-H69/LX4, P-gp hyperexpressive) which had an IC_{50} of 1.346 mg/ml.

3.2.3. *Sutherlandia frutescens*

In a study conducted by Stander et al. (2009), an *S. frutescens* aqueous extract reduced the cell viability of a human breast adenocarcinoma (MCF-7) cell line to 26% and a human non-tumorigenic epithelial mammary gland (MCF-12A) cell line to 49%, after 72 h treatment with 10 mg/ml. The current study indicated a 50% cell viability reduction of the non-cancerous LLC-PK1 cell line after 96 h exposure to 8.017 mg/ml *S. frutescens* crude aqueous extract solution, which corresponds well with the MCF-12A findings. The anticancer effects of the *S. frutescens* extract on the panel of SCLC cell lines were apparent at concentrations much lower than the concentration found to be cytotoxic, with IC_{50} values of 0.358 mg/ml (H69V) 0.494 mg/ml (NCI-H69/LX4) and 1.406 mg/ml (H69AR). This indicated severe resistance of the MRP1 hyperexpressing H69AR cell line to the *S. frutescens* aqueous extract solution.

3.2.4. *Xysmalobium undulatum*

The plant extract with the highest anticancer potential in this study was *X. undulatum,* with the lowest IC_{50} value in the LLC-PK1 cell line (0.010 mg/ml). This suggested significant cytotoxicity. However, it had relatively good chemotherapeutic effects on the drug sensitive SCLC cell line (H69V), with an IC_{50} value of 0.004 mg/ml. The hyperexpressed efflux transporters in the H69AR and NCI-H69/LX4 lines, however, inferred high levels of resistance to the effects of the *X. undulatum* extract, and IC_{50} values (0.020 mg/ml for H69AR and 0.015 mg/ml for NCI-H69/LX4) would probably be cytotoxic to non-cancerous cells. These results may negate the use of this plant extract in MDR cancer.

Roy et al. (2005) evaluated the cytotoxicity of several phytochemicals isolated from *Asclepias curassavica*, belonging to the *Asclepiadaceae* botanical family. The IC_{50} values (mg/ml) for one of the isolated compounds, uzarigenin, on different cancer cell lines were found to be 0.00024 (A549), 0.0019 (MDA-MB-231), 0.00169 (MCF-7) and 0.00012 (HepG2). Uzarigenin is also a known constituent of *X. undulatum* (Vermaak et al., 2014). This data supports the conclusion that

X. undulatum could potentially have anticancer effects, in spite of the risk of cytotoxicity for normal tissues when used at higher concentrations.

Table 1: The IC_{50} values determined for the selected medicinal plant materials after 96 h treatment in each selected cell line obtained from the MTT assay

Medicinal plant	Parameters	Cell models			
		LLC-PK1	**H69V**	**H69AR**	**NCI-H69/LX4**
A. vera (gel material)	IC_{50} (mg/ml)*	0.374	0.027	0.227	0.201
	95% confidence interval (mg/ml)#	0.349 - 0.400	0.023 - 0.031	0.203 - 0.253	0.166 - 0.237
	Cancer Selectivity		13.830	1.649	1.860
	Resistance Ratio			8.387	7.435
A. vera (precipitated polysaccharides)	IC_{50} (mg/ml)*	1.201	0.179	0.719	1.346
	95% confidence interval (mg/ml)#	1.113 - 1.351	0.152 - 0.208	0.640 - 0.807	0.961 - 2.218
	Cancer Selectivity		6.702	1.670	0.893
	Resistance Ratio			4.017	6.702
S. frutescens	IC_{50} (mg/ml)*	8.017	0.538	1.406	0.494
	95% confidence interval (mg/ml)#	7.900 - 8.168	0.486 - 0.588	1.318 - 1.485	0.418 - 0.567
	Cancer Selectivity		14.901	5.704	16.235
	Resistance Ratio			2.613	0.918
X. undulatum	IC_{50} (mg/ml)*	0.010	0.004	0.020	0.015
	95% confidence interval (mg/ml)#	0.006 - 0.014	0.003 - 0.005	0.017 - 0.023	0.012 - 0.016
	Cancer Selectivity		2.322	0.483	0.660
	Resistance Ratio			4.810	3.518

**IC_{50} is the concentration which results in a cell viability reduction of 50% relative to an untreated control (n = 3).*

#n = 3

3.2.5. Selectivity and resistance ratios

The IC_{50} values were also used to determine the selectivity for cancer of the plant extracts, as well as the level of resistance to each extract (Table 1). The *S. frutescens* extract resulted in the highest cancer selectivity ratio, whereas *X. undulatum* had the lowest cancer selectivity ratio towards the panel of cells. This suggests the crude *S. frutescens* extract to be more selective for cancerous tissue compared to the *X. undulatum* extract. The *A. vera* gel demonstrated higher cancer selectivity against the H69V and NCI-H69/LX4 cell lines than the *A. vera* polysaccharides, suggesting that additional active phytochemicals are present in the gel which are not found in the precipitated polysaccharides.

If the resistance ratio is > 1, it is an indication that the plant material is less effective against the drug resistant cell lines. On the other hand, if a ratio is < 1, the extract could potentially be considered as treatment in MDR cancer (Hall et al., 2009). Overall, the *S. frutescens* extract demonstrated promising anticancer efficacy against the P-gp hyperexpressive cell line (NCI-H69/LX4), considering a resistance ratio of only 0.918, while the *A. vera* gel was least effective (8.387 for H69AR and 7.435 for NCI-H69/LX4) against chemoresistant cell lines. The *A. vera* gel was also less effective against the efflux transporter hyperexpressive cell lines than the *A. vera* precipitated polysaccharides, suggesting that some of the active phytochemicals in the gel extract could be substrates for both P-gp and MRP1 efflux transporters.

Higher IC_{50} values for an extract in the hyperexpressive cell lines, compared to a lower IC_{50} value for the chemosensitive cell line may indicate some of the active components in an extract to be substrates for the associated transporter. This was observed for all of the plant extracts to some degree, except for the *S. frutescens* extract in the P-gp hyperexpressing cell line, NCI-H69/LX4, suggesting that several active components in the extracts are substrates for P-gp and MRP1 efflux transporters.

3.3. Advanced anticancer efficacy evaluation

All cell models were treated with the IC_{50} concentrations of each plant material determined for the chemosensitive H69V cell line. The H69V cell line was also treated with the respective plant materials' IC_{75} concentrations, while the two resistant SCLC cell lines were also treated with their respective IC_{50} concentrations for each plant extract. The intracellular ATP and extracellular AK content of the various cell models were measured following treatment for 96 h with the plant materials, at the selected concentrations relative to the daily wet biomass. Intracellular ATP levels are directly linked to mitochondrial activity, and is therefore an indicator of cell viability. Conversely, the presence of extracellular AK serves as a marker of cell death (Single et al., 1998; Leist et al., 1999).

3.3.1. *Aloe vera* gel material

The outcomes following administration of the *A. vera* gel material to the various SCLC, as well as the non-cancerous LLC-PK1, cell lines are graphically depicted in Figure 2. A significant reduction in the intracellular ATP content was found, as well as a significant increase in the extracellular AK released from the non-cancerous LLC-PK1 cell line (Figure 2A) following treatment with 0.027 mg/ml gel (H69V IC_{50}) when compared to the untreated control group.

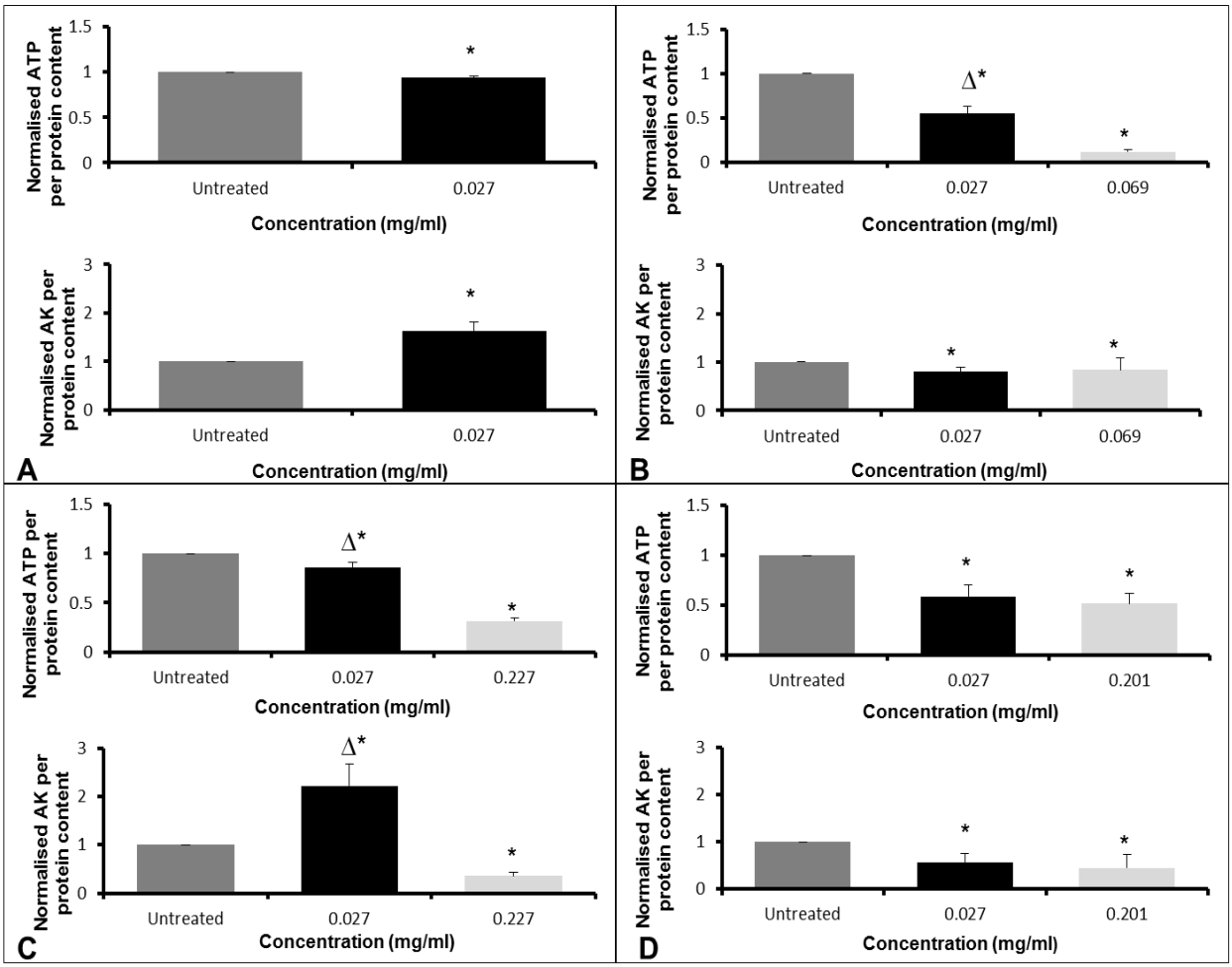


Figure 2: Normalised intracellular adenosine triphosphate (ATP) per protein content and extracellular adenylate kinase (AK) per protein content of the (A) LLC-PK1, (B) H69V, (C) H69AR and (D) NCI-H69/LX4 cell lines after treatment with *A. vera* gel material for 96 h (* indicates statistical significance between the samples and the untreated control ($p < 0.05$), and Δ indicates significant differences between the samples, $n = 6$; error bars = standard deviation (SD)).

These findings suggest a higher loss in cell viability of the non-cancerous cell line at the specified concentration than was suggested by the MTT assay. Treatment of the chemosensitive H69V line with 0.027 (H69V C_{50}) and 0.069 mg/ml (H69V C_{75}) *A. vera* gel material, is presented in Figure 2B, showing a statistically significant concentration dependent decrease in intracellular ATP levels

(0.550 and 0.116 respectively) when compared to the untreated control group (ratio of 1.000, normalised ATP per protein content). In contrast to the LLC-PK1 cell line (ratio of 1.000, normalised AK per protein content), the AK release in the H69V cells were also significantly decreased for both concentrations (0.803 and 0.835, respectively). This suggested that apoptosis already occurred prior to the time point of the assay. This is expected, since the chemosensitive H69V cell line was more sensitive to the *A. vera* gel than the LLC-PK1 non-cancerous control, as shown by the MTT data.

In Figure 2C, a significant concentration dependent loss of intracellular ATP levels in the MRP1 hyperexpressive H69AR cell line was noticed for both concentrations of *A. vera* gel, to 0.862 and 0.317 normalised ATP per protein content, respectively. Treatment with the IC_{50} value of *A. vera* gel for the chemosensitive cell line (H69V; 0.027 mg/ml) resulted in a significant ($p < 0.05$) increase in extracellular normalised AK per protein content (2.223), suggesting cell stress and apoptosis. However, the higher H69AR IC_{50} concentration of 0.227 mg/ml caused the normalised AK per protein content levels to decrease significantly (0.360) compared to the untreated control (1.000), suggesting apoptosis had occurred at an earlier time point. The results suggested a decreaced toxicity response in the resistant H69AR cell line, which could indicate that the active phytochemicals in the gel are MRP1 substrates. This is further supported by the faster cell death observed in the resistant cell line at a 10-fold higher concentration of *A. vera* gel (0.227 mg/ml), possibly due to saturation of the MRP1 efflux transporters.

Statistically significant reductions in both the intracellular ATP and extracellular AK levels were observed in the resistant NCI-H69/LX4 cell line, relative to the untreated control (Figure 2D). This, once again, suggested that apoptosis already occurred earlier and that possible necrosis started to occur. However, no marked differences were seen between the two experimental groups (represented by H69V IC_{50} of 0.027 mg/ml and NCI-H69/LX4 IC_{50} 0.201 mg/ml), suggesting a plateau of the anticancer effect of the gel. The results did not indicate any resistance to the *A. vera* gel anticancer effects in the NCl-H69/LX4 P-gp hyperexpressed cell line, suggesting that the anticancer components in *A. vera* gel are probably not P-gp efflux transporter substrates.

The advanced screening of the anticancer potential of *A. vera* gel supported the MTT findings of efficacy against the chemosensitive SCLC cell model. However, it appeared that the MTT assay may have underestimated the cytotoxic potential of the *A. vera* gel on the non-cancerous cell line. Similar to the MTT data, *A. vera* gel anticancer efficacy was found to be influenced by MRP1 hyperexpression, but not as significantly by P-gp hyperexpression as was suggested by the MTT assay.

3.3.2. *Aloe vera* precipitated polysaccharides

Figure 3 presents the normalised intracellular ATP per protein content and extracellular AK per protein content of the different cell lines after treatment with *A. vera* precipitated polysaccharides for 96 h. The H69V IC_{50} concentration of 0.179 mg/ml *A. vera* polysaccharides resulted in a statistically significant decrease in intracellular ATP levels (0.654 ratio normalised ATP per protein content) and increase in extracellular AK levels (5.516 normalised AK per protein content) in the non-cancerous LLC-PK1 cell line after 96 h treatment, as seen in Figure 3A. This is typical of apoptosis, and is more extensive than indicated by the MTT data. In Figure 3B, the *A. vera* polysaccharides also demonstrated a statistically significant concentration dependent depletion of the intracellular ATP levels (0.338 and 0.203 normalised ATP per protein content, respectively) in the chemosensitive H69V cell line, when treated with the H69V IC_{50} and IC_{75} concentrations of 0.179 mg/ml and 0.428 mg/ml. Although no marked effect could be seen at 0.179 mg/ml, the AK levels of the chemosensitive cell line was reduced significantly at 0.428 mg/ml (0.356) compared to the untreated control (1.000 normalised AK per protein content). The results indicated possible necrosis, with apoptosis probably occurring earlier than the 96 h. This supports the cancer selectivity of *A. vera* precipitated polysaccharides determined with the MTT assay.

The polysaccharides resulted in a significant loss of cell viability in the MRP1 hyperexpressive H69AR cell line (Figure 3C). This was evident from the concentration dependent decline in intracellular ATP levels. The normalised AK levels were also found to differ statistically from the untreated control (1.000 normalised AK per protein content), although it was increased following treatment with 0.179 mg/ml (1.457), but decreased with 0.791 mg/ml (0.187). The metabolic activity (ATP levels) of the H69V cell line (0.338 normalised ATP per protein content) was reduced to a greater extent than in the H69AR cell line at the H69V IC_{50} dosage of 0.179 mg/ml. This supports the conclusions from the MTT data, suggesting slight resistance of the H69AR cell model which could indicate that some of the active phytochemicals in the polysaccharide fraction may be subject to MRP1 efflux.

Similar results as observed in the H69AR cell line were obtained following treatment of the resistant NCI-H69/LX4 cell line with *A. vera* precipitated polysaccharides. It was also comparable with the results obtained after treatment of the NCI-H69/LX4 cell line with the *A. vera* gel material. The statistically significant reduction in both the ATP and AK levels were concentration dependent (Figure 3D), signifying loss in cell viability.

Overall, the *A. vera* precipitated polysaccharides seemed to have good anticancer efficacy against both the chemosensitive, and the resistant cell lines. However, the cytotoxicity of the polysaccharides in non-cancerous cells could be a cause for concern due to reduced cell viability of the LLC-PK1 cells detected at 0.179 mg/ml.

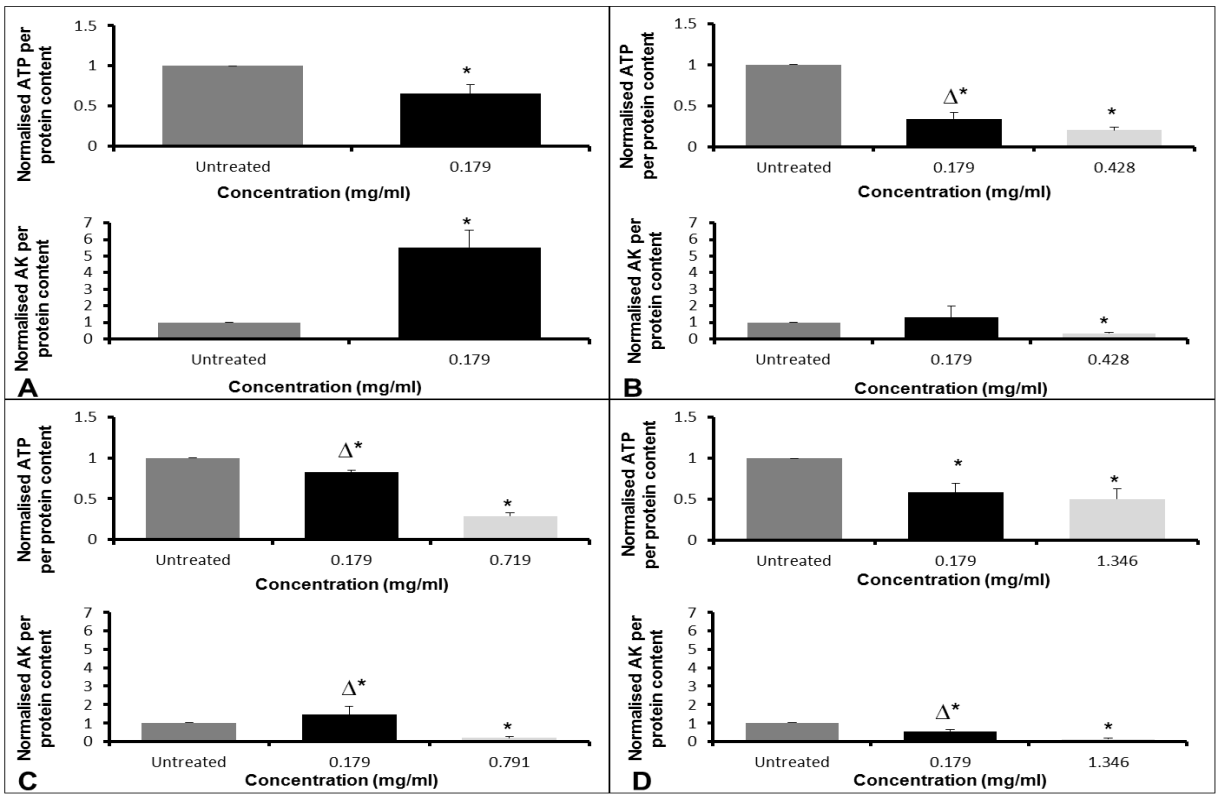


Figure 3: Normalised intracellular adenosine triphosphate, extracellular adenylate kinase and protein levels of the (A) LLC-PK1, (B) H69V, (C) H69AR and (D) NCI-H69/LX4 cell lines after administration of *A. vera* presipitated polysaccharide concentrations. (* indicates statistical significance between the samples and the untreated control ($p < 0.05$), and Δ indicates significant differences between the samples, $n = 6$; error bars = standard deviation (SD)).

3.3.3. *Sutherlandia frutescens crude aqueous extract*

Results from the treatment of the various cell lines with crude aqueous *S. frutescens* extract are depicted in Figure 4. In the non-cancerous LLC-PK1 cell line, a cytotoxic effect was indicated by the significant reduction in the ATP (0.567 normalised ATP per protein content, compared to untreated control normalised to 1.000) and increase in the AK (2.147 normalised AK per protein content, compared to untreated control normalised to 1) levels following treatment with the H69V IC_{50} concentration of 0.538 mg/ml (Figure 4A).

When administered to the chemosensitive H69V cell line, *S. frutescens* (H69V IC_{50} concentration of 0.538 and H69V IC_{75} concentration of 0.874 mg/ml) resulted in a statistically significant concentration dependent reduction of both the ATP and AK levels when compared to the untreated control (Figure 4B). However, no significant ATP and AK level differences were observed between the two tested concentrations.

In Figure 4C, significant and concentration dependant decrease in intracellular ATP levels was apparent in the experimental groups (H69V IC_{50} concentration of 0.538 mg/ml and H69AR IC_{50}

concentration of 1.406 mg/ml), compared to the untreated H69AR cell line. Data from the extracellular AK quantification showed a statistically significant increase (1.327 normalised AK per protein content, compared to untreated control normalised to 1.000) when 0.538 mg/ml *S. frutescens* was administered to the MRP1 hyperexpressive cell line. However, at 1.406 mg/ml *S. frutescens* decreased the extracellular AK significantly (0.676 normalised AK per protein content). This observation could be a result of two different modes of cell death, namely apoptosis at 0.538 mg/ml, and possible necrosis at 1.406 mg/ml *S. frutescens* in the resistant H69AR cell line. Furthermore, a lower effect was observed at 0.538 mg/ml *S. frutescens* in the MRP1 hyperexpressive cell line, than at the same concentration in the chemosensitive H69V cell line. This supports the MRP1-mediated resistance seen in the MTT data, and also suggests the active components in the *S. frutescens* crude aqueous extract to be substrates for the MRP1 efflux transporter.

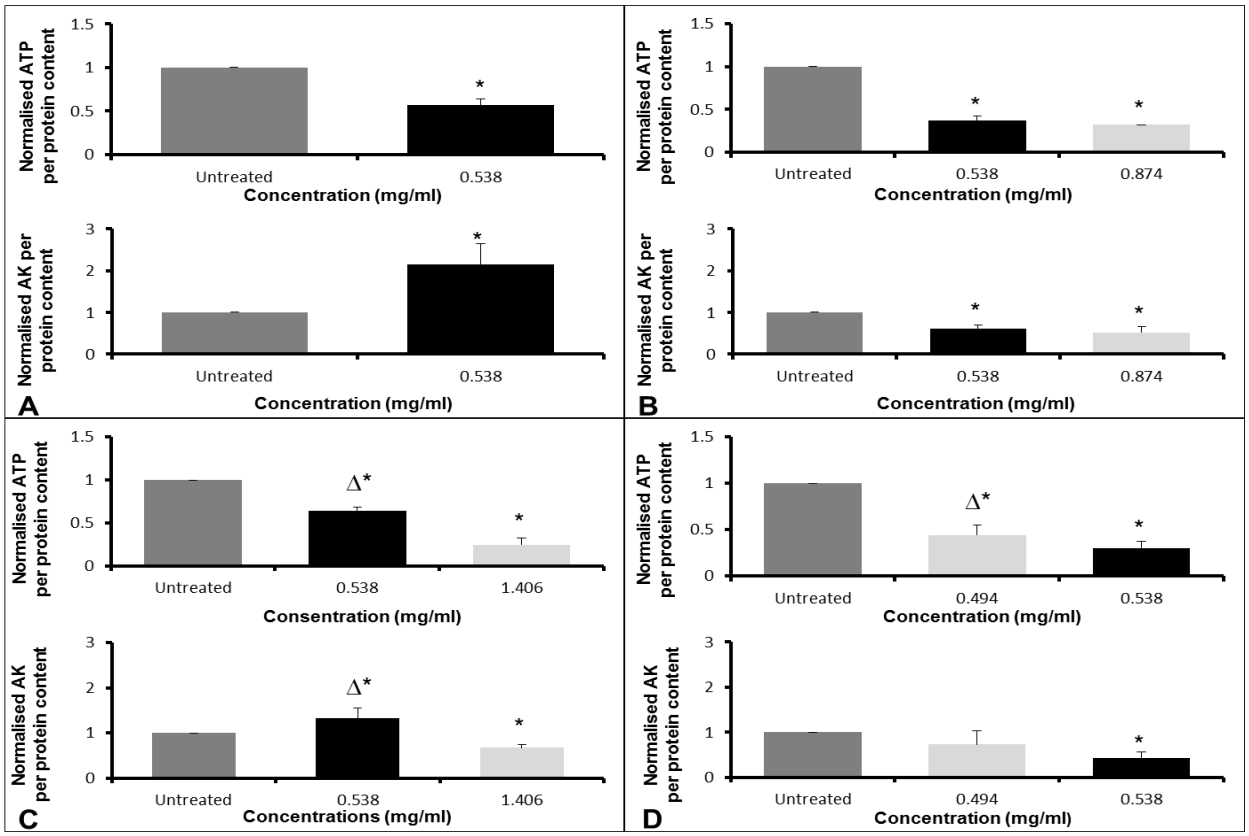


Figure 4: Normalised intracellular adenosine triphosphate, extracellular adenylate kinase and protein levels of the (A) LLC-PK1, (B) H69V, (C) H69AR and (D) NCI-H69/LX4 cell lines after administration of *S. frutescens.* (* indicates statistical significance between the samples and the untreated control ($p < 0.05$), and Δ indicates significant differences between the samples, $n = 6$; error bars = standard deviation (SD)).

Possible necrosis was also seen following treatment of the resistant NCI-H69/LX4 cell line with 0.494 mg/ml and 0.538 mg/ml *S. frutescens* (H69V IC_{50} concentration and NCI-H69/LX4 IC_{50}

concentration, respectively), as indicated in Figure 4D. A statistically significant decrease in intracellular ATP compared to the untreated control, was present in a concentration dependent manner. There was also a significant decrease in the AK levels at 0.538 mg/ml.

The data indicated that the *S. frutescens* extract reduced the general production of ATP in both the non-tumorigenic and the SCLC panel of cells, suggesting both cytotoxic effects and anticancer efficacy. However, the extent of viability reduction in the SCLC cells appeared higher than for the LLC-PK1 line, suggesting that anticancer activity may be possible at non-cytotoxic concentrations of *S. frutescens.*

3.3.4. *Xysmalobium undulatum* crude aqueous extract

In Figure 5 the results are depicted for the various SCLC cell lines, as well as the non-cancerous LLC-PK1 cell line, after treatment with *X. undulatum* aqueous extract. Data from the non-cancerous cell line (H69V IC_{50} of 0.004 mg/ml *X. undulatum*), showed significantly reduced ATP (0.786) and increased AK levels (2.252 normalised ATP per protein content) compared to the untreated control (normalised to 1.000) (Figure 5A). An overall loss in cell viability was detected with indications of apoptosis, and this is indicative of cytotoxicity. Data in Figure 5B, however, also indicate some anticancer efficacy for *X. undulatum* in the chemosensitive H69V cell model, since there was a significant and concentration dependent decrease in ATP (0.522 and 0.308, respectively) combined with an increase in AK levels (1.657 and 2.327, respectively) relative to the untreated control (1.000), at 0.004 mg/ml and 0.017 mg/ml (H69V IC_{75} concentrations). These results showed that, in spite of the cytotoxic effects of the *X. undulatum* crude extract, some active components did alter the cell viability of the SCLC cell lines.

Similarly, the crude *X. undulatum* extract also resulted in the statistically significant decrease of ATP and increase of AK levels when administered to the MRP1 hyperexpressive H69AR cell line, as shown in Figure 5C. The loss of cell viability in the resistant cell line, compared to the untreated control and the chemosensitive H69V cell lines, did not support the MRP1-related resistance seen in the MTT data. The same loss in cell viability was seen when *X. undulatum* was administered to the NCI-H69/LX4 cell line at 0.004 mg/ml and 0.015 mg/ml (H69V IC_{50} and H69 NCI-H69/LX4 IC_{50} concentrations, respectively). In Figure 5D, a similar decrease in ATP levels was indicated (0.709 and 0.529 normalised ATP per protein content, compared to untreated control normalised to 1.000) following treatment of the P-gp hyperexpressive cell line, again in a concentration-dependent manner. The AK levels, however, were also decreased significantly (0.733 and 0.645 normalised AK per protein content, compared to untreated control normalised to 1.000). The reduction in ATP levels also proved to be statistically significant when the two experimental groups were compared. The 0.004 mg/ml *X. undulatum* did not decrease the ATP content of the NCI-H69/LX4 cell line to a greater extent than in the chemosensitive H69V cell line, suggesting some resistance by the P-gp hyperexpressing cell line as indicated by the MTT data as well.

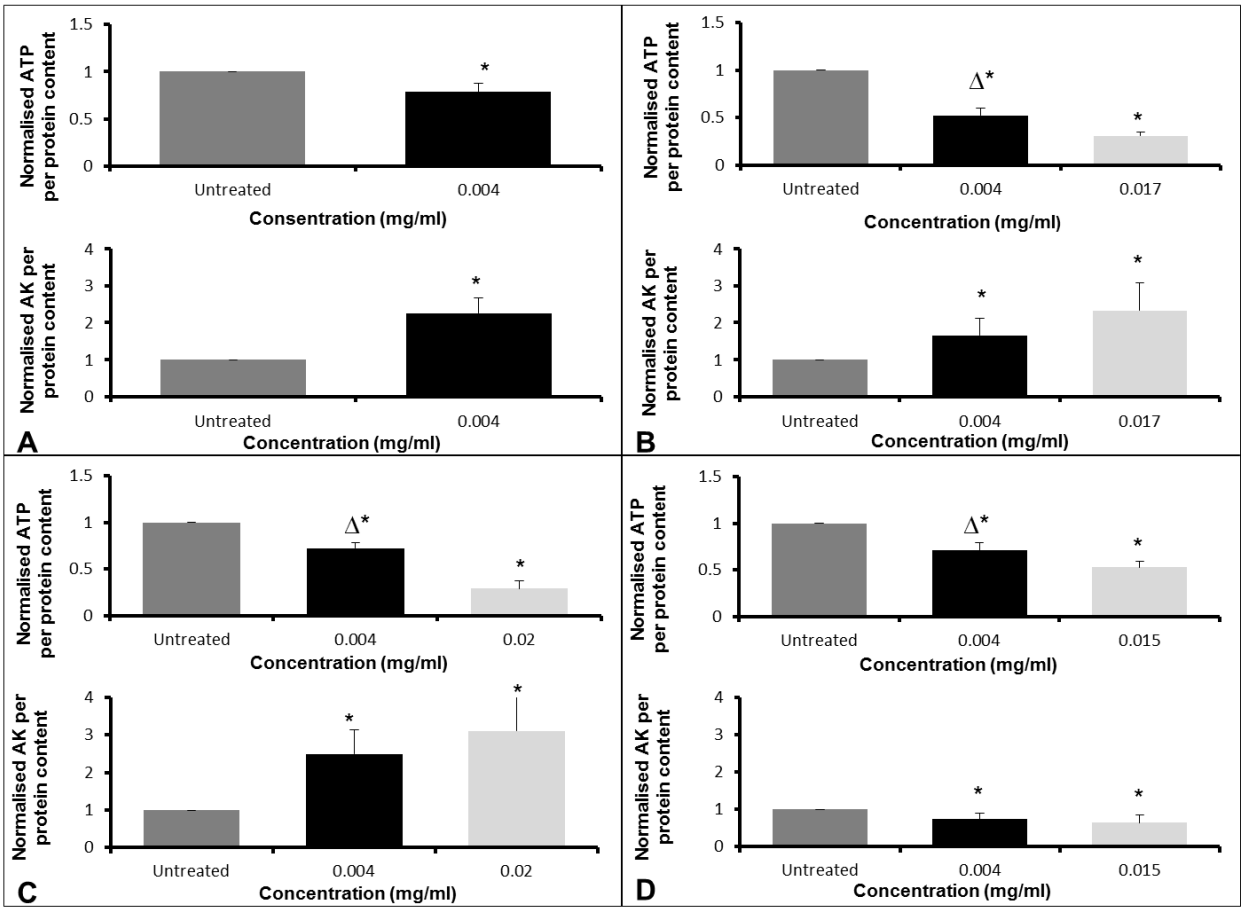


Figure 5: Normalised intracellular adenosine triphosphate, extracellular adenylate kinase and protein levels of the (A) LLC-PK1, (B) H69V, (C) H69AR and (D) NCI-H69/LX4 cell lines after administration of *X. undulatum.* (* indicates statistical significance between the samples and the untreated control ($p < 0.05$), and Δ indicates significant differences between the samples, $n = 6$; error bars = standard deviation (SD)).

The *X. undulatum* crude aqueous extract showed potential as a treatment source for SCLC, even in cancer resistance due to MRP1 hyperexpression. However, the cytotoxic properties of the extract are a concern, as well as the possible P-gp mediated resistance.

4. Conclusion

In summary, all four plant materials altered the cell viability of the selected SCLC cell lines to some extent, although they also had some indications of cytotoxicity in a non-cancerous cell line. The data obtained with the *A. vera* gel material suggested the presence of additional bioactive phytochemicals, not present in the precipitated polysaccharides, and the precipitated polysaccharides seemed to alter the intracellular ATP levels of the non-cancerous LLC-PK1 cell line to a greater extent than the *A. vera* gel. The gel also reduced viability of the chemosensitive H69V model to a larger extent at a lower concentration than the polysaccharides. Therefore, it is suggested

that the cytotoxic components in *A. vera* are concentrated in the polysaccharide fraction, and some active anticancer components are located in the gel and not in the polysaccharides. It is also suggested that some active anticancer components in *A. vera* are substrates for MRP1 mediated efflux.

The *S. frutescens* extract demonstrated the highest calculated cancer selectivity ratio of all the tested plant material on all three SCLC cell models, which supports the traditional use of this plant in cancer management. The active components in *S. frutescens* may, however, be substrates for MRP1 mediated efflux and could therefore be subjected to resistance by this mechanism. The *X. undulatum* extract reduced the cell viability of the SCLC cells, however, cytotoxicity could negate its use in cancer management. The anticancer efficacy of this crude extract was also altered by the hyperexpression of MRP1 and P-gp efflux transporters.

Although some findings of the preliminary screening with the MTT assay did not correspond exactly with the more sensitive ATP and AK cell viability evaluation, most effects were consistent between these two assay methods. In general, the MTT assay appeared to underestimate the cytotoxic potential of the plant materials. All of the plant extracts evaluated in this study had very good efficacy against the chemosensitive SCLC cell line, although the cytotoxic effects of some could be detrimental to its use as a treatment option. However, the active components may be isolated in an effort to increase efficacy, while decreasing cytotoxicity. The anticancer phytochemicals present in most of the selected plant materials investigated exhibited signs of being substrates for the MRP1 and P-gp efflux transporters. This may potentially also be exploited as efflux transporter inhibitors through competitive inhibition, to be prescribed in combination with allopathic chemotherapeutics to reduce resistance. These therapies may work in a synergistic manner and thereby increase activity against MDR cancer, but this needs to be confirmed in future studies.

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Author contributions: C. Gouws, C. Willers, C. Calitz, JH. Hamman designed the experiments; RA. Swanepoel, JH. Hamman prepared the plant extracts; RA. Swanepoel, C. Willers maintained and seeded the cells; RA. Swanepoel performed the *in vitro* assays; RA. Swanepoel, C. Willers, C. Gouws, C. Calitz processed and analysed the data; RA. Swanepoel, C. Gouws, C. Willers wrote the first draft of the paper; C. Gouws, C. Willers, C. Calitz, JH. Hamman revised and prepared the paper for publishing. AM. Viljoen assisted in characterising the *S. frutescens* and *X. undulatum* extracts.

6. Conflict of interest

The authors of this article declare no conflict of interest, financial or otherwise.

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Chapter 6

Concluding remarks and future recommendations

6.1. Introduction

Multidrug resistant (MDR) small cell lung cancer cells (SCLC) demonstrate resistance to a wide spectrum of anticancer drugs. This leads to reduced therapeutic efficacy and insufficient cancer management, contributing to the high mortality rate in lung cancer patients. The adenosine triphosphate (ATP) -binding cassette (ABC) efflux transporters frequently associated with MDR are promising treatment target to overcome resistance, since inhibition of these transporters may increase the intracellular accumulation of anticancer drugs and thereby increase treatment efficacy. South Africa has a vast ethno-botanical heritage, and can potentially form a cornerstone in the search for alternative anticancer drugs. Three medicinal plants traditionally used in the treatment of cancer, namely *Aloe vera*, *Sutherlandia frutescens* and *Xysmalobium undulatum,* were included in this study. The anticancer treatment efficacy of *A. vera* gel material, precipitated polysaccharides of the *A. vera* gel material and the aqueous extracts of *S. frutescens* and *X. undulatum* dried plant material was screened in a panel of SCLC cell lines (chemosensitive and chemoresistant) and a non-cancerous cell line. Furthermore, the potential of these plant material suspensions to overcome MDR in SCLC were investigated. This efficacy evaluation included a preliminary screening of a series of concentrations of each solution with the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay, followed by statistical evaluation to determine the IC_{50} and IC_{75} values for each plant material on each cell line. Subsequently, these concentrations were used to treat the cell models for 96 h, followed by a more sensitive anticancer efficacy evaluation.

6.2. Chemical characterisation of the plant material

Since the chemical composition of a plant can differ significantly due to factors such as time of harvest, region of growth, rainfall and time since harvest, it is important to not only confirm the identity of the plant material, but to also obtain a chemical profile of the phytoconstituents in the prepared material. The marker molecules in the *A. vera* gel material, as well as the precipitated polysaccharides, were identified by means of nuclear magnetic resonance (H-NMR) spectroscopy, shown in Figures 4.1 and 4.2. These molecules and their abundance in the *A. vera* material are also summarised in Tables 4.1 and 4.2. The SU1 marker molecule in the *S. frutescens* aqueous extract, a cycloartane-like triterpene glycoside, was quantified as 10.1 µg/mg dried plant material, and the liquid chromatography-mass spectrometry chromatogram is presented in Figure 4.3. The ultra-

performance liquid chromatography analysis of the *X. undulatum* aqueous extract confirmed the presence of the marker molecule, uzarin (Figure 4.4).

6.3. Preliminary anticancer efficacy screening

The MTT assay was employed as a preliminary screening tool to identify the concentration for each plant material extract resulting in 50% viability reduction (IC_{50}), relative to an untreated control following treatment for 96 h (Table 4.3). From these values, cancer selectivity and resistance ratio were calculated for each phytomedicine on each cell line.

The *A. vera* gel material and precipitated polysaccharide fraction both showed anticancer efficacy towards the panel of chemosensitive and chemoresistant SCLC cells. The *A. vera* gel material demonstrated a higher cancer selectivity ratio of 13.830 for the chemosensitive H69V cell line, compared to the 6.702 ratio of the *A. vera* precipitated polysaccharides. The efficacy of the precipitated polysaccharides, however, was less influenced by MRP1 hyperexpression than P-gp expression, as indicated by the 1.177 resistance ratio determined for the H69AR (MRP1 hyperexpressive) cell line compared to the 6.702 resistance ratio for the NCI-H69/LX4 (P-gp hyperexpressive) cell line (Figure 4.11. and 4.12.). Overall, the MTT data suggested the possible use of the *A. vera* gel material to treat chemosensitive SCLC, while the *A. vera* precipitated polysaccharides are suggested to be more useful as MDR SCLC treatment.

The *S. frutescens* aqueous extract induced the highest cancer selectivity ratio of 14.901 on the chemosensitive cell line, whereas the *X. undulatum* aqueous extract resulted in the lowest ratio (2.322). It was evident that the *S. frutescens* extract could potentially be useful as a SCLC treatment, especially to overcome resistance due to P-gp hyperexpression (resistance ratio of 0.918 in the NCI-H69/LX4 cell line). Although the *X. undulatum* extract demonstrated anticancer efficacy towards both resistant SCLC cell lines (Figure 4.19 and 4.20), the IC_{50} values in these lines (0.0199 For H69AR and 0.015 for NCI-H69/LX4) were similar to the IC_{50} concentration in the LLC-PK1 cell line (0.010 mg/ml) suggesting possible cytotoxicity in non-cancerous cells.

6.4. Advanced anticancer efficacy evaluation

The IC values determined following the MTT assays were further investigated for their effects on cell viability in the SCLC and LLC-PK1 cell lines. The calculated IC_{50} concentration of each extract on the chemosensitive H69V cell line was applied on all the cell lines, to clarify the overall anticancer efficacy of each medicinal plant. The effects of treatment of each resistant cell line with its own IC_{50} concentration, on the intracellular ATP and extracellular AK levels were compared to the response after treatment with the chemosensitive H69V cell line's IC_{50} concentration. This comparison indicated the impact of the hyperexpressed efflux transporters on the efficacy of the extracts. The selected cell lines were treated daily with the calculated IC concentrations relative to the wet biomass, for a 96 h period.

6.4.1. Anticancer efficacy of *Aloe vera* gel material

The *A. vera* plant has a complex phytochemical composition (e.g. anthraquinones, carbohydrates, inorganic compounds), of which a combination of bioactive constituents are responsible for the observed health benefits (Hamman, 2008). The *A. vera* gel material used in the current study included phytochemicals such as aloverose, glucose and malic acid (Table 4.1). The *A. vera* gel material induced apoptosis in the H69V cell line (Chapter 5: Figure 2B), and the metabolic activity (normalised ATP per protein content) of this chemosensitive cell line was reduced to a greater extent than in the non-cancerous LLC-PK1 cell line (Chapter 5: Figure 2A). This suggests the gel material to be relatively cancer selective at 0.027 mg/ml.

The hyperexpressed MRP1 efflux transporters in the H69AR cell line may have contributed to a reduction of the anticancer efficacy of the *A. vera* gel material, since the H69V IC_{50} concentration (0.027 mg/ml) reduced the normalised ATP per protein content in the H69V cell line to a greater extent than in the MRP1 hyperexpressing H69AR cell line (Chapter 5: Figure 2). The normalised ATP per protein content levels in the NCI-H69/LX4 were reduced more than in the H69AR cell line, suggesting the phytochemicals in the *A. vera* gel material may be possible P-gp substrates, but may have a higher affinity for the MRP1 efflux protein.

6.4.2. Anticancer efficacy of *Aloe vera* precipitated polysaccharides

The bioactivity of acemannan, a polysaccharide present in *A. vera*, includes inhibition of tumour growth through immunomodulation (Xie *et al.*, 2016). Polysaccharides have been suggested to have anticancer efficacy between 400 and 5 KDa (Im *et al.*, 2005). The precipitated polysaccharides used in this study demonstrated anticancer efficacy in the SCLC cell lines at different concentrations (Chapter 5: Figure 3). The increased normalised AK per protein content levels seen in the LLC-PK1 cell line when compared to the untreated control, suggested the presence of apoptosis in the non-cancerous cell line. The normalised ATP per protein content in the H69AR and NCI-H69/LX4 cell lines showed significant reduction compared to the untreated control (Chapter 5: Figures 3C and 3D), indicating an alteration in the metabolic activity of the MDR SCLC cell lines and possible apoptosis. The *A. vera* polysaccharide fraction, therefore, showed not only anticancer efficacy but also possible cytotoxicity. The phytochemicals in the precipitated polysaccharides of the *A. vera* gel material are suggested to be possible MRP1 substrates. This observation is supported by comparing the alteration in metabolic activity (ATP levels) of 0.179 mg/ml on both the H69V (0.338) and H69AR (0.824) cell line.

6.4.3. Anticancer efficacy of *Sutherlandia frutescens* crude aqueous extract

One of the major phytochemicals in *S. frutescens* is a cycloartane-like triterpene glycoside, named SU1, which was quantified as 10.1 µg/mg dried plant material in the extract prepared. Some of the triterpenoids in *S. frutescens* have demonstrated structurally related features similar to cycloartane-like triterpenoids, which have been found to act in a cancer chemopreventative manner (Van Wyk & Albrecht, 2008). Aqueous *S. frutescens* extracts have previously been illustrated to have anticancer efficacy by inducing autophagy and apoptosis (Stander *et al.*, 2009; Vorster *et al.*, 2012). A possible apoptotic mechanism suggested is the occurrence of a flip-flop trans-locating effect of phosphotidyl serine (Chinkwo, 2005).

The data presented in this study indicated the *S. frutescens* aqueous extract altered the normalised ATP and AK per protein content levels of the various SCLC cells compared to each untreated control (Chapter 5: Figures 4B, 4C and 4D), suggesting anticancer efficacy. The metabolic activity of the chemosensitive H69V cell line, as suggested by the intracellular ATP levels, was reduced more than in the MRP1 hyperexpressive H69AR cell line, suggesting some efflux of the active components of the *S. frutescens* extract by the MRP1 transporters. However, the H69AR cell line still underwent possible apoptosis at 0.538 mg/ml, while possible necrosis of the cells occurred at 1.406 mg/ml (Chapter 5: Figure 4C). The H69V IC_{50} concentration of 0.538 mg/ml reduced both the ATP and AK levels of the P-gp hyperexpressing NCI-H69/LX4 cell line more than for the MRP1 hyperexpressing H69AR cell line (Chapter 5: Figures 4C and 4D). This supports the conclusion that the anticancer phytochemicals in the *S. frutescens* extract are MRP1 substrates, yet, the influence of P-gp on the anticancer efficacy cannot be excluded. Possible cytotoxicity was also seen following treatment with 0.538 mg/ml *S. frutescens* extract, since normalised AK levels increased and normalised ATP levels decreased in the LLC-PK1 cell line compared to the untreated control (Chapter 5: Figure 4A).

6.4.4. Anticancer efficacy of *Xysmalobium undulatum* crude aqueous extract

Some cardenolides (e.g. uzarin, xysmalobin) in *X. undulatum* are suggested as P-gp efflux transport inhibiters, contributing to the anticancer effects of plant material containing such cardenolides (Krishna *et al.*, 2015). In another study (Calitz *et al.*, 2018), an aqueous *X. undulatum* extract demonstrated antiproliferating effects on a hepatocellular carcinoma cell line (HepG2/C3A). The data presented in the current study showed the *X. undulatum* extract to alter the normalised ATP and AK levels of both the SCLC and the LLC-PK1 cell lines, when compared to the untreated controls (Chapter 5: Figure 5). The possible non-tumorigenic cell cytotoxicity was demonstrated by a reduction in normalised ATP per protein and increased normalised AK per protein levels following treatment of the LLC-PK1 cell line with 0.004 mg/ml, suggesting apoptosis. The *X. undulatum* extract also induced apoptosis in the H69V and H69AR cell lines as indicated by decreased normalised ATP and increased normalised AK levels, relative to the untreated controls.

The *X. undulatum* extract did not appear to reduce the cell viability of the resistant NCI-H69/LX4 cell line as much, compared to the chemosensitive cell line. This is supported by comparing the normalised ATP levels (0.522) on the chemosensitive cell line with the ATP levels (0.709) of the P-gp hyperexpressed cell line after a 96 h treatment with the 0.004 mg/ml (H69V IC_{50}) concentration. Therefore the cytotoxic phytochemicals in the crude aqueous *X. undulatum* extract are suggested to be P-gp efflux transporter substrates.

6.5. Final conclusion

The different plant materials evaluated in this study have demonstrated varying degrees of anticancer efficacy against the various SCLC cell lines, although cytotoxicity was also indicated to some extent in the non-cancerous cell line. The anticancer effects observed were for the most part dose dependent, and frequently influenced by the hyperexpression of MRP1 and P-gp efflux transporters (as described in Chapter 4). The *S. frutescens* and *X. undulatum* aqueous extracts and the *A. vera* gel material and precipitated polysaccharides consists of a complex mix of phytochemicals, other than the reported marker molecules, which could contribute to the anticancer efficacy demonstrated. The anticancer phytochemicals in the crude plant material may also be MRP1 or P-gp substrates.

The results of this study validates the traditional use of the selected plant material to treat cancer. However, the study also indicated potential risk with prolonged or high-dosage use of the plant material due to potential cytotoxicity. It is, therefore, imperitive that further studies should be performed to find suitable concentrations of the various plant materials, where beneficial anticancer effects are attained, with minimal cytotoxic adverse events.

6.6. Future recommendations

The current data demonstrated the anticancer potential of the three selected medicinal plants. These findings can be further investigated through various approaches.

One approach involves combining the plant material (containing of possible substrates for MDR transporters) with allopathic chemotherapeutic drugs, to evaluate possible synergism or antagonism. These transporter substrates may potentially increase intracellular drug accumulation through competing for the specific binding areas of the efflux transporters. Furthermore, demographically different sources of plant material from different provinces in southern Africa may be included to determine the influence of harvesting and seasonal changes on the anticancer efficacy. The current study focused on SCLC and LLC-PK1 cell lines. In future, this panel of models can be expanded to include a non-small cell lung cancer cell line (NCSLC) and other non-cancerous cell lines to further evaluate the possible cytotoxicity and anticancer efficacy of the selected plants.

Another approach involves isolation of the specific bioactive phytochemicals responsible for the anticancer effects. The precipitated polysaccharides can also be separated into different molecular weight fractions by means of a Sephacryl S400 HR column. The fractions can then be used for *in vitro* anticancer screening. Changing the extraction liquid used for the *S. frutescens* and *X. undulatum* extracts from water to organic solvents may result in the extraction of different phytochemicals which are not present in an aqueous extract.

The two-dimensional (2D) *in vitro* study demonstrated anticancer potential of these plants. It would be advisable to develop three-dimensional (3D) *in vitro* models of the panel of SCLC cells, since it may provide more physiologically relevant results. A 3D cell model *in vitro* study could be applied to confirm the findings from the 2D screening, or to identify possible mechanisms of action. The inclusion of proteomics analysis, as well as caspase-3 and reactive oxygen species (ROS) detection can also contribute to the elucidation of a possible anticancer mechanism.

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Appendix A

The author guidelines of *Current Cancer Drug Targets*

Current Cancer Drug Targets

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- Bard, M.; Woods, R.A.; Bartón, D.H.; Corrie, J.E.; Widdowson, D.A. Sterol mutants of *Saccharomyces cerevisiae*: chromatographic analyses. *Lipids*, **1977**, *12*(8), 645-654.
- Zhang, W.; Brombosz, S.M.; Mendoza, J.L.; Moore, J.S. A high-yield, one-step synthesis of o-phenylene ethynylene cyclic trimer *via*precipitation-driven alkyne metathesis. *J. Org. Chem*., **2005**, *70*, 10198-10201.

Book reference

- Crabtree, R. H. *The Organometallic Chemistry of the Transition Metals*, 3rd Ed.; Wiley & Sons: New York, **2001**.

Book chapter reference

- Wheeler, D.M.S.; Wheeler, M.M. Stereoselective Syntheses of Doxorubicin and Related Compounds In: *Studies in Natural Products Chemistry*; Atta-ur-Rahman, Ed.; Elsevier Science B. V: Amsterdam, **1994**; Vol. 14, pp. 3-46.

Conference proceedings

- Jakeman, D.L.; Withers, S.G. E. In: *Carbohydrate Bioengineering: Interdisciplinary Approaches*, Proceedings of the 4th Carbohydrate Bioengineering Meeting, Stockholm, Sweden, June 10-13, 2001; Teeri, T.T.; Svensson, B.; Gilbert, H.J.; Feizi, T., Eds.; Royal Society of Chemistry: Cambridge, UK, **2002**; pp. 3-8.

URL(WebPage)

- **]** National Library of Medicine. Specialized Information Services: Toxicology and Environmental Health. sis.nlm.nih.gov/Tox/ToxMain.html (Accessed May 23, **2004**).

Patent

- **7]** Hoch, J.A.; Huang, S. Screening methods for the identification of novel antibiotics. U.S. Patent 6,043,045, March 28, **2000**.

Thesis

- Mackel, H. *Capturing the Spectra of Silicon Solar Cells*. PhD Thesis, The Australian National University: Canberra, December **2004**.

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All authors must strictly follow the guidelines below for preparing illustrations for publication in ***Current Cancer Drug Targets***. If the figures are found to be sub-standard, then the manuscripts will be rejected.

The authors are expected to submit good quality figure(s) in PDF, PPT, MS Word, TIFF or JPEG versions, which, if required, should be improved yourself or by professional graphic designers of your organization/ country. You may even consider approaching our contracted service providers Eureka Science for Graphics Enhancement Services.

The Graphics Designing team at Eureka Science can assist in improving the quality of your images at affordable rates. Eureka Science has contracted special rates with us of US $125 for the improvement of up to five figures, with any additional figures being charged at US $20 each.

The quality of Graphic Enhancement Services offered by Eureka Science can be viewed at http://www.eureka-science.com/images/Binder1.pdf, along with valuable feedback on their services at http://www.eureka-science.com/testimonials.php. You may contact Eureka Science at info@eureka-science.com

Note: Availing Graphics Enhancement Services do not guarantee acceptance of the manuscript for publication. The final acceptance/decision on the manuscript is taken by the EiC.

Guideline for figures/illustrations

Illustrations must be provided according to the following guideline:

- Illustrations should be embedded in the text file, and must be numbered consecutively in the order of their appearance. Each figure should include only a single illustration which should be cropped to minimize the amount of space occupied by the illustration.
- If a figure is in separate parts, all parts of the figure must be provided in a single composite illustration file.
- Photographs should be provided with a scale bar if appropriate, as well as high-resolution component files.

Scaling/resolution

Line Art image type is normally an image based on lines and text. It does not contain tonal or shaded areas. The preferred file format should be TIFF or EPS, with the color mode being Monochrome 1-bit or RGB, in a resolution of 900-1200 dpi.

Halftone image type is a continuous tone photograph containing no text. It should have the preferred file format TIFF, with color mode being RGB or Grayscale, in a resolution of 300 dpi.

Combination image type is an image containing halftone, text or line art elements. It should have the preferred file format TIFF, with color mode being RGB or Grayscale, in a resolution of 500-900 dpi.

Formats

Illustrations may be submitted in the following file formats

- **Illustrator**
- **EPS** (preferred format for diagrams)
- **PDF** (also especially suitable for diagrams)
- **PNG** (preferred format for photos or images)
- **Microsoft Word** (version 5 and above; figures must be a single page)
- **PowerPoint** (figures must be a single page)
- **TIFF**
- **JPEG** (conversion should be done using the original file)
- **BMP**
- **CDX** (ChemDraw)
- **TGF** (ISISDraw)

Bentham Science Publishers does not process figures submitted in GIF format.

For TIFF or EPS figures with considerably large file size restricting the file size in online submissions is advisable. Authors may therefore convert to JPEG format before submission as this results in significantly reduced file size and upload time, while retaining acceptable quality. JPEG is a 'lossy' format, however. In order to maintain acceptable image quality, it is recommended that JPEG files are saved at High or Maximum quality.

Zipit or Stuffit tools should not be used to compress files prior to submission as the resulting compression through these tools is always negligible.

Please refrain from supplying:

1. Graphics embedded in word processor (spreadsheet, presentation) document.
2. Optimized files optimized for screen use (like GIF, BMP, PICT, WPG) because of the low resolution.
3. Files with too low a resolution.
4. Graphics that are disproportionately large for the content.

Technical requirements for graphic/ figure submissions.

Requirement
Width = 8.5 inches (In-between the required size)
Height = 11 inches (In-between the required size)
Pixels/Inches = 300 (minimum dpi)
All figures should be in vector scale (except half tone, photograph.)

Image conversion tools

There are many software packages, many of them freeware or shareware, capable of converting to and from different graphics formats, including PNG.

General tools for image conversion include Graphic Converter on the Macintosh, Paint Shop Pro, for Windows, and ImageMagick, available on Macintosh, Windows and UNIX platforms.

Bitmap images (*e.g.* screenshots) should not be converted to EPS as they result in a much larger file size than the equivalent JPEG, TIFF, PNG or BMP, and poor quality. EPS should only be used for images produced by vector-drawing applications such as Adobe Illustrator or CorelDraw. Most vector-drawing applications can be saved in, or exported as, EPS format. If the images were originally prepared in an Office application, such as Word or PowerPoint, original Office files should be directly uploaded to the site, instead of being converted to JPEG or another format of low quality.

Color figures/illustrations

- The cost for each individual page of color figures/plates/illustrations is **US$ 997**.
- Color figures should be supplied in CMYK and not RGB colors.

Note for authors To maintain publication quality, figures submitted in colour will be published in colour only.

Chemical structures

Chemical structures must be prepared in ChemDraw/CDX and provided as separate file.

Structure drawing preferences
[As according to the ACS style sheet]

Drawing Settings	
Chain angle	120°
Bond spacing	18% of width
Fixed length	14.4 pt (0.500cm, 0.2in)
Bold width	2.0 pt (0.071cm, 0.0278in)
Line width	0.6 pt (0.021cm, 0.0084in)
Margin width	1.6 pt (0.096cm)
Hash spacing	2.5 pt (0.088cm, 0.0347in)
Text Settings	
Font	Times New Roman / Helvetica
Size	10 pt
Under the Preference Choose	
Units	points
Tolerances	3 pixels
Under Page Setup Use	
Paper	US letter
Scale	100%

Tables

- Data Tables should be submitted in Microsoft Word table format.

- Each table should include a title/caption being explanatory in itself with respect to the details discussed in the table. Detailed legends may then follow.
- Table number in bold font *i.e*. Table **1**, should follow a title. The title should be in small case with the first letter in caps. A full stop should be placed at the end of the title.
- Tables should be embedded in the text exactly according to their appropriate placement in the submitted manuscript.
- Columns and rows of data should be made visibly distinct by ensuring that the borders of each cell are displayed as black lines.
- Tables should be numbered in Arabic numerals sequentially in order of their citation in the body of the text.
- If a reference is cited in both the table and text, please insert a lettered footnote in the table to refer to the numbered reference in the text.
- Tabular data provided as additional files can be submitted as an MS Excel spreadsheet.

Authors and institutional affiliations

The names of the authors should be provided according to the previous citations or as the authors would want them to be published along with the institutional affiliations, current address, telephone, cell & fax numbers and the email address. Email address must be provided with an asterisk in front of the name of the principal author. The corresponding author(s) should be designated and their complete address, business telephone and fax numbers and e-mail address must be stated to receive correspondence and galley proofs. Also it is suggested to regularly update the profile on SCOPUS and other databases.

Changes to authorship

Authors must provide a final list of authors at the time of submission, ensuring the correct sequence of the names of authors, which will not be considered for any addition, deletion or rearrangement after final submission of the manuscript. If a change is essentially required, it can only be done on Editor's approval, for which the Editor must receive the following from the corresponding author

(a) the reason for change in the author list and the sequence

(b) written confirmation from all co-authors that they agree with the addition, removal or rearrangement. In the case of addition or removal of authors, confirmation from the author being added or removed is mandatory.

Only in exceptional circumstances will the Editor consider the addition, deletion or rearrangement of authors after the manuscript has been accepted. While the Editor considers the request, publication of the manuscript will be suspended. If the manuscript has already been published in an online issue, any requests approved by the Editor will result in a corrigendum.

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Generally, the editorial decisions are not reverted. However, authors who think that their manuscript was rejected due to a misunderstanding or mistake may seek an explanation for the decision. Appeals must give sound reasoning and compelling evidence against the criticism raised in the rejection letter. A difference of opinion as to the interest, novelty, or suitability of the manuscript for the journal will not be considered as an appeal. The EIC and other relevant editors will consider the appeal and the decision thereafter taken by the journal will be deemed final. Acceptance of the manuscript is not guaranteed even if the journal agrees to reconsider the manuscript, and the reconsideration process may involve previous or new reviewers or editors and substantive revision.

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Manuscripts containing language inconsistencies will not be published. Authors should seek professional assistance for correction of grammatical, scientific and typographical errors before submission of the revised version of the article for publication. Professional editing services may also be sought by the team available at Bentham Science.

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The corresponding author will be solely responsible for ensuring that the revised version of the manuscript incorporating all the submitted corrections receives the approval of all the co-authors of the manuscript.

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Reviewing and promptness of publication

All papers submitted for publication are immediately subjected to preliminary editorial scrutiny by the Editor-in-Chief regarding their suitability. The Editor-in-Chief determines if the manuscript

(a) falls within the scope of the journal and

(b) meets the editorial criteria of *Bentham Science Publishers* in terms of originality and quality.

Manuscripts that appear to be suitable are then subjected to single/ double blind peer-review by, usually two, neutral eminent experts. The services of eminent international experts are sought through invitations to conduct the peer-review of a submitted manuscript, keeping in view the scope of the manuscript and the expertise of the reviewers. The identity of the reviewers is not disclosed to the authors. The anonymity of reviewers ensures objective and unbiased assessment of the manuscript by the reviewers.

Before sending the manuscripts to reviewers, *Bentham Science* seeks consent from potential reviewers about their availability and willingness to review. Correspondence between the editorial office of the journal and the reviewers is kept confidential. The reviewers are expected to provide their reports in a timely fashion since a prompt review leads to timely publication of a manuscript which is beneficial not only to the authors but to the scientific community as well.

The editorial process and peer-review workflow for each journal are taken care of by a team of Senior Editors, Editorial Advisory Board Members (EABMs) and dedicated Journal managers who have the requisite expertise in their specific fields.

The Editor-in-Chief may recommend the acceptance or rejection of a manuscript after considering the opinions of the independent referees, or he/she may take assistance and advice from other experts in the field, if needed.

The Editor-in-Chief and Senior Editors of a journal have the right to select reviewers for a particular manuscript considering the knowledge and experience of the reviewers.

After reviewing of the manuscript by at least two independent experts, in addition to the assessment of the Editor, the decision is relayed to the authors, which may be categorized as:

- Accept without changes
- Revisions Required
- Reject

Bentham Science requests not to have the manuscripts peer-reviewed by those experts who may have competing interest with the author(s) of a submitted manuscript. It is not possible for Editors to be aware of all competing interests; it is therefore expected that the reviewers would inform the Editor-in-Chief/Handling Editor if they notice any potential competing interest during the course of review of a manuscript. Moreover, the reviewers are expected to inform the Editors or editorial office of the journal if they have a conflict of interest in carrying out the review of a manuscript submitted by any author/contributor of the manuscript.

Papers which are delayed by the authors in revision for more than 30 days are required to be re-submitted as a new submission. Papers accepted for publication are typeset and proofs are dispatched to authors for any corrections prior to final publication.

All peer-reviewing will be conducted *via* the Internet to facilitate rapid reviewing of the submitted manuscripts. Every possible effort will be made to assess the manuscripts quickly with the decision being conveyed to the authors in due course.

Plagiarism prevention

Bentham Science Publishers uses the iThenticate software to detect instances of overlapping and similar text in submitted manuscripts. iThenticate software checks content against a database of periodicals, the Internet, and a comprehensive article database. It generates a similarity report, highlighting the percentage overlap between the uploaded article and the published material. Any instance of content overlap is further scrutinized for suspected plagiarism according to the publisher's Editorial Policies. Bentham Science allows an overall similarity of 20% for a manuscript to be considered for publication. The similarity percentage is further checked keeping the following important points in view

Low text similarity

The text of every submitted manuscript is checked using the Content Tracking mode in iThenticate. The Content Tracking mode ensures that manuscripts with an overall low percentage similarity (but which may have a higher similarity from a single source) are not overlooked. The acceptable limit for similarity of text from a single source is 5%. If the similarity level is above 5%, the manuscript is returned to the author for paraphrasing the text and citing the original source of the copied material.

It is important to mention that the text taken from different sources with an overall low similarity percentage will be considered as a plagiarized content if the majority of the article is a combination of copied material.

High text similarity

There may be some manuscripts with an overall low similarity percentage, but a higher percentage from a single source. A manuscript may have less than 20% overall similarity but there may be 15 % similar text taken from a single article. The similarity index in such cases is higher than the approved limit for a single source. Authors are advised to thoroughly rephrase the similar text and properly cite the original source to avoid plagiarism and copyright violation.

Types of plagiarism

We all know that scholarly manuscripts are written after thorough review of previously published articles. It is therefore not easy to draw a clear boundary between legitimate representation and plagiarism. However, the following important features can assist in identifying different kinds of plagiarized content. These are:

- Reproduction of others words, sentences, ideas or findings as one's own without proper acknowledgement.
- Text recycling, also known as self-plagiarism. It is an author's use of a previous publication in another paper without proper citation and acknowledgement of the original source.
- Paraphrasing poorly: Copying complete paragraphs and modifying a few words without changing the structure of original sentences or changing the sentence structure but not the words.
- Verbatim copying of text without putting quotation marks and not acknowledging the work of the original author.
- Properly citing a work but poorly paraphrasing the original text is considered as unintentional plagiarism. Similarly, manuscripts with language somewhere between paraphrasing and quoting are not acceptable. Authors should either paraphrase properly or quote and in both cases, cite the original source.
- Higher similarity in the abstract, introduction, materials and methods, and discussion and conclusion sections indicates that the manuscript may contain plagiarized text. Authors can easily explain these parts of the manuscript in many ways. However, technical terms and sometimes standard procedures cannot be rephrased; therefore Editors must review these sections carefully before making a decision.

Plagiarism in published manuscripts

Published manuscripts which are found to contain plagiarized text are retracted from the journal website after careful investigation and approval by the Editor-in-Chief of the journal. A 'Retraction Note' as well as a link to the original article is published on the electronic version of the plagiarized manuscript and an addendum with retraction notification in the journal concerned.

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Appendix B

The author guidelines of *Journal of Ethnopharmacology*

Journal of Ethnopharmacology

Author information pack

Description

The Journal of Ethnopharmacology is dedicated to the exchange of information and understandings about people's use of plants, fungi, animals, microorganisms and minerals and their biological and pharmacological effects based on the principles established through international conventions. Early people confronted with illness and disease, discovered a wealth of useful therapeutic agents in the plant and animal kingdoms. The empirical knowledge of these medicinal substances and their toxic potential was passed on by oral tradition and sometimes recorded in herbals and other texts on materia medica. Many valuable drugs of today (e.g., atropine, ephedrine, tubocurarine, digoxin, reserpine) came into use through the study of indigenous remedies. Chemists continue to use plant-derived drugs (e.g., morphine, taxol, physostigmine, quinidine, emetine) as prototypes in their attempts to develop more effective and less toxic medicinals.
In recent years the preservation of local knowledge, the promotion of indigenous medical systems in primary health care, and the conservation of biodiversity have become even more of a concern to all scientists working at the interface of social and natural sciences but especially to ethnopharmacologists. Recognizing the sovereign rights of States over their natural resources, ethnopharmacologists are particularly concerned with local people's rights to further use and develop their autochthonous resources.

Accordingly, today's ethnopharmacological research embraces the multidisciplinary effort in the:

- Documentation of indigenous medical knowledge,
- Scientific study of indigenous medicines in order to contribute in the long-run to improved health care in the regions of study, as well as
- Search for pharmacologically unique principles from existing indigenous remedies.

The Journal of Ethnopharmacology publishes original articles concerned with the observation and experimental investigation of the biological activities of plant and animal substances used in the traditional medicine of past and present cultures. The journal will particularly welcome interdisciplinary papers with an ethnopharmacological, an ethnobotanical or an ethnochemical approach to the study of indigenous drugs. Reports of anthropological and ethnobotanical field studies fall within the journal's scope. Studies involving pharmacological and toxicological mechanisms of action are especially welcome. Clinical studies on efficacy will be considered if contributing to the understanding of specific ethnopharmacological problems. The journal welcomes review articles in the above mentioned fields especially those highlighting the multi-disciplinary nature of ethnopharmacology.
Commentaries are by invitation only.

Audience

Ethnopharmacologists, Medicinal Chemists, Pharmacologists, Toxicologists, Anthropologists, Pharmacognosists, Ethnobotanists, Economic Botanists, Ethnobiologists

Impact factor

2017: 3.115 © Clarivate Analytics Journal Citation Reports 2018

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Introduction

The Journal of Ethnopharmacology is dedicated to the exchange of information and understandings about people's use of plants, fungi, animals, microorganisms and minerals and their biological and pharmacological effects based on the principles established through international conventions. Early people, confronted with illness and disease, discovered a

wealth of useful therapeutic agents in the plant and animal kingdoms. The empirical knowledge of these medicinal substances and their toxic potential was passed on by oral tradition and sometimes recorded in herbals and other texts on materia medica. Many valuable drugs of today (e.g., atropine, ephedrine, tubocurarine, digoxin, reserpine) came into use through the study of indigenous remedies. Chemists continue to use plant-derived drugs (e.g., morphine, taxol, physostigmine, quinidine, emetine) as prototypes in their attempts to develop more effective and less toxic medicinals.

Please note that figures and tables should be embedded in the text as close as possible to where they are initially cited. It is also mandatory to upload separate graphic and table files as these will be required if your manuscript is accepted for publication.

Classification of your paper

Please note that upon submitting your article you will have to select at least one classification and at least three of the given keywords. You can preview the list of classifications and keywords (here). This information is needed by the Editors to more quickly process your article. In addition to this, you can submit free keywords as described below under "Keywords".

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The Editors and Editorial Board have developed the "Rules of 5" for publishing in JEP. We have produced five clear criteria that each author needs to think about before submitting a manuscript and setting the whole process of editing and reviewing at work. Click here.
For more details on how to write a world class paper, please visit our Pharmacology Author Resources page.

Authors are encouraged to submit video material or animation sequences to support and enhance your scientific research. For more information please see the paragraph on video data below.

Types of paper

The Journal of Ethnopharmacology will accept the following contributions:

1. Original research articles - whose length is not limited and should include Title, Abstract, Methods and Materials, Results, Discussion, Conclusions, Acknowledgements and References. As a guideline, a full length paper normally occupies no more than 10 printed pages of the journal, including tables and illustrations.
2. Short Communications - whose average length is not more than 4 pages in print (approx. 2000-2300 words, including abstract and references). A maximum of 2 illustrations (figures or tables) is allowed. See paragraph below for description and format.
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5. Book reviews - Books for review should be sent to the Reviews Editor.
6. Commentaries - invited, peer-reviewed, critical discussion about crucial aspects of the field but most importantly methodological and conceptual-theoretical developments in the field and should also provide a standard, for example, for pharmacological methods to be used in papers in the Journal of Ethnopharmacology. The scientific dialogue differs greatly in the social / cultural and natural sciences, the discussions about the common foundations of the field are ongoing and the papers published should contribute to a transdisciplinary and multidisciplinary discussion. The length should be a maximum of 2-3 printed pages or 2500 words. Please contact the Reviews Editor j.ethnopharmacol@pharmacy.ac.uk with an outline.
7. Conference announcements and news.

Submission checklist

Please click here to download the Submission Checklist. This is a mandatory file during submission. Upload the completed checklist and choose the file type as "Checklist".

You can use this list to carry out a final check of your submission before you send it to the journal for review. Please check the relevant section in this Guide for Authors for more details.

Ensure that the following items are present:

One author has been designated as the corresponding author with contact details:

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All necessary files have been uploaded:

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- All tables (including titles, description, footnotes)
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Supplemental files (where applicable)

Further considerations

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Please see our information pages on Ethics in publishing and Ethical guidelines for journal publication.

Policy and ethics

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Appendix C

Certificate of analysis of commercial plant material

Certificate of analysis for *Sutherlandia frutescens* (SFFW790)

AfriNatural

African Botanicals, Cold Pressed Oils & Butters

CERTIFICATE OF ANALYSIS

Sutherlandia frutescens - Milled

Herbapulv sic (400 micron)

Batch number: SFFW790
Manufacturing date: Mar 2017
Re-test date: Mar 2020

Characteristic	Specification	Result
Appearance	Green	Yes
Odour& Taste	Typical	Yes
Moisture	< 12%	Yes
Sieve test	95% < 400 micron	Yes
Foreign matter	< 2%	Yes
TLC	Compare to reference	
Heavy Metals		
Lead	< 3 mg / Kg	Not tested
Cadmium	< 1 mg / Kg	Not tested
Mercury	< 0.1 mg / Kg	Not tested
Arsenic	< 3 mg / Kg	Not tested
Microbiological		
TMA	< 1 000 000 CFU's / g	Not tested
Yeast &Moulds	< 10 000 CFU's / g	Not tested
E. coli	Absent	
Staph. Aureus	Absent	
Salmonella	Absent	

Normally not irradiated.

WA Joubert

Executives: Adolf Joubert (CEO) & Annemarie Rautenbach
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Certificate of analysis for *Xysamalobium undulatum* (XU174)

Afrinatural Holdings

Brings you the rich knowledge and wisdom of Africa

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e-mail info@afrinatural.com

CERTIFICATE OF ANALYSIS

Xysmalobium undulatum (Uzara) - Milled:

Radix (400 micron)

Batch number: XU174
Manufacturing date: Oct 2014
Re-test date: Oct 2017

Characteristic	Specification	Result
Appearance	Beige to light brown	
Odour & Taste	Typical	
Moisture	< 12%	
Sieve test	95% < 400 micron	
Foreign matter	< 2%	
TLC	Compare to reference	
Heavy Metals		
Lead	< 3 mg / Kg	
Cadmium	< 1 mg / Kg	
Mercury	< 0.1 mg / Kg	
Arsenic	< 3 mg / Kg	
Microbiological		
TMA	< 1 000 000 CFU's / g	
Yeast & Moulds	< 10 000 CFU's / g	
E. coli	Absent	
Staph. Aureus	Absent	
Salmonella	Absent	

WA Joubert

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Appendix D

Supplementary data for Chapter 4

Seeding density of the selected cell lines

Table D.1: Estimated confluency of the H69V cell line over a period of 120 h when seeded at different concentrations

Cells/well ($0.32cm^2$)	Hours after seeding				
	24h	48h	72h	96h	120h
3000	25	25	30	30	40
4000	30	35	35	45	50
5000	30	40	45	50	60
6000	30	45	50	60	70
7000	35	50	55	70	80
8000	40	50	60	70	85
9000	50	60	65	80	90
10000	50	75	80	90	100

Table D.2: Estimated confluency of the H69AR cell line over a period of 120 h when seeded at different concentrations

Cells/well ($0.32cm^2$)	Hours after seeding				
	24h	48h	72h	96h	120h
16000	15	30	45	60	80
18000	25	40	50	65	85
20000	30	50	60	80	90
22500	35	55	70	90	100
25000	50	65	80	100	ND
27500	55	75	85	100	ND
30000	60	80	90	100	ND
32500	70	85	90	100	ND

Table D.3: Estimated confluency of the LLC-PK1 cell line over a period of 120 h when seeded at different concentrations

Cells/well ($0.32cm^2$)	Hours after seeding				
	24h	**48h**	**72h**	**96h**	**120h**
2000	**25**	**25**	**30**	**35**	**40**
2500	**30**	**30**	**40**	**40**	**45**
3000	**35**	**40**	**40**	**45**	**50**
3500	**40**	**45**	**45**	**50**	**60**
4000	**45**	**45**	**50**	**55**	**60**
5000	**50**	**55**	**60**	**65**	**70**
6000	**55**	**60**	**65**	**75**	**80**
7000	**60**	**65**	**70**	**80**	**90**

NCI-H69/LX4 cell line

Seeing as this cell line forms aggregates in suspension, and doesn't adhere to the tissue culture flask surface, the confluency determination used for adherent cell lines is not applicable. Therefore, this cell line is sub-cultured when the clusters start to become dense (darker colour) in the centre.

Usually, a T75 cm^2 flask of this cell line that can be cultured for 6-7 days has ± 8 x 10^4 cells/cm^2 before being sub-cultured. This calculates to approximately 25 600 cells/well for a 96-well of 0.32 cm^2. Since an exposure period of only 96 h was used in this project, it means that at least 30 000 cells/well were required for such a short culturing period.

Table D.4: The statistically determined cell viability reduction (IC_{75}), for the various plant extracts

		Cell models			
Medicinal plant	**Parameters**	LLC-PK1	H69V	H69AR	NCI-H69/LX4
A. vera (gel)	IC_{75} (mg/ml)	0.594	0.069	0.432	0.441
	IC_{75} range (mg/ml)[#]	0.550.648	0.061-0.078	0.367-0.472	0.399-0.490
A. vera (polysaccharides)	IC_{75} (mg/ml)	1.588	0.428	1.228	2.527
	IC_{75} range (mg/ml)[#]	1.340 -1.966	0.390-0.472	1.114-1.369	1.820-4.288
S. frutescens	IC_{75} (mg/ml)	8.707	0.8735	2.026	1.020
	IC_{75} range (mg/ml)[#]	8.503-8.996	0.811-0.939	1.961-2.092	1.017-1.180
X. undulatum	IC_{75} (mg/ml)	0.067	0.017	0.043	0.021
	IC_{75} range (mg/ml)[#]	0.051-0.087	0.014-0.020	0.040-0.046	0.018-0.024

[#] *The IC_{75} range indicates a 95% confidence interval (n = 3)*

Appendix E

Supplementary data for Chapter 5

The normalised protein content of each plant extract over a 96 h period

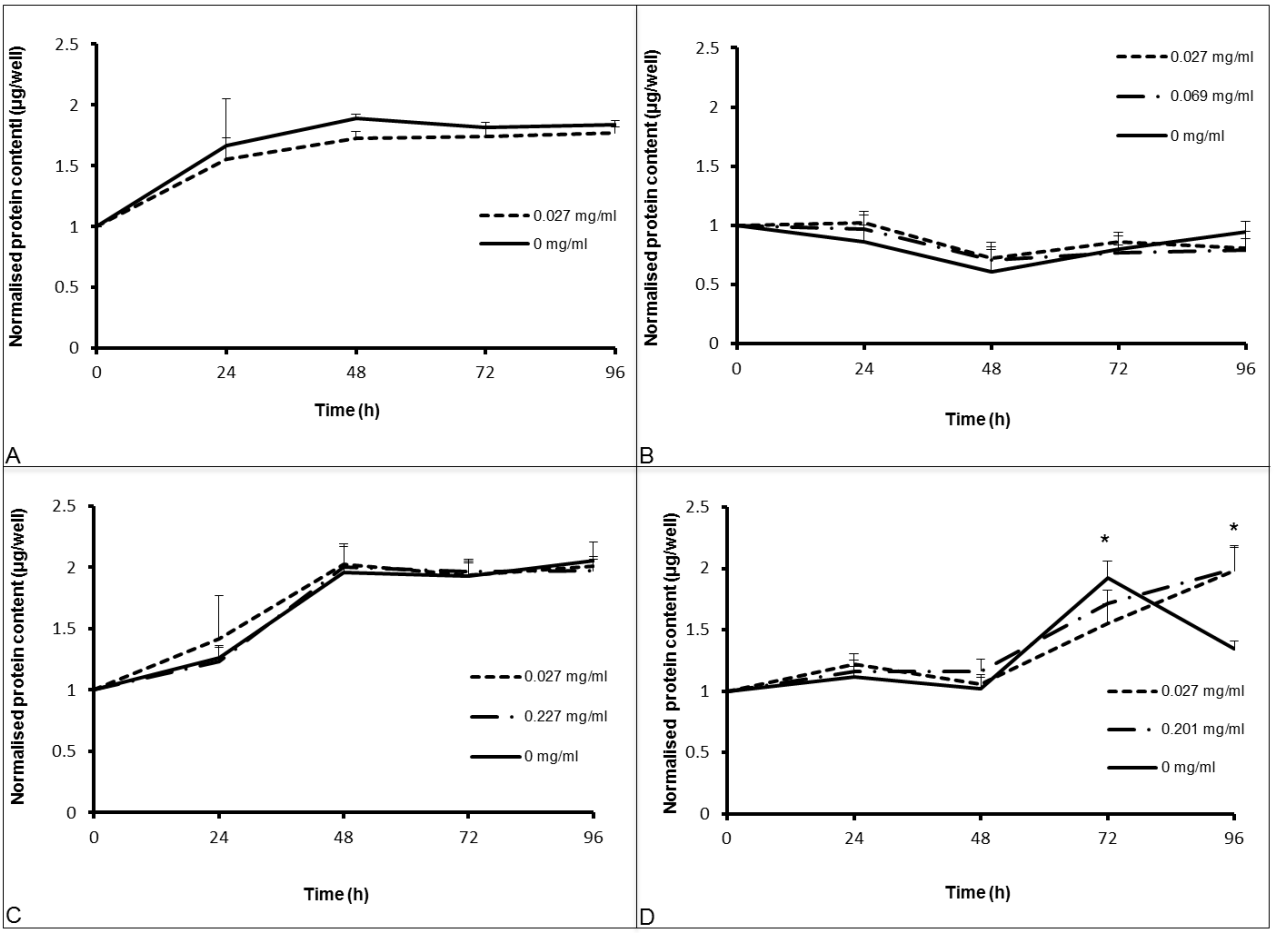


Figure E.1: Normalised protein content of the (A) LLC-PK1, (B) H69V, (C) H69AR and (D) NCI-H69/LX4 cell lines after treatment with *A. vera* gel material for 96 h. (* indicates statistical significance between the samples and the untreated control ($p < 0.05$), $n = 6$, error bars = standard deviation (SD)).

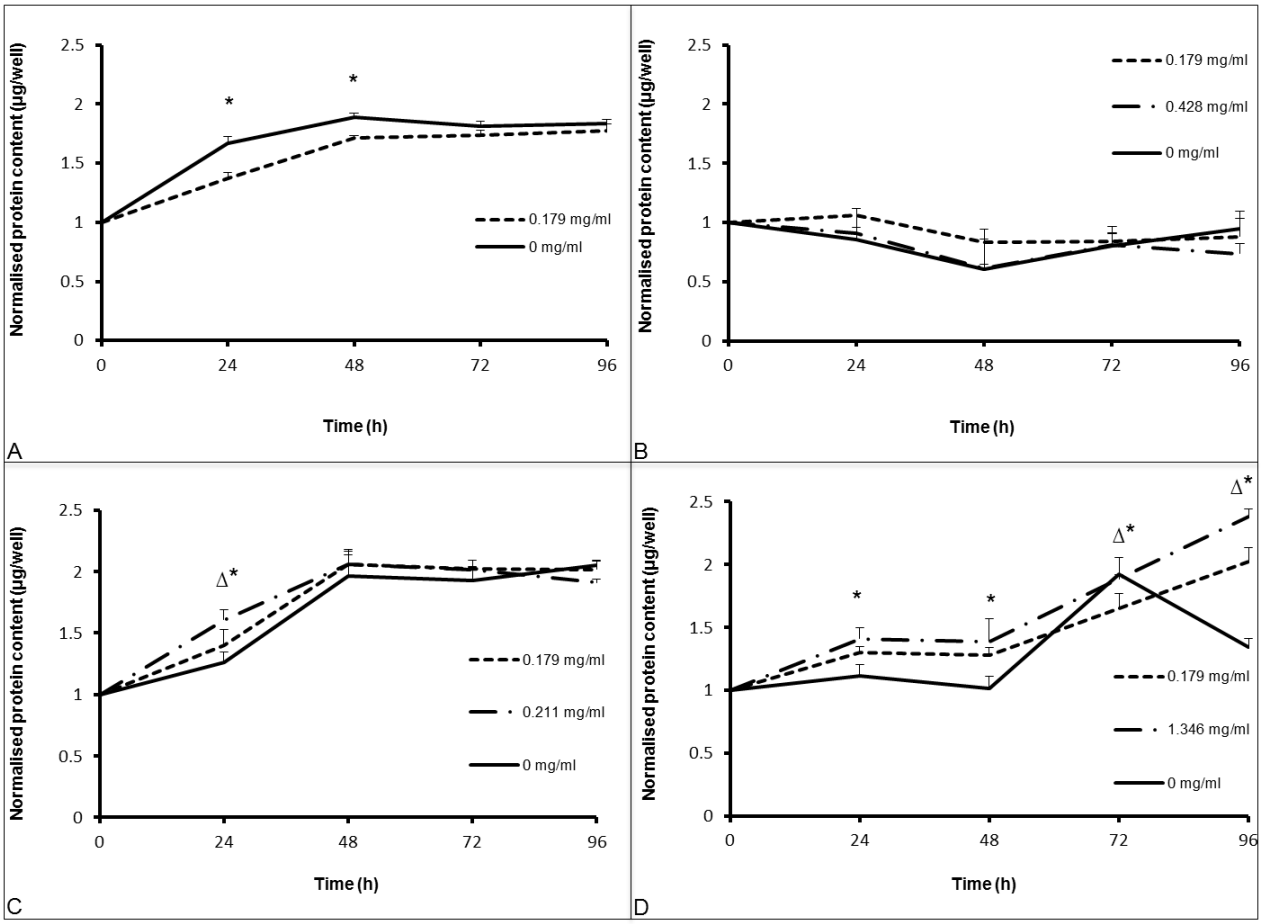


Figure E.2: Normalised protein content of the (A) LLC-PK1, (B) H69V, (C) H69AR and (D) NCI-H69/LX4 cell lines after administration of *A. vera* polysaccharide concentrations. (* indicates statistical significance between the samples and the untreated control ($p < 0.05$), and Δ indicates significant differences between the samples, $n = 6$; error bars = standard deviation (SD)).

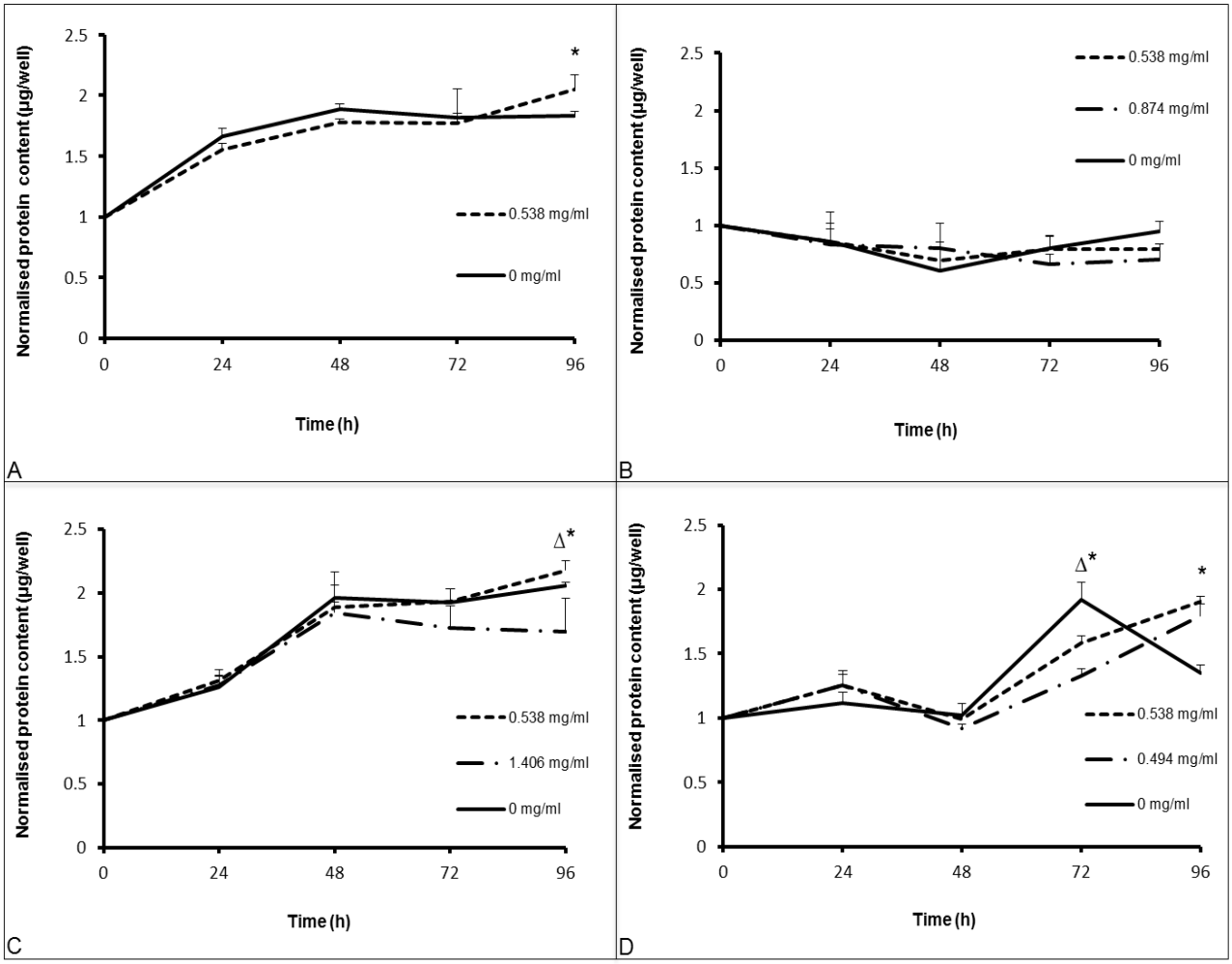


Figure E.3: Normalised protein content of the (A) LLC-PK1, (B) H69V, (C) H69AR and (D) NCI-H69/LX4 cell lines after administration of *S. frutescens.* (* indicates statistical significance between the samples and the untreated control ($p < 0.05$), and Δ indicates significant differences between the samples, $n = 6$; error bars = standard deviation (SD)).

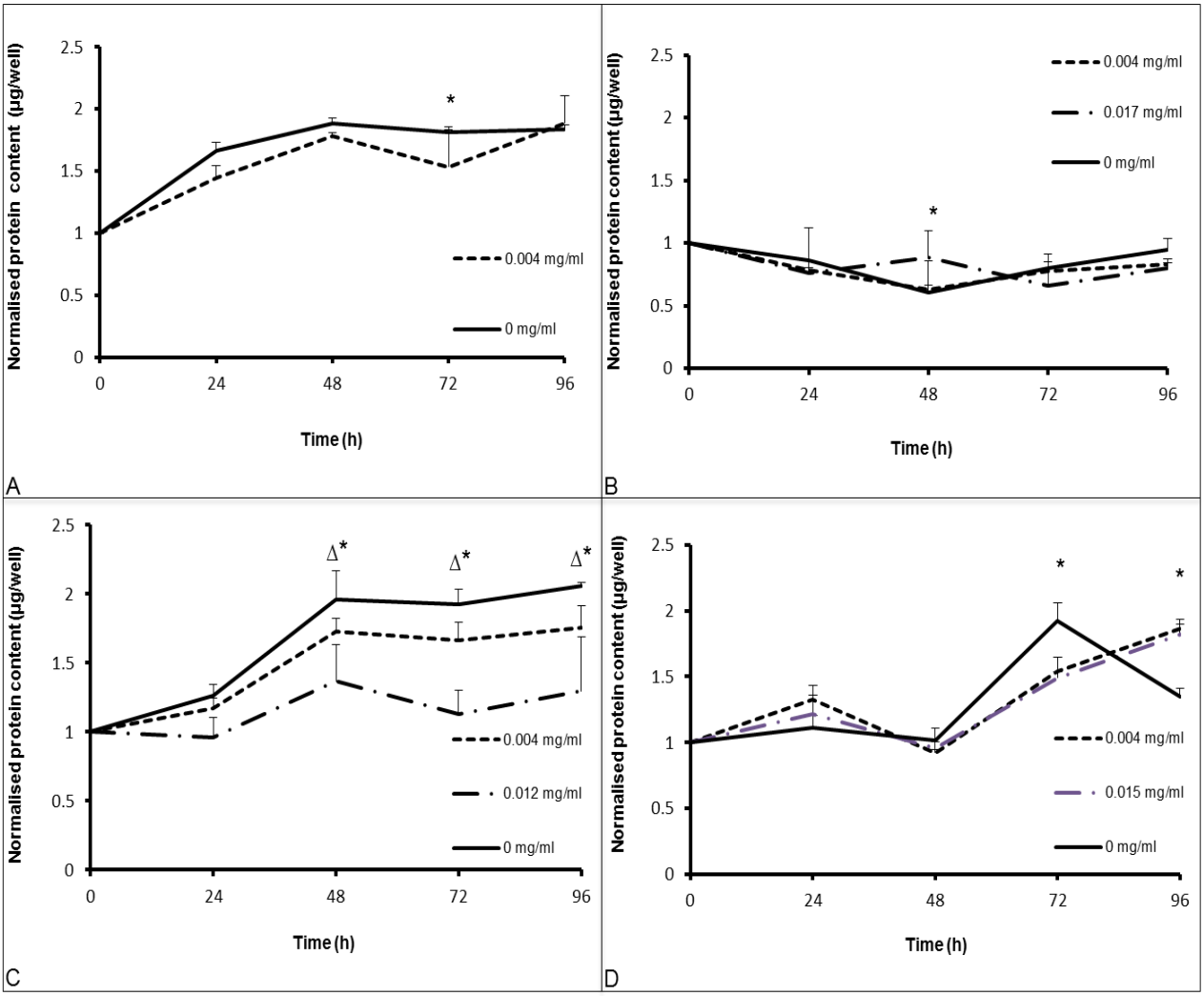
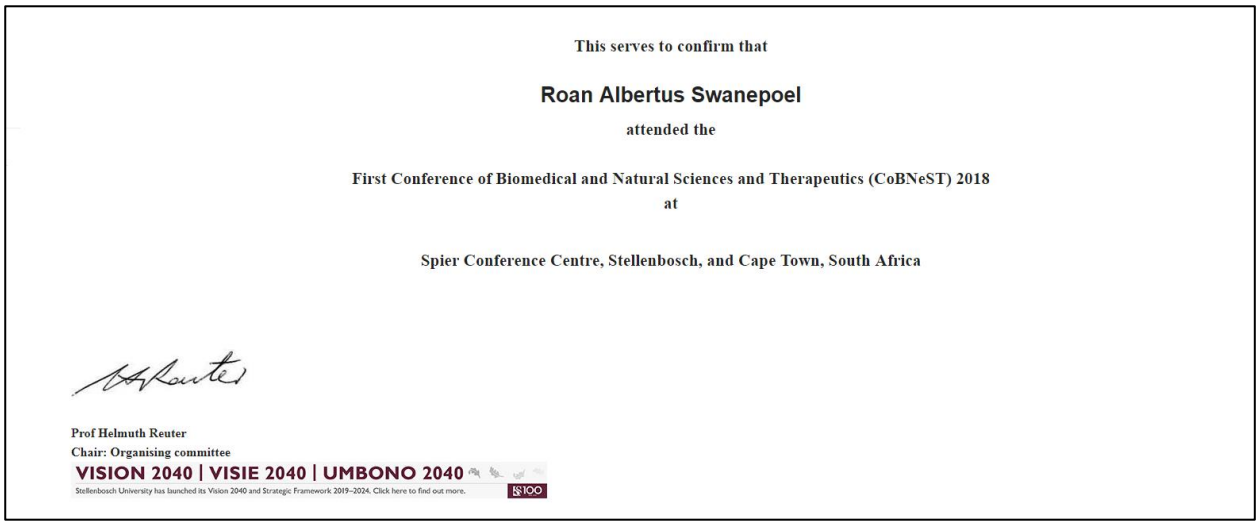


Figure E.4: Normalised protein content of the (A) LLC-PK1, (B) H69V, (C) H69AR and (D) NCI-H69/LX4 cell lines after administration of *X. undulatum.* (* indicates statistical significance between the samples and the untreated control ($p < 0.05$), and Δ indicates significant differences between the samples, $n = 6$; error bars = standard deviation (SD)).

Appendix F

Proof of participation in the First Conference of Biomedical and Natural Sciences and Therapeutics (CoBNeST)

Certificate of participation



This serves to confirm that

Roan Albertus Swanepoel

attended the

First Conference of Biomedical and Natural Sciences and Therapeutics (CoBNeST) 2018

at

Spier Conference Centre, Stellenbosch, and Cape Town, South Africa

Prof Helmuth Reuter
Chair: Organising committee

VISION 2040 | VISIE 2040 | UMBONO 2040

Stellenbosch University has launched its Vision 2040 and Strategic Framework 2019–2024. Click here to find out more.

Accepted abstract which was orally presented at the conference

***In vitro* efficacy evaluation of selected medicinal plant extracts against multidrug resistant small cell lung cancer**

Swanepoel, R.A. [1], Willers, C. [1], Hamman, J.H. [1] and Gouws, C. [1]

[1]*Centre of Excellence for Pharmaceutical Sciences, North-West University, Private Bag X6001, Potchefstroom 2520, South Africa*

Cancer is ranked as one of the leading causes of death globally. The reduced therapeutic outcome in cancer management can be ascribed to increased cancer metastasis and multidrug resistance (MDR) occurrences. Efflux transporters can decrease the intracellular accumulation of chemotherapeutic drugs in cancer tissues, which may result in sub-therapeutic levels leading to acquired MDR. There is an urgent need to identify novel treatment approaches to combat multidrug-resistance in cancer. Several traditional plants are believed to cure, prevent or manage cancer, although their anti-cancer efficacy remains to be confirmed. In this study, aqueous extracts of three

traditional plants namely *Aloe vera* (gel and polysaccharides), *Sutherlandia frutescens* and *Xysmalobium undulatum* were screened for potential *in vitro* anti-cancer efficacy against selected small cell lung cancer (SCLC) cell lines. These SCLC cell models included a drug-sensitive line (H69V), a MDR line with hyper-expressed multidrug resistance-associated protein 1 (MRP1) efflux transporters (H69AR), as well as a MDR line which hyper-expressed P-glycoprotein (P-gp) transporters (NCI-H69/LX4). A non-tumorigenic cell line (LLC-PK1) was also included to evaluate the cytotoxic effects of the selected plants on normal tissue. The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay was used as preliminary screening method to determine the relative reduction in cell viability (IC) of each cell line after a 96 h exposure period to the various plant extracts. The intracellular adenosine triphosphate (ATP) and extracellular adenylate kinase (AK) levels, which are indicative of cell viability, were measured following dose related treatment according to wet biomass with the selected plant extracts. From the results obtained, it was evident that all three plant extracts reduced the cell viability of the SCLC cells in a concentration-dependent manner, signifying anti-cancer efficacy. The phytochemicals present in these plant extracts may be MRP1 and P-gp inhibitors, which can be considered for combination therapies to enhance intracellular drug accumulation.