Medicinal uses of *Galenia africana*: A study of the antimicrobial, antifungal and anticancer properties

Candidate

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KEYWORDS

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Acute oral toxicity

Acute dermal toxicity

Episkin skin irritation

Dermal sensitization

Staphylococcus aureus

Methicillin-resistant *Staphylococcus aureus*

Candida albicans

Candida glabrata

Antimicrobial testing

Checkerboard assay

Sensititre susceptibility testing

MTT (3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium bromide) assay

MCF-7 cells

KMST-6 cells

RT² Profiler PCR Array

INTELLECTUAL PROPERTY

Some of the data presented in this dissertation is under patent review and forms background intellectual property for the project **Indigenous Botanical Adjuvant Technologies** which is funded by the **Technology Innovation Agency (TIA)** on behalf of the **South African Department of Science and Technology.**

ABSTRACT

Medicinal uses of *Galenia africana*: A study of the antimicrobial, antifungal and anticancer properties

Over the years, microorganisms have become resistant to commonly used antimicrobial agents leading to multidrug resistance. This is believed to occur even with new classes of therapeutic agents thus creating a challenge on the global healthcare system. The study of medicinal plants allows for their possible use as alternative therapeutic agents. *Galenia africana* (*G. africana*) is a South African medicinal plant with numerous health benefits.

The purpose of this study was to evaluate the potential antimicrobial, antifungal and anticancer properties of the ethanolic extract of *G. africana*. Prior to evaluating these properties, *in vitro* and *in vivo* acute toxicity studies were conducted to assess the toxicity profile of *G. africana*.

The toxicity profile of the *G. africana* extract was evaluated using acute toxicity studies conducted in animal and reconstituted human *epidermis* skin models. The results of the acute oral and dermal toxicity studies revealed that the median lethal dosage (LD_{50}) for *G. africana* extract in Sprague-Dawley rats was considered to exceed 2000 mg/kg. In the dermal sensitization study, the stimulation index (SI) values for the mice treated with the *G. africana* extract at concentrations of 25% (50 mg/ml), 50% (100 mg/ml), and 100% (200 mg/ml), when compared to the control group, were 1.3, 0.9 and 1.3, respectively which did not result in an SI value of ≥ 3 in any group. Hence, it did not elicit a hypersensitivity response. In the irritation test, the *G. africana* (concentrate) and *G. africana* (in-use dilution) extracts were non-irritant on the reconstituted human *epidermis*.

The antimicrobial activity of *G. africana* was evaluated against two *Staphylococcus aureus strains* namely methicillin-resistant (MRSA: ATCC 33591) and methicillin-sensitive/susceptibile (MSSA: ATCC 25923). Results of the broth microdilution assay showed that *G. africana* exerted good antimicrobial activity against the two strains with a minimum inhibitory concentration (MIC) of 3.12 mg/ml for both strains. Results of the minimum bactericidal concentration (MBC) determination showed that *G. africana* was bactericidal against both strains (MBCs of 3.125 and 6.25 mg/ml for MSSA and MRSA, respectively). In addition, the checkerboard assay demonstrated that the combination of *G. africana* and ampicillin had an additive effect against MSSA and an indifference result against MRSA (fractional inhibitory concentration indices (FICI) of 0.64 against MSSA and 1.002 against MRSA). The combination of *G. africana* with several antimicrobial agents in the Sensititre susceptibility test also showed a reduction in the MICs of a number of the antimicrobial agents.

Investigation of the antifungal activity of *G. africana* was done by evaluating its activity against *Candida* species (*Candida albicans* and *Candida glabrata*, ATCC 90028 and ATCC 26512, respectively). Results of the broth microdilution assay showed that *G. africana* possessed antifungal activity against the two *Candida* strains (MIC of 6.25 mg/ml for both *Candida* strains). Minimum fungicidal concentration (MFC) determination revealed that *G. africana* was fungicidal against both *Candida* strains (6.25 and 12.5 mg/ml for *C. albicans* and *C. glabrata*, respectively). Results of the checkerboard assay revealed that the combination of *G. africana* and fluconazole had a strong synergistic effect against *C. albicans* and an indifference result against *C. glabrata* when interpreted by the FICI (0.36 and 1.002 for *C. albicans* and *C. glabrata*, respectively). Results of the Sensititre susceptibility test also showed a reduction in the MICs of the antifungal agents when combined with *G. africana*. Microscopic analysis, using scanning

electron microscopy, showed that there was cell damage including a decrease in cell size after *G. africana* treatment for both *Candida* species.

Evaluation of the anticancer properties of *G. africana* was done by investigating its cytotoxic potential in human breast cancer (MCF-7) and normal fibroblast (KMST-6) cell lines. The ability of *G. africana* to affect gene expression, apoptosis and cell cycle processes was also evaluated in MCF-7 cell lines. It was observed that *G. africana* had a greater anticancer effect against the MCF-7 than the KMST-6 cells as determined by the IC_{50} values. Results of the effect of *G. africana* on the gene expression, apoptosis and cell cycle of MCF-7 cells showed that genes, such as *BCL2L11*, *CASP2* and *CASP7/9*, that are involved in apoptosis were up-regulated. It was also observed that genes involved in angiogenesis such as *ANGPT1* and *ANGPT2* were down-regulated. In addition, genes involved in cell cycle processes, such as *ACLY*, *ACSL4* and *PFKL*, were down-regulated.

Collectively, these results suggested that *G. africana* possessed antimicrobial, antifungal and anticancer activities against medically important microorganisms as well as breast cancer. Furthermore, toxicity analysis results revealed that *G. africana* was not toxic when tested using animal and human skin models. This provides important information for the development of new therapeutic agents.

DECLARATION

I, Tiza Lucy Ng'uni, hereby declare that the dissertation titled "*Medicinal uses of Galenia africana: A study of the antimicrobial, antifungal and anticancer properties*" submitted for the PhD degree in Medical Biosciences at the University of the Western Cape is my own work and has not been previously submitted for any degree or examination at this or any other university. All sources of information I have used or cited have been indicated and acknowledged by complete references.

Full name: Tiza Lucy Ng'uni Date: 7th August, 2017

Signed: 

DEDICATION

This dissertation is dedicated to my beautiful and wonderful daughter, Angel Mwansa Ng'uni and my amazing and beloved mother Catherine Ng'uni. Words cannot describe what you mean to me. Even though I have been so far away from you, the thought of you has got me through some tough times. I thank God for you each and every day. Your encouragement, support and love are things I would not trade for anything and hold them very dear to my heart. You are a big blessing to me. May God always be with you and bless you. I love you very much.

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LIST OF PUBLICATIONS

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Tiza Ng'uni, Jeremy A. Klaasen and Burtram C. Fielding. *Galenia africana* stimulates apoptosis in breast cancer (MCF-7) cells in a caspase-dependent pathway. (In preparation).

LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
%	Percent
°C	Degrees celsius
5FC	Flucytosine
ABC	ATP-binding cassette
ACLY	ATP citrate lyase
ACSL4	Acyl-CoA synthetase long-chain family member 4
ADCC	Antibody-dependent cell-mediated cytotoxicity
AMR	Amtimicrobial resistance
ANGPT1	Angiopoietin 1
ANGPT2	Angiopoietin 2
ANOVA	Analysis of variance
ASR	Age standardized rate
ATP	Adenosine triphosphate
ATP	Adenosine 3/tri phosphates
BCL2L11	BCL2-like 11 (apoptosis facilitator)
BCRP	Breast cancer resistance protein
BIRC3	Baculoviral IAP repeat containing 3
BMI1	BMI1 polycomb ring finger oncogene
C. albicans	*Candida albicans*
C. glabrata	*Candida glabrata*
C/M	Cells and media
CA	Community-associated

CAMHB	Cation Adjusted Muller Hinton Broth
CASP2	Caspase 2, apoptosis-related cysteine peptidase
CASP7	Caspase 7, apoptosis-related cysteine peptidase
CASP9	Caspase 9, apoptosis-related cysteine peptidase
CCND3	Cyclin D3
CDH2	Cadherin 2, type 1, N-cadherin (neuronal)
cDNA	Complementary deoxyribonucleic acid
CFU	Colony forming unit
CLSI	Clinical and Laboratory Standards Institute
cm^2	Square centimeter
CPD	Critical point dryer
C_T	Threshold cycle
CTLA-4	Cytotoxic T-lymphocyte-associated antigen 4
CV	Cell viability
CYP51	Cytochrome P450-dependent enzyme 14α-lanosterol demethylase
DDB2	Damage-specific DNA binding protein 2, 48kDa
DDIT3	DNA-damage-inducible transcript 3
DDR	DNA damage response
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPM	Disintegrations per minute
DSP	Desmoplakin
ECVAM	European Centre for the Validation of Alternative Methods

EF2	Translation elongation factor 2
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
EMT	Epithelial-mesenchymal transition
ER+	Estrogen receptor positive
ERG1	Squalene monooxygenase
ERG2	D_7–D_8 isomerase
ERG24	D_{14}-reductase
FASLG	Fas ligand (TNF superfamily, member 6)
FDA	Food and Drug Administration
FGF2	Fibroblast growth factor 2 (basic)
FIC	Fractional Inhibitory Concentration
FICI	Fractional Inhibitory Concentration Index
FLC	Fluconazole
FLT1	Fms-related tyrosine kinase 1 (vascular endothelial growth factor/vascular permeability factor receptor)
G. africana	*Galenia africana*
G6PD	Glucose-6-phosphate dehydrogenase
G6PD	Glucose-6-phosphate dehydrogenase
GADD45G	Growth arrest and DNA-damage-inducible, Gamma
GHS	Global Harmonized System
GLP	Good laboratory practice
GLP	Good laboratory practice

GOF	Gain-of-function mutations
HA	Hospital/healthcare-associated
HER2	Human epidermal growth factor receptor 2
HNSCC	Head and neck squamous cell carcinoma
H (h)	Hour
I	Intermediate
IAP	Inhibitors of apoptosis proteins
IE	Infective endocarditis
IFN	Interferons
IGF1R	Insulin-like growth factor 1 receptor
IGFBP3	Insulin-like growth factor binding protein 3
IGFBP3	Insulin-like growth factor binding protein 3
IGFBP5	Insulin-like growth factor binding protein 5
IGFBP7	Insulin-like growth factor binding protein 7
IL2	Interleukin-2
IMS	Inter-membrane space
INT	*p*-iodonitrotetrazolium chloride
IUPAC	International Union of Pure and Applied Chemistry
Kg	Kilogram
KMST-6	Normal fibroblasts
LA	Livestock-acquired
LAKs	Lymphokine-activated killer cells
LCB	Lactophenol cotton blue
LD_{50}	Median lethal dosage

LDHA	Lactate dehydrogenase A
LLNA	Local Lymph Node Assay
MABs	Monoclonal antibodies
MABs	Monoclonal antibodies
MAD	Mutual Acceptance of Data
MAPK14	Mitogen-activated protein kinase 14
MBC	Minimum bactericidal Concentration
MCF-7	Breast cancer cell line
MFC	Minimum Fungicidal Concentration
MFS	Major facilitator superfamily
MHA	Muller Hinton Agar
MIC	Minimum Inhibitory Concentration
Min	Minute
ml	Milliliter
mm	Milimeter
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSSA	Methicillin-sensitive *Staphylococcus aureus*
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAC	Non-*albicans Candida*
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
NC	Negative control
NK	Natural killer cells
NS	Non-susceptible

OCLN	Occludin
OD	Optical density
OECD	Organization for Economic Co-operation and Development
PBS	Phosphate buffered saline
PcG	Polycomb Group
PD1	Programmed cell death protein 1
PDL1	PD1 ligand
PFKL	Phosphofructokinase, liver
PINX1	PIN2/TERF1 interacting, telomerase inhibitor 1
PlGF	Placenta growth factor
PPP1R15A	Protein phosphatase 1, regulatory (inhibitor) subunit 15A
QC	Quality Control
qPCR	Quantitative Polymerase Chain Reaction
R	Resistant
RhE	Reconstructed human *epidermis*
RIN	RNA integrity number
RIN	RNA integrity number
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT PCR	Real-Time Polymerase Chain Reaction
S	Susceptible
S or Sec	Second
S. aureus	*Staphylococcus aureus*

SD	Standard deviation
SDD	Susceptible dose dependent
SDS	Sodium dodecyl sulfate
SEM	Scanning Electron Microscopy
SGA	Sabouraud Glucose Agar
SI	Stimulation index
SNAI1	Snail homolog 1 (Drosophila)
SOD1	Superoxide dismutase 1, soluble
STMN1	Stathmin 1
TAA	Tumor-associated antigens
TCR	T cell receptor
TEM	Transmission electron microscopy
TG	Test guideline
TILs	Tumorinfiltrating lymphocytes
TNF	Tumor necrosis factor
Tregs	Tumor-infiltrating regulatory T cells
TSA	Tumor-specific antigens
UQCRFS1	Ubiquinol-cytochrome c reductase, Rieske iron-sulfur polypeptide 1
VC	Vehicle treated control
VEGF-A	Vascular endothelial growth factor A
w/v	Weight per volume
WEE1	WEE1 homolog (*S. pombe*)
WHO	World Health Organization
YNBG	Yeast Nitrogen Base Glucose

µg/ml	Micrograms per milliliter
µl	Microlitre
µl/well	Microliters per well
µm	Micrometer
µM	Micromolar

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1 CHAPTER 1: General introduction

1.1 Background

Multidrug resistance has been described in microorganisms such as bacteria and fungi as well as in diseases such as cancer (Housman, Byler *et al.* 2014). Antimicrobial resistance (AMR) develops mainly as a result of misuse of antimicrobial agents, patients not adhering to treatment plans and patients stopping treatment once they experience side effects (Ayukekbong, Ntemgwa *et al.* 2017). Drug resistance in cancer normally develops by means of various processes such as drug efflux, DNA damage repair, drug target alteration, cell death inhibition and drug inactivation (Housman, Byler *et al.* 2014).

The multidrug resistance crisis is worsened by the fact that a large number of pharmaceutical companies prefer to produce drugs for the treatment of chronic diseases, such as diabetes and hypertension, as this is more profitable for them. In addition, these companies believe that resistance to newly developed antimicrobial agents would still develop thus investing in this type of research would yield less profit (Brandenburg and Schürholz 2015). The increasing threat of drug resistance prompted researchers to explore the use of medicinal plants as alternative therapeutic agents for the treatment of diseases. Numerous studies have been conducted on medicinal plants to evaluate their effect on drug resistant microorganisms as well as cancer (Graidist, Martla *et al.* 2015; Anyanwu and Okoye 2017).

Medicinal plants have been extensively used around the world for their nutritional value and ability to treat various diseases. A large proportion of the population worldwide, approximately 60-80%, still depends on medicinal plants to treat diseases that are frequently encountered in the communities (Nile, Nile *et al.* 2017). They are also the main sources of bioactive compounds

which are incorporated in or used as herbal supplements and drugs (Ekor 2014). The bioactive compounds present in most medicinal plants include nitrogen compounds (such as alkaloids, amines and betalains), phenolic compounds (such as flavonoids, phenolic acids, tannins, stilbenes and lignans), vitamins, carotenoids as well as other compounds with a wide range of biological activities (Cai, Sun *et al.* 2003; Cai, Luo *et al.* 2004).

For years, medicinal plants and plant products have been used as herbal medicines and are incorporated in complementary and alternative medicine (Hashempur, Heydari *et al.* 2015; Nile, Nile *et al.* 2017). The current need for treatment options that limit and manage the spread of drug resistant microorganisms and subsequently treat numerous infections is the basis for the continuous research being conducted on medicinal plants and plant products.

1.2 Aims and objectives of the study

The aim of this study was to evaluate the South African medicinal plant, *G. africana*, for its potential antimicrobial, antifungal and anticancer properties. This study was also aimed at investigating the toxicity profile of this medicinal plant. The current study was formulated with several objectives as follows:

1. To assess the toxicity profile of *G. africana* in animal and reconstructed human *epidermis* models.
2. To evaluate the antimicrobial activity of *G. africana* alone as well as its interaction with antimicrobial drugs against methicillin-sensitive (MSSA) and methicillin-resistant (MRSA) *Staphylococcus aureus* strains.
3. To evaluate the anti-*Candida* activity of *G. africana* alone and in combination with antifungal drugs against *Candida albicans* and *Candida glabrata*.

4. To investigate the cytotoxicity of *G. africana* alone and in combination with doxorubicin in human breast cancer (MCF-7) and normal fibroblast (KMST-6) cell lines.

1.3 Study rationale

Multidrug resistance is a growing concern worldwide and the need to find ways to combat it is of utmost importance. The rate at which resistance develops is greater than the rate at which new drugs are being produced and this puts a strain on the healthcare sector. It is for this reason that alternative treatment options are being explored, in the form of medicinal plants, as a way of alleviating the burden created by multidrug resistant microorganisms. Medicinal plants have been used in the treatment of diseases and the study of these plants and plant products creates a platform to assess their potential as therapeutic agents. Nonetheless, despite their use as herbal medicines there is need for toxicology studies to be conducted to assess the safety and potential adverse effects associated with them. It is for this reason that the South African medicinal plant was selected in order to evaluate its potential beneficial health effects.

1.4 The use of medicinal plants as therapeutic agents

Natural products are still being used in drug development and they play a fundamental role in this industry. Approximately a third of the most common drugs originate from plants and plant-based compounds (Ma, Zheng *et al.* 2009). It has been shown that at least 190 clinical trials are dedicated to the study of pure or combined plant compounds to assess their ability to treat a variety of diseases (Joray, Trucco *et al.* 2015). The World Health Organization states that approximately 70-95% of people in developing countries rely on medicinal plants for the treatment of most diseases. Herbal medicine is frequently used in rural areas because it is often the only treatment option available to them (Mabona and Van Vuuren 2013). Most people use

medicinal plants because they are considered to be cheaper alternatives, readily available and the indigenous knowledge makes them safer options for the local population (Street, Stirk *et al.* 2008).

In Southern Africa, plants that are used for skincare, such as *Harpagophytum procumbens*, *Lobostemon fruticosus*, *Trichilia emetic* and *Warburgia salutaris*, and being considered for commercialization still have not undergone phytochemical, toxicity and pharmacological efficacy testing (Vermaak, Kamatou *et al.* 2011). Furthermore, it has been observed that the skincare industry is incorporating a number of medicinal plants, such as Rooibos (*Aspalathus linearis*), *Aloe ferox* and *Aloe vera*, in most of their products (Mabona and Van Vuuren 2013). Medicinal plants predominantly found in Southern African have also been shown to have the ability to treat skin infections caused by pathogens such as *Staphylococci* species and *Candida albicans* (Mabona and Van Vuuren 2013). Studies conducted on medicinal plants used for the treatment of skin infections showed that they caused adverse effects which included phytodermatitis, an increased risk of photosensitization and allergic reactions (Reuter, Merfort *et al.* 2010). It has been shown that about 27 million South Africans rely on the use of medicinal plants for the treatment of a large number of infections (Street, Stirk *et al.* 2008). Even though South Africa has a variety of medicinal plants and affluent biodiversity, most of the plant species have not been extensively researched and validated (Springfield, Eagles *et al.* 2005).

Medicinal plants used to treat numerous are usually considered to be safe and not toxic. This belief is as a result of their long term use in treating diseases and the indigenous knowledge past down from previous generations. Nevertheless, studies have shown that a large number of medicinal plants, currently being used as traditional medicines, have the ability to be mutagenic and carcinogenic (Fennell, Lindsey *et al.* 2004). The ability of medicinal plants to be potentially

toxic is generally as a result of the lack of proper identification, preparation of herbal formulations and due to poorly trained people administering these herbal medicines (Nasri and Shirzad 2013). It is for this reason that toxicity studies should be conducted, on medicinal plants, as they provide important information on their safety which is vital for their potential use as therapeutic agents (Manaharana, Chakravarthib *et al.* 2014).

1.5 *Galenia africana*

1.5.1 Description

Galenia africana (*G. africana*, commonly known as "kraalbos" or "geelbos") (Van Wyk, De Wet *et al.* 2008) belongs to the Aizoaceae family and is a fragrant, woody sub-shrub, that can grow to heights of 0.5-1 meters. Its leaves are green, about 5 cm long, hairless and arranged in a distinct fashion. The immature leaves are green but as they mature, they tend to turn yellow (Van der Lugt, Schultz *et al.* 1992). The tip of the twigs is the site at which inflorescence develops with the presence of a number of small yellow flowers. The flowers are approximately 1.5 mm wide with their blooming season usually being October and December. These flowers are yellowish green and are born in large loose heads (Figure 1.1) (Le Roux, Kotze *et al.* 1994).

Figure 1.1: Pictures showing a *G. africana* shrub and the plant during flowering (Picture credits: Prof JA Klaasen, University of the Western Cape).

1.5.2 Distribution and ecology

Initially, *G. africana* was predominantly found in the Namaqualand region. However, over the years it has been found to be common in the Western and Southern Karoo (Figure 1.2) (Kellerman, Coetzer *et al.* 1988). This plant is mainly found in areas around the kraals, road sides as well as trampled veld (De Kock 1928; Kellerman, Coetzer *et al.* 1988). Not only can the presence of *G. africana* indicate that an area has been disturbed, this plant is also the first to colonize areas that were once disturbed or damaged. Even when other plant species are scarce in the veld, due to overgrazing, *G. africana* is the only plant species that is able to grow and thrive. When animals have to migrate between regions due to the changes in winter and summer rainfall, they become hungry and tend to feed on any plants they come across along the way. Nonetheless, a number of these plants are normally not suitable for the animals. In addition, farmers at times keep their livestock within or around pens for extended times. This causes the animals to become very hungry and as a result, they end up grazing on poisonous plants (Van Aardt 2004).

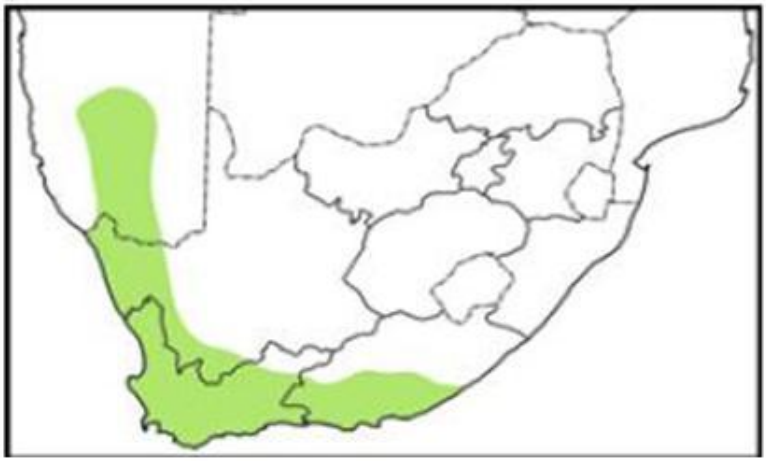

Figure 1.2: Distribution of *G. africana* in South Africa (Mukind and Eagles 2010).

1.5.3 Indigenous knowledge and medical uses

A number of health benefits associated with *G. africana* have been described. It has been documented that the indigenous Khoi-San (Hottentots) tribe of South Africa chewed on the plant in order to alleviate toothaches (Watt and Breyer-Brandwijk 1962; Mativandlela, Muthivhi *et al.* 2009). *G. africana* is also used in the treatment of venereal disease, skin diseases and relieving of eye inflammation. It has been shown that ointments prepared by frying *G. africana* with other medicinal plants in butter have been used to treat wounds, particularly on the legs of women (Watt and Breyer-Brandwijk 1962). The aerial parts of *G. africana* have also been used to treat asthma, coughs including tuberculosis. The extensive ability of *G. africana* to treat various ailments demonstrates that it possesses numerous health benefits (Watt and Breyer-Brandwijk 1962; Mativandlela, Muthivhi *et al.* 2009; De Beer and Van Wyk 2011).

1.5.4 Toxicity in animals

Based on information gathered from farmers, if the plant and leaves are green, it is edible, pleasant and not poisonous. However, in the summer when the leaves are yellow and the plant is dry and woody, it is poisonous and not edible (Le Roux, Kotze *et al.* 1994). Furthermore, they state that the yellow leaves contain elevated amounts of acidic material which destroys the stomach lining when ingested by animals which eventually leads to death (Kellerman, Coetzer *et al.* 1988). In cases of severe drought, animals tend to feed on this plant which leads to liver damage and a condition known as 'waterpens' or 'water belly'. This condition is commonly seen in animals such as sheep and goats (De Kock 1928; Kellerman, Coetzer *et al.* 1988). Waterpens is associated with severe abdominal expansion, weight loss and death. In addition, animals also fail to stand and tend to lay flat on the ground as a result of this condition (Van der Lugt, Schultz *et al.* 1992). It has been reported that ingestion of the plant by animals caused hepatic lesions which were thought to be compatible with cyanotic indurations of the liver believed to be caused by congestive heart failure (Van der Lugt, Schultz *et al.* 1992). The appearance of the liver in diseased animals depends on the extent of the disease. The liver may appear smaller or larger than normal, it may have a grayish-blue to yellowish-brown color, the morphology can either be unchanged or have nodular hyperplasia and/or certain parts of the live may become atrophic or hypertrophic (Kellerman, Coetzer *et al.* 1988). Researchers believe that the liver lesions are caused by an unknown toxin which enables the plant to be hepatotoxic (Van der Lugt, Schultz *et al.* 1992).

1.5.5 Antimicrobial and cytotoxicity studies

Studies have shown that whole plant extracts of *G. africana* display antifungal characteristics against *Botrytis cinerea* (Vries, El Bitar *et al.* 2005). This pathogen causes gray mould on numerous agricultural and horticultural crops of economic importance such as fruits and flowers which leads to loss of revenue. This antifungal activity of *G. africana* makes it a potential agent for use as a fungicide (Fielding, Knowles *et al.* 2015). Medicinal plants are used across the world for the treatment of various diseases as they possess numerous health beneficial compounds such as alkaloids, tannins, tepenoids and flavonoids (Cha, Lee *et al.* 2014). Furthermore, a study evaluating the antimycobacterial activity of South African medicinal plants revealed that *G. africana* displayed the greatest antimycobacterial activity against *Mycobacterium smegmatis* and *Mycobacterium tuberculosis* with MICs of 0.78 and 1.2 mg/ml, respectively. Fractionation of the *G. africana* ethanolic extract revealed the presence of a flavone called 5,7,2'-trihydroxyflavone. This fraction displayed MICs of 0.031 and 0.10 mg/ml against *M. smegmatis* and *M. tuberculosis*, respectively (Mativandlela, Meyer *et al.* 2008).

In vitro cytotoxicity studies showed that *G. africana* had an IC_{50} value of 118.2 µg/ml when tested against vero cells (mammalian kidney cells) (Mativandlela, Muthivhi *et al.* 2009). In addition, analysis of whole blood cultures using lactate dehydrogenase leakage (LDH) revealed that *G. africana* was not toxic at a concentration of 625 µg/ml and had an IC_{50} value of 975 µg/ml. Analysis of LDH leakage in human breast adenocarcinoma cell lines (MCF-7) showed an IC_{50} value of 1127 µg/ml (Shoko 2010). Evaluation of the environmental toxicity of *G. africana* was done using the *Vibrio fischeri* bioluminescent test which produced EC_{50} values of 1 and 0.7 µg/ml at exposure periods of 15 and 30 min, respectively. Another test known as the *Daphnia*

pulex test was employed to assess acute toxicity of *G. africana*. Results showed EC_{50} values of 40 and 30 µg/ml at exposure periods of 24 and 48 h, respectively (Pool, Klaasen *et al.* 2009).

1.5.6 Chemistry

Phytochemical analysis of an 80% (ethanol: water) *G. africana* extract conducted by Ticha and colleagues revealed that this extract was mostly composed of flavonoids. It was shown that the purified fractions of *G. africana* contained flavonoids that were structurally identical to chrysin, sakuranetin and pinocembrin (Ticha, Klaasen *et al.* 2015). Furthermore, analysis of a 20% (w/v) *G. africana* extract (80% ethanol: water) revealed that it consisted of approximately 61 compounds. The majority of these compounds are currently found in the human diet. The chemical composition of *G. africana* as well as the CAS number (where applicable), the International Union of Pure and Applied Chemistry (IUPAC) name, relative concentration and the percentage match against the spectral database are outlined in Table 1.1. The Table also includes the seven groups into which the chemical compounds where assigned. These groups included aliphatic, aliphatic triterpenoid (at), carbonyl, fatty acid, flavonoid, tocopherol and other (Gledhill and Holmes 2011)

Table 1.1: Chemical components of *G. africana*.

#	Cas No	Name	% Conc	% Match	Group
1	544-76-3	hexadecane	0.65	98	aliphatic
2	593-45-3	octadecane	0.67	96	aliphatic
3	502-69-2	hexahydrofarnesyl acetone	2.79	97	at
4	1117-52-8	farnesyl acetone	0.43	95	at
5	628-97-7	ethyl hexadecanoate	1.09	99	fatty acid
6	544-35-4	linoleic acid ethyl ester	0.34	99	fatty acid
7	1191-41-9	ethyl linolenate	0.64	97	fatty acid
8	124-04-9	hexanedioic acid (adipic acid)	6.04	95	fatty acid
9	629-99-2	pentacosane	0.30	95	aliphatic

10	88-99-3	o- phthalic acid	0.96	91	other
11	630-01-3	hexacosane	0.38	98	aliphatic
12	646-31-1	tetracosane	0.32	98	aliphatic
13	24634-95-5	ethyltetracosanate	0.20	93	fatty acid
14	111-02-4	squalene	0.21	94	at
15	630-03-5	nonacosane	0.35	99	aliphatic
16	77-96-19-2	2-heptacosane	0.19	96	aliphatic
17	638-68-6	triacontane	0.22	99	aliphatic
18	593-49-7	heptacosane	2.02	98	aliphatic
19	103-30-0	trans-stilbene	0.49	97	flavonoid
20	120-51-4	benzylbenzoate	0.51	97	other
21	10191-41-0	(+/-)-alpha-tocopherol	0.52	95	tocopherol
22		alpha tocopherolquinone	0.39	93	tocopherol
23		vitamin e acetate	0.58	93	tocopherol
24	4602-84-0	farnesol	1.20	74	at
25	122-57-6	4-phenylbut-3-en-2-one	0.08	97	carbonyl
26	140-10-3	*trans*-cinnamic acid	0.96	98	flavonoid
27	544-63-8	tetradecanoic acid (myristic acid)	0.06	96	fatty acid
28	1002-84-2	pentadecanoic acid	0.42	99	fatty acid
29	502-69-2	6,10,14-trimethyl-2-pentadecanone	<LOQ	99	aliphatic
30	57-10-3	hexadecanoic acid (palmitic acid)	0.66	98	aliphatic
31	1019-41-0	vitamin e	<LOQ	98	tocopherol
32	6538-02-9	ergostanol	0.14	86	flavonoid
33	92-48-8	6-methylcoumarin	0.13	96	flavonoid
34	305-01-1	6,7-dihydroxy-2-chromenone	0.51	59	flavonoid
35	126-33-0	sulfolane	3.36	91	other
36	629-59-4	tetradecane	0.35	98	aliphatic
37	7786-61-0	2-methoxy-4-vinyl phenol	0.32	90	carbonyl
38	784-62-3	thioflavone	0.92	86	others
39	1776-30-3	chalcone	0.97	20	flavonoid
40		2,6,10,14 tetramethylheptadecane (pristane)	1.02	98	at
41	100-52-7	benzaldehyde	0.08	97	carbonyl
42	119-84-6	3,4 dihydrocoumarin	0.04	94	flavonoid
43		1-(3-methylphenyl)-2-(2‘-hydroxyphenoxy) ethanone	0.05	83	carbonyl

44		1-(2‘,4‘-dihydroxyphenyl)-3-phenylpropan-1-one	2.92	97	carbonyl
45		2s-5-hydroxyflavanone	0.82	83	flavonoid
46	100-52-7	benzaldehyde	0.09	97	carbonyl
47	65-85-0	benzoic acid	0.36	95	carbonyl
48	119-84-6	3,4 dihydrocoumarin	0.53	96	flavonoid
49		1-(2‘,4‘dihydroxyphenyl)-3-phenylpropan-1-one	0.51	97	carbonyl
50	100-52-7	benzaldehyde	0.31	97	carbonyl
51		benzoic acid	1.05	93	carbonyl
52		3,4 dihydrocoumarin	0.57	94	flavonoid
53		1-(2‘,4‘-dihydroxyphenyl)-3-phenylpropan-1-one	0.66	97	carbonyl
54	1776-30-3	2‘, 4‘-dihydroxychalcone	14.00	-	flavonoid
55	666-86-7	7-hydroxyflavone	10.20	-	flavonoid
56	531-95-3	equol	3.87	-	flavonoid
57	480-39-7	pinocembrin	23.10	-	flavonoid
58	67604-48-2	narengenin	0.44	-	flavonoid
59	480-44-4	acacetin	0.40	-	flavonoid
60		chrysin or aesculetin	0.54	-	flavonoid
61	520-28-5	tectochrysin	7.07	-	flavonoid

At: Aliphatic triterpenoid; LOQ: Limit of quantification; Conc: Concentration

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2 CHAPTER 2: Literature review

2.1 Assessing the potential toxicity of medicinal plants

2.1.1 Introduction

Medicinal plants have been used for centuries around the world for the treatment of various diseases. According to the World Health Organization (WHO), approximately 80% of people in Africa and Asia rely on herbal medicines for the treatment of diseases. This can be seen in developed countries as well where 70-80% of people rely on complementary or alternative medicine (Araújoa, Barcellosb *et al.* 2017). With the increase in the use of complementary and alternative medicine, it has become imperative to assess the safety of medicinal plants (Neergheen-Bhujun 2013). Medicinal plants are normally used as crude extracts and thus contain a wide range of compounds. However, the biological activities of these compounds are normally unknown (Konan, Bacchi *et al.* 2006). In addition, substances such as digitoxines, cyanogenic glycosides and strychnine, which are considered to be very toxic, can be found in plants. Thus, plant species have to be assessed for safety before they can be confidently used for medicinal purposes (Araújoa, Barcellosb *et al.* 2017). This has led to numerous studies being carried out on medicinal plants to assess their safety, efficacy as well as validate their traditional uses (Menegati, de Lima *et al.* 2016).

The organization of Economic Co-operation and Development (OECD) is an economic organization comprising the governments of 34 countries. It was established in 1961 to enhance economic development and world trade (Thybaud, Lorge *et al.* 2017). In 1981, the chemical testing program was formed in order to help countries harmonize methods used in the assessment of chemical safety as well as good laboratory practice (GLP). However, the methods used to test

the toxicity of chemicals are physically demanding and expensive (Thybaud, Lorge *et al.* 2017). In addition, it was noted that numerous countries were conducting toxicological tests on similar types of chemicals. It was for this reason that the OECD council implemented the Mutual Acceptance of Data (MAD). The MAD stated that test data produced by member countries, that followed the OECD test guidelines (TGs) and principles of GLP, would be accepted across all member countries and used to safeguard human health and the environment as well as for regulatory purposes (Thybaud, Lorge *et al.* 2017).

Nevertheless, the OECD TGs are not complete testing procedures but are instead intended to offer assurance of test results. However, the test results might differ depending on the test chemical or member country. Furthermore, each member country's regulatory authority is responsible for outlining the tests required for the registration or demonstration of safety of test chemicals (Thybaud, Lorge *et al.* 2017). The OECD TGs incorporate both *in vivo* and *in vitro* assays. The frequently used TGs have undergone revisions, over the years, to allow for modern scientific knowledge, improved techniques as well as improved knowledge of the assay limitations. The aim of these revisions was to offer well-rounded testing methods, improve data interpretation and statistics and guarantee consistency among guidelines (Thybaud, Lorge *et al.* 2017). In addition, animal welfare was also taken into consideration when revising the TGs for *in vivo* tests by reducing the number of animals used in studies without compromising the quality of the data produced. The revisions also incorporated the use of animals of a single sex as opposed to both sexes, reviewed the number of animals in each group and reduced the need for simultaneous positive controls. The revision of TGs is an on-going process that relies on current scientific knowledge and improved techniques (Thybaud, Lorge *et al.* 2017).

2.1.2 *In vitro and in vivo toxicity studies*

2.1.2.1 *Acute Oral Toxicity – Acute Toxic Class Method*

2.1.2.1.1 *Animal selection*

The acute oral toxicity study is carried out using a set of guidelines outlined by OECD No. 423 and is aimed at providing adequate information on the acute toxicity of a test substance using the smallest number of animals per group (OECD 2001). The animals in the experimental groups receive the test substance through oral administration at one of the pre-defined doses established by the Global Harmonized System (GHS) based on LD_{50} values (OECD 1998). These guidelines define the quantity of a given chemical or substance that an animal can consume that would kill 50% of that animal or test model and this is referred to as the Lethal Dose (LD_{50}).

The animals used in this study are usually rats though other rodents can be used (OECD 2001). They are normally 3 per group and can be confined to one sex (usually females) (Roll, Höfer-Bosse *et al.* 1986). Female rats are generally used because literature reviews of LD_{50} tests prove that females are more sensitive. Nonetheless, the variation in sensitivity between male and female rats is minimal (Lipnick, Cotruvo *et al.* 1995). The guidelines state that doses should not induce noticeable pain and distress to the animals and if such happens then they should be killed humanely (OECD 2000; OECD 2001). The strains of animals should be those generally used in the laboratory and they should be young adults in a healthy state. The females must be nulliparous and not pregnant. At dosing, the age of each animal must be between 8 and 12 weeks and their weights should be within acceptable limits (OECD 2001).

2.1.2.1.2 *Management of animals and dose preparation*

The animal housing should be kept under the following standard conditions: temperatures of 22°C (± 3°C); humidity of 50-60% (it can be a minimum of 30% but not more than 70%) and artificial lighting of 12 h of light and 12 h of dark (OECD 2001). The usual laboratory diets can be used and drinking water can be given to the animals without limitations. Animals can be placed in cages according to their respective doses. However, numbers should be kept small enough to ensure that the desired observations are easily seen (OECD 2001). The selection of animals is random and marks are placed on them for easy identification. They are placed in cages for a minimum of 5 days before commencement of dosing to enable the animals to adjust to the laboratory surroundings (OECD 2001).

Doses should be administered in a constant volume and the maximum volume that can be administered is dependent on the size of the animal to be tested. Regulatory authorities state that neat or undiluted forms of liquid mixtures should be evaluated for potential risk. Dose formulations can be prepared using water or other solvents. However, if water is not used to prepare the dose formulations, then the vehicle should be analyzed to determine the toxicological properties. Dose formulations should be made up just before they are administered to the animals except in situations where preparations have shown to be stable over a period of time (OECD 2001).

2.1.2.1.3 *Procedure, observations and data reporting*

The test substances are given as a single dose by gavage through a stomach tube or an appropriate intubation cannula. Before dosing, animals should not receive any food but can be given water (rats should not receive food overnight whereas mice should not receive food for 3-4

h). After fasting, the weight of the animals should be recorded followed by the administration of the test substance. Food can then be given after 3-4 h and 1-2 h for rats and mice, respectively (OECD 2001). Four fixed dose levels of 5, 50, 300 and 2000 mg/kg body weight have been outlined from which the initial dose to be used can be chosen. In cases where information pertaining to the test substance is not available, it is suggested that an initial dose of 300 mg/kg body weight should be used. Before animals can be treated with the next dose, it is imperative to ensure that the animals receiving the lower dose survive (OECD 2001).

Animal observations are conducted on an individual basis at least once in the first 30 min following dosing and throughout the first 24 h. Daily observations are then made for a period of 14 days except when the animals have to be humanely killed. However, observations depend on the toxic reactions of the animals to the test substance as well as the duration of time it takes for them to recover (OECD 2001). Some toxic reactions tend to appear late (delayed) and hence it is essential to note the time at which the signs first appear and when they fade away (Chan and Hayes 1994). Once the study is finished, the surviving animals are weighted and then humanely killed (OECD 2001). A gross necropsy is conducted on all tested animals including the ones that died during the study or those that were taken out of the study on animal welfare grounds. Every gross pathological variation observed in each animal must be documented (OECD 2001).

Data on each animal used in the study should be made available. The summary of the data should be presented in a table format which should indicate the test group, number of animals used, number of animals exhibiting signs of toxicity, number of animals that died in the course of the test or those that were humanely killed, the time that each animal died and the necropsy findings (OECD 2001). Furthermore, a test report should be included that consists of information

pertaining to the test substance, vehicle, test animals, test conditions and all results obtained after completion of the study (OECD 2001).

2.1.2.2 *Acute Dermal Toxicity*

The guidelines outlined by the OECD for testing chemicals are occasionally being reviewed due to advances in the science field as well as animal welfare concerns. Originally, the acute dermal toxicity guideline 402 was implemented in 1987. This method is a version of the acute toxic class method (OECD 2001) and uses the same number and sex of test animals. The system of ranking test substances or chemicals is the same as that used for other acute toxicity testing procedures (such as TG 420, 423 and 425) (OECD 2015a). Before this study can be carried out, information on the possible human exposure of the test chemical and the anticipated use should be supplied. This information is necessary in validating the need to perform the acute toxicity study as well as in selecting a suitable starting dose (OECD 2015a). A widespread assessment of mixture products such as pesticides, biocides and other formulations revealed that in 99% of the cases, the dermal LD_{50} was $\geq$ 2000 mg/kg body weight (OECD 2015a).

2.1.2.2.1 *Animal selection*

The principle is similar to that of the acute oral toxicity study in that the test chemical is administered to animals of a single sex in a series of steps using suitable fixed doses (OECD 2015a). The animals of choice in this study are adult rats. Analysis of acute oral (Lipnick, Cotruvo *et al.* 1995) and acute inhalation toxicity studies (Warbrick, Dearman *et al.* 2002; Price, Stallard *et al.* 2011) revealed that in most cases the difference between sexes is small, although females have shown to be somewhat more sensitive in instances where there are differences. Thus, this study also suggests the use of female rats. The rats should be in a healthy state, the

strain should be that normally used in laboratory settings, they should not be pregnant and nulliparous. The animals must be at least 8-10 weeks old with a weight of between 200-300 g. In addition, the skin of the animals should not be damaged (OECD 2015a).

2.1.2.2.2 *Management of animals and preparation*

The housing and feeding conditions of the acute dermal toxicity study are the same as those outlined for the acute oral toxicity. The animals are allowed to adjust to the laboratory conditions for no less than 5 days before commencement of the study. Animals are placed in study groups in a random fashion and marked for easy recognition. About 24 h prior to conducting the study, the fur from the animal's dorsal region of the trunk is removed through clipping or shaving. This should be done carefully to prevent bruising of the animal's skin which might change the permeability. About 10% of the dorsal region must be exposed for the application of the test chemical and the animal's weight should be considered when choosing this region (OECD 2015a).

2.1.2.2.3 *Procedure, observations and data reporting*

The experimental groups consist of 2-3 animals and the test chemical is applied to each animal systematically. The three fixed level doses from which the initial dose can be selected are 50, 200 or 1000 mg/kg body weight which are derived from the GHS Categories for acute dermal toxicity. However, if information on the test chemical is lacking, it is advised that the starting dose should be 2000 mg/kg body weight on the grounds of animal welfare (OECD 2015a). The chemical must be evenly applied on test area (about 10% of the total body surface area). Porous gauze dressing and non-irritating tape must be used to ensure that the test chemical remains in contact with the skin for the entire 24 h test period. The gauze dressing must be properly secured

to make sure that the animals do not remove it and subsequently consume the test chemical. It is advisable to keep the animals in separate cages so that they do not end up consuming the chemicals on other animals. Liquid test chemicals are normally used without being diluted. Once the testing period is over, the animals are taken back to group housing (OECD 2015a).

Observations of the animals must be conducted for a minimum of 14 days. The criteria for conducting observations are similar to those of the acute oral toxicity. However, great care must be taken in the first 2-6 h after application of test chemical. The skin, fur, eyes and mucous membranes must be observed for any changes. The respiratory, circulatory, autonomic and central nervous systems in addition to the somatomotor activity and behavior patterns must also be monitored (OECD 2015a). Evaluation of the body weight and necropsy is done in the same way as that outlined in the acute oral toxicity.

Data should be presented in a table form and reported in the same way as that outlined for acute oral toxicity. The test report should include the entire aspects outlined in the acute oral toxicity testing guideline relating to the test chemical, vehicle, test animals, test conditions and results (OECD 2015a).

2.1.2.3 *Skin Sensitization: Local Lymph Node Assay (LLNA)*

The first TG used to establish skin sensitization in mice (LLNA; TG 429) was approved in 2002 (OECD 2002). The next TG was approved in 2010. The LLNA is an *in vivo* procedure that assesses the immunological processes occurring as a result of chemical stimulation in the initial stages of sensitization and can be used to evaluate dose responses (OECD 2010). The principle of this test is that chemicals that cause sensitization stimulate the production/proliferation of lymphocytes in the lymph nodes located in the area the test chemical is applied. Lymphocyte

production is relative to the amount (dose) and strength of the test substance and allows for the sensitization to be measured (OECD 2010).

2.1.2.3.1 *Animal selection, management and dose preparation*

The preferred animals for this assay are mice. This assay uses young adult female, nulliparous and non-pregnant CBA/Ca or CBA/J mice. During commencement of the study, mice ought to be 8-12 weeks old and their weight must not go beyond 20% of the mean weight. Nonetheless, additional strains and sex of mice can be used provided the reasons for doing so are justified (OECD 2010). The mice should be kept in groups except in situations where mice have to be housed separately. The temperature and humidity conditions are similar to those specified in the acute oral toxicity study (22 ± 3ºC and 50-60%, respectively). Artificial lighting of 12 h light/day cycles should be employed. Animals are fed using the usual laboratory diets and the supply of water should be unlimited (OECD 2010).

The selection of animals is done in a random manner and marks are placed on them to make the identification process easier. Before dosing, the animals should be maintained in their cages for no less than 5 days for them to acclimatize to the laboratory environment. The skin of all the animals must be checked for the presence of any skin lesions before the test substance can be applied (OECD 2010). When solid test chemicals are used, dissolving or re-suspending them in solvents/vehicles should be done before they are applied to the ear of the mice. Test substances that are liquid in nature can either be diluted or applied neat. Fresh preparations of the test substance should be made for dosing (OECD 2010).

2.1.2.3.2 *Procedure and observations*

Doses of test chemicals are chosen from a set of concentrations such as 0.5%, 1%, 2.5%, 5%, 10%, 25%, 50% and 100%. The measurement of lymphocytes proliferation is done by evaluating the mean proliferation values of the test group and vehicle treated control (VC) group and comparing them to each other (OECD 2010). The proportion (ratio) of the mean proliferation values of the treated group and the VC group are used to calculate the stimulation index (SI), which must be ≥ 3 for the test substance to be classified as a skin sensitizer. Radioactive labeling is employed in determining the elevation in the number of cells produced in the lymph nodes (OECD 2010).

Positive (several doses) and negative (vehicle of the test substance only) controls are included in the assay to assess the proficiency of the assay. This is done by evaluating the reproducibility of the results obtained after application of the test substance. However, the vehicle of choice should not affect the test results and should be chosen based on its ability to increase the solubility of the test substance and improve the concentration yield. The assay must be performed with at least four animals in each dose group and at least three concentrations of the test substance (OECD 2010). A preliminary test can be carried out if information regarding maximum dose levels is lacking to determine the most suitable dose.

Clinical observations should be done for each mouse. Care must be taken when monitoring the mice for systemic toxicity or local irritation and observations for each animal must be documented. Body weights of each animal must be determined and reported at the beginning of testing and at the time the animals are being killed (OECD 2010).

2.1.2.3.3 *Data reporting*

The compiled data should be presented in a table format showing the proliferation (disintegrations per minute, DPM) values for each animal, group mean DPM values, SD or SEM values as well as the mean SI values for each dose group in comparison to the VC group (OECD 2010). The following information should be included: characteristics of the test substance; formulation and composition of the test and control substances; justification for vehicle selection; information relating to test animals; dose selection criteria for vehicle and test substance; toxicity measurement methods and reasons for protocol deviation. The test report can also include any statistical analysis carried out (OECD 2010).

2.1.2.4 *In vitro skin irritation: reconstructed human epidermis test method*

The *in vitro* skin irritation test is used to evaluate the effect of chemicals on the human skin. Reconstructed human *epidermis* (RhE) models are used to assess this effect as its biochemical and physiological characteristics are similar to those of the human skin. Non-transformed keratinocytes (derived from human skin cells) are used as models for this test. In addition, it encompasses the early stages of the inflammatory response that result when cells and tissues are damaged due to trauma to specific areas (*in vivo* irritation responses). This test guideline was initially implemented in 2010 but updated in 2013 and 2015 to include extra methods (OECD 2015b).

The test chemicals can either be solids (soluble or insoluble in water), liquids (aqueous or non-aqueous), semi-solids or waxes. However, solids should be ground to a fine powder prior to being applied. The use of gases and aerosols has not yet been evaluated in a validation study (Welss, Basketter *et al.* 2004). Test chemicals with the ability to absorb light in the same range

as MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and/or capable of reducing MTT may impede the cell viability measurements. When these chemicals are assessed, the experiments need to employ controls that will be used to correct these factors. In cases where classifying a test chemical is clear, one test run consisting of three replicate tissues is adequate (OECD 2015b).

2.1.2.4.1 *Principle and procedure of test*

The test chemical is applied to the surface of a three-dimensional RhE model. This model consists of human-derived keratinocytes that have undergone differentiation to create a multilayer consisting of the basal, spinosum and granular layers as well as a multilayered *stratum corneum* with lipid classes similar to those found *in vivo*. Chemicals that cause skin irritation tend to penetrate the skin and injure the layers underneath such as the keratinocytes and other skin cells thus causing an inflammatory response. Inflammatory mediators released during this inflammatory response can also exert their action on cells and other structures found in the *dermis* (OECD 2015b). Thus, RhE-based test methods are used to evaluate the initial events that occur during skin irritation such as cell/tissue damage through cell viability (Hoffmann 2006; Spielmann, Hoffmann *et al.* 2007).

Cell viability is assessed or measured by the ability of viable cells, through the use of enzymes, to covert the MTT into a blue/purple formazan salt/precipitate (Mosmann 1983). During the MTT assay, the tissue sample is placed into a suitable MTT solution (concentration of 0.3 to 1 mg/ml) for a period of 3 h. Isopropanol (or other solvents with the same characteristics) is then used to remove the precipitate from the tissue. The precipitated MTT formazan can be measured by standard absorbance measurements (optical density, OD) or an HPLC/UPLC-

spectrophotometry procedure (OECD 2015b). Negative and positive controls should be included and the results generated by these controls must be reproducible (OECD 2015b).

Each test run must include a minimum of three replicates for every chemical tested as well as each control. With regard to both liquid and solid test chemicals, adequate amounts of the test chemical must evenly be applied in order to cover the epidermis surface (range of 26-83 µl/cm^2 or mg/cm^2). Nevertheless, solid chemicals require that the *epidermis* surface be dampened with either de-ionized or distilled water prior to the chemical being applied (OECD 2015b). This enhances chemical and epidermal surface contact. Once testing is completed, the test chemical must be washed off with care using aqueous buffer or 0.9% sodium chloride (NaCl). The duration of chemical exposure and incubation temperatures differ depending on the RhE test method employed with exposure times ranging between 15 and 60 min and temperatures of between 20 and 37°C. Optimization of these parameters can be done based on the fundamental properties of the test methods (OECD 2015b).

The classification of a chemical as being an irritant is done by assessing the potential of the chemical to reduce cell viability below the outlined threshold levels (i.e. ≤ 50%, for UN GHS Category 2). However, chemicals can also generate cell viabilities that are over the stated threshold levels, in this case, they can be regarded as non-irritants (i.e. > 50%, No Category) (OECD 2015b). The RhE models must not contain any bacteria, viruses, mycoplasma or fungi and should be able to represent *in vivo* conditions (OECD 2015b).

2.1.2.4.2 *Data reporting*

The following information should be included: OD values; tissue/cell viabilities of the test chemical; experiments that were repeated (if any) and mean ± SD for every run. Any

observations made must be documented (OECD 2015b). Information on the test chemical and control substances must also be included. The information should pertain to physical and chemical properties such as chemical identification, purity and physical appearance. The storage conditions and type of RhE models should also be indicated (OECD 2015b).

2.2 Drug resistance in *Staphylococcus aureus* and use of medicinal plants

2.2.1 Introduction

Staphylococcus aureus (*S. aureus*) is a Gram-positive coccus that belongs to the Staphylococcaceae family. It is a normal flora of the skin and mucosa but has the ability to colonize other parts of the body through cuts and abrasions causing various illnesses as a result of its virulence factors (Stefani, Chung *et al.* 2012). The various diseases associated with *S. aureus* infections range from mild skin infections to more severe and life-threatening conditions, such as osteomyelitis, endocarditis, and sepsis (Azeez-Akande 2010). With the growing trend of multi-resistant strains, antimicrobial therapy against *S. aureus* infections has become an increasing challenge (Reiß, Pané-Farré *et al.* 2012). The ability of bacteria to develop resistance to a wide range of antibiotics has led to various studies being carried out to evaluate alternative sources of antimicrobial agents (Fischbach and Walsh 2009). These alternative sources include natural plant products, which are generating a tremendous amount of interest due to their potential to cure a number of diseases and conditions. Numerous studies have been carried out that outline these various abilities (Payne, Gwynn *et al.* 2007; Amenu 2014; Farooqui, Khan *et al.* 2015; Haroun and Al-Kayali 2016).

Drug resistant bacteria contribute to the deaths of millions of people worldwide. Methicillin-resistant *Staphylococcus aureus* (MRSA) is one such bacterium that is of major concern. MRSA

is the pathogen usually associated with both hospital-acquired (HA-MRSA) and community-acquired (CA-MRSA) infections (Deleo, Otto *et al.* 2010). It has been established that MRSA is resistant to approximately 23 antibiotics and is one of the leading causes of death in the elderly and immunocompromised patients (Dubey, Rath *et al.* 2012). It causes a wide variety of infections which include extensive deep tissue (arthritis, osteomyelitis and renal and breast abscesses) and skin and soft tissue (carbuncles and furuncles) infections (Daniyan, Galadima *et al.* 2011).

Three main mechanisms are responsible for antimicrobial resistance and these are; enzymatic inactivation of the drug (Davies 1994) target site modification (Spratt 1994) and drug extrusion by efflux which is facilitated by efflux pump genes that code for efflux pump proteins. These proteins are responsible for the extrusion of antibiotics, thereby allowing the pathogens to evade the effect of the antimicrobial agents (Lomovskaya and Bostian 2006).

2.2.2 Global prevalence and incidence rates of MRSA

MRSA strains were initially identified in the United Kingdom (UK) in 1961. This was after they developed resistance to methicillin following its introduction (Jevons, 1961). Since then, MRSA epidemics and endemics have been reported worldwide (Ayliffe 1997; Chambers 2001; Fridkin, Hageman *et al.* 2003). This eventually led to the assumption that *S. aureus* epidemiology was changing (Boyce 1998; Herold, Immergluck *et al.* 1998). MRSA infections are causing problems for hospitals and healthcare facilities as well as numerous communities worldwide (Bratu, Eramo *et al.* 2005; Kuehnert, Hill *et al.* 2005; Mulvey, MacDougall *et al.* 2005). Reports from different countries around the world indicated that there was an increase in the prevalence of MRSA infections and population risk. Data obtained from North America as well as the Centers for

Disease Control and Prevention (CDC), in the United States of America (USA), showed that the incidence of MRSA has increased over the years (Hughes 1987; Garner, Jarvis *et al.* 1988; Horan, Culver *et al.* 1988; Mulvey, MacDougall *et al.* 2005; Azeez-Akande 2010). Additional studies carried out at different hospitals in USA revealed that the prevalence of MRSA rose from 6% in 1998 to 50% in 2002 (Azeez-Akande 2010).

Data published from other countries has also shown an increase in the prevalence of MRSA infections. In hospital wards in France, for instance, a study carried out revealed that the prevalence of MRSA was 33% - 62% (in relation to *S. aureus* isolates) (Mangeney, Bakkouch *et al.* 1995). Studies carried out in Taiwan indicated a rise in MRSA prevalence from 1981-1986 (Hsueh, Chen *et al.* 2002; Hsueh, Liu *et al.* 2002). Other studies carried out in Japan (Lotus, Imamura *et al.* 1995) and the Republic of Korea (Woojoo and Seunchill 1999) have demonstrated MRSA prevalence of 54% and 70%, respectively. However, reports on MRSA prevalence in Africa have been very few. Nonetheless, in Sudan, a study conducted at a Khartoum hospital revealed a prevalence of 11% (Musa, Shears *et al.* 1999). Studies carried out between 1996 and 1997 in Nigeria, Cameroon, Kenya and Algeria showed MRSA prevalence rates of 21 -30% (Nigerian, Cameroon and Kenya) while Algeria had a lower rate of less than 10% (Kesah, Ben Redjeb *et al.* 2003).

Other studies have also reported increased MRSA incidence in Ghana (Odonkor, Newman *et al.* 2012), Ethiopia (Geyid and Lemeneh 1991), Kenya (Omari, Malonza *et al.* 1997), Nigeria (Rotimi, Orebamjo *et al.* 1987; Okesola, Oni *et al.* 1999), Senegal (Sow, Wade *et al.* 1998), South Africa and Sudan (van den Ende and Rotter 1986; Peddie, Donald *et al.* 1988; Gardee and Kirby 1993; Musa, Shears *et al.* 1999).

2.2.3 Treatment of *S. aureus* infections

Penicillin was usually the drug of choice in the treatment of *S. aureus* infection following its discovery. Unfortunately, resistance to penicillin was reported in many countries following its introduction as early as 1942. In 1944, Kirby reported penicillinase-producing strains (Kirby 1944). Oxacillin or flucloxacillin have been used in first-line therapy and are penicillinase-resistant beta-lactam antibiotics. These drugs were given in combination with gentamicin to treat serious infections such as endocarditis (Korzeniowski and Sande 1982; Bayer, Bolger *et al.* 1998) . However, the use of gentamicin posed some controversy as its use lead to kidney damage (Cosgrove, Vigliani *et al.* 2009). Antimicrobial susceptibility testing, severity and site of infection normally determine the duration and type of treatment administered (Bamberger and Boyd 2005). Drugs such as intravenous nafcillin, oxacillin (Bactocill) and oral dicloxacillin (Dynapen) have been administered to patients that are not allergic to penicillin (Bamberger and Boyd 2005). Cephalosporins such as cephalexin (Keflex) and intravenous cefazolin (Ancef) have also been given as substitutes.

Vancomycin is considered to be the best treatment option in MRSA infections. However, it has been shown that the use of vancomycin has a down side. This is because its absorption in the gastrointestinal tract is rather poor, it has slow bactericidal activity and also has numerous side effects (Levine, Fromm *et al.* 1991; Gould 2008; Rasmussen, Fowler *et al.* 2011). Vancomycin is administered intravenously due to the low absorption experienced when administered orally. Nonetheless, treatment failure has been reported and some studies have actually shown that treatment with β-lactams was more beneficial compared to treatment with vancomycin (Chang, Peacock *et al.* 2003; Stryjewski, Szczech *et al.* 2007).

Resistance mechanism of *S. aureus* Teicoplanin, a glycopeptide, is another drug that has been used to treat *S. aureus* infections and is administered intravenously or intramuscularly. A number of studies have revealed that its effectiveness is similar to that of vancomycin (Rolston, Nguyen *et al.* 1994; Yalaz, Cetin *et al.* 2004). Other drugs such as tigecycline, linezolid (Zyvox), daptomycin (Cubicin) and telavancin have also been used in treating *S. aureus* infections. Tigecycline is used in the treatment of complex skin infections and has bacteriostatic properties (Rasmussen, Fowler *et al.* 2011). Linezolid is administered orally or intravenously and has been used to treat pneumonia and skin and soft tissue infections caused by *S. aureus* strains (Lin, Zhang *et al.* 2008; Tascini, Gemignani *et al.* 2009). In addition, it has demonstrated to have bacteriostactic activities (Bamberger and Boyd 2005). Daptomycin has shown bactericidal properties in "*in vitro*" studies and is used to treat complicated skin and soft tissue infections (Arbeit, Maki *et al.* 2004) and is administered intravenously (Bamberger and Boyd 2005). The FDA approved the use of telavancin for the treatment of infections of the skin and skin-structures (Rasmussen, Fowler *et al.* 2011).

2.2.4 Resistance mechanism of *S. aureus*

Antimicrobial agents have been used to treat various bacterial infections since their discovery, and it was believed that they would contribute to the purge of these infections. Nevertheless, diseases considered to have been eradicated or controlled in the past are recurring as a result of drug resistance (Levy and Marshall 2004). The emergence of multi-drug resistant bacteria is affecting the effectiveness of existing drugs and thus inhibiting the proper and efficient treatment of these infections worldwide. This in turn increases both medical and socio-economic expenses (Maranan, Moreira *et al.* 1997; Carbon 1999; Bratu, Eramo *et al.* 2005). Resistance to new

antimicrobial agents is rapid and widespread, indicating that this would be the trend even with the new families of antimicrobial agents, thereby limiting their shelf life and effectiveness (Coates, Hu *et al.* 2002).

Antimicrobial resistance results from three main strategies which include enzymatic inactivation of the drug (Davies 1994), target site modification (Spratt 1994), and drug extrusion by efflux (Lomovskaya and Bostian 2006). During target site modification, chemical modifications of the antibiotic target site decrease the affinity of the antibiotic to its binding site (Lambert 2005). In enzymatic inactivation, hydrolytic and transferase enzymes render the antibiotic inactive by degrading the antibiotic, as well as modifying it through acetylation, adenylation or phosphorylation (Over, Gur *et al.* 2001). Efflux pump proteins, coded for by efflux pump genes, are responsible for the extrusion of antibiotics, thus allowing the pathogens to evade antimicrobial effects (Lomovskaya and Bostian 2006).

2.2.4.1 *Methicillin resistance*

Methicillin was introduced in 1961 but methicillin-resistance by some isolates followed shortly after its introduction (Jevons 1961). This resulted in limited action and reduced effectiveness of therapeutic agents against infections caused by methicillin-resistant isolates (Cosgrove, Sakoulas *et al.* 2003). The *mecA* gene is responsible for conferring methicillin resistance (Katayama, Ito *et al.* 2000). Methicillin-sensitive *Staphylococcus aureus* (MSSA) can become methicillin-resistant by acquiring the *mecA* gene, which is the methicillin-resistance determinant (Hiramatsu, Cui *et al.* 2001). The mortality rate from severe MRSA infection was found to be as high as 10-34% (Tumbarello, de Gaetano Donati *et al.* 2002).

2.2.4.2 *β-lactam resistance*

β-lactams include broad-spectrum penicillins, cephalosporins, monobactams and carbapenems. The mechanism of antimicrobial resistance by a large number of bacteria is through the degradation of the antibiotics by means of chromosomal-or-plasmid encoded β-lactamases (Bush, Calmon *et al.* 1995). Resistance to carbapenems is achieved by mutations of genes that code for porin OprD proteins which are the main route of antibiotic uptake (Trias and Nikaido 1990). Resistance to beta- lactam antibiotics is believed to occur in two ways; (i) by the production of an enzyme beta- lactamase, which subsequently causes inactivation of the beta- lactam antibiotics and (ii) by the expression of transpeptidases, which are resistant to antibiotic activity. The *mecA* gene, which codes for the penicillin-binding protein PBP2a, is responsible for the antibiotic resistance exhibited by *S. aureus* (Archer and Climo 2001; Finan, Archer *et al.* 2001; McKinney, Sharma *et al.* 2001).

Penicillin was introduced in the early 1940s. Nevertheless, penicillin-resistant staphylococcal strains were detected in both hospitals and communities shortly after its introduction (Rammelkamp and Maxon 1942). As the late 1960s approached, over 80% of both hospital-acquired and community-acquired staphylococcal isolates were penicillin-resistant. This trend of resistance, initially appearing in hospitals and then extending to the community, is a pattern now evident with new antimicrobial agents (Chambers 2001)

2.2.4.3 *Tetracycline resistance*

Tetracycline has been widely used in the treatment of both Gram-negative and Gram-positive bacterial infections, as well as mycoplasma, rickettsiae, and protozoan parasites (Chopra, Lacey *et al.* 1974; Chopra 1975; Markham and Neyfakh 2001). Apart from its therapeutic use in

humans and animals, tetracycline has been erratically used in animal husbandry for growth promotion, leading to extensive tetracycline resistance in both Gram-negative and Gram-positive bacteria (including *S. aureus*). Tetracycline resistance is achieved by two main mechanisms which are active efflux by tetracycline-specific pumps/transporters called Tet-pumps (Markham and Neyfakh 2001), and ribosomal protection (Schnappinger and Hillen 1996). Tet-pumps are generally spread among Gram-negative and Gram-positive bacteria (Roberts 1994).

2.2.4.4 *Aminoglycoside resistance*

The mode of action of aminoglycosides is the inhibition of protein synthesis by binding to the bacterial ribosomes, thereby resulting in their death. This action is mediated by protonated amine and/or hydroxyl interactions with the ribosomal RNA of the bacterial 30S ribosomal subunit (Lomovskaya and Watkins 2001). Nonetheless, the need to identify other compounds that are capable of resisting modification by aminoglycoside modifying enzymes is essential in order to improve the action of these therapeutic agents (Miller, Sabatelli *et al.* 1997). Aminoglycoside resistance is believed to be as a result of two main mechanisms which are: specific enzymatic inactivation and by modifying enzyme resistance impermeability. These are considered to be the most common mechanisms by which resistance occurs (Lomovskaya and Watkins 2001).

Ribosomal mutations and active efflux of the drug out of the bacteria also contribute to aminoglycoside resistance (Macmaster, Zelinskaya *et al.* 2010). These enzymes exert their function by covalently attaching either a phosphate, nucleotide or acetyl moiety to either the amine and/or the alcohol key functional group of the antibiotic. This causes a reduction in the ribosomal binding affinity due to the change in charge. ANT (4') IA aminoglycoside adenylytransferase 4' IA is one of the aminoglycoside modifying enzymes found in *S. aureus*

(Porter, Green *et al.* 2010; Revuelta, Corzana *et al.* 2010). It attaches an adenyl moiety to 4'hydroxyl group of a number of aminoglycosides such as kamamycin and gentamicin.

In staphylococcal species, it has been found that these bacteria tend to possess numerous aminoglycoside modifying enzymes that lead to multiple genotypes and complex phenotypes (Miller, Sabatelli *et al.* 1997). Aminoglycosides were some of the very few antimicrobial agents that were not affected by the active extrusion brought about by multidrug resistance pumps (MDR). Unfortunately, that is currently not the case (Moore, DeShazer *et al.* 1999).

2.2.4.5 *Quinolone resistance*

When first introduced in the 1980s, the intended use of fluoroquinolones was to treat Gram-negative bacterial infections. However, because of their ability to treat Gram-positive bacterial infections, they were also employed in the treatment of infections brought about by staphylococcal species (Gootz and Brighty 1996). Unfortunately, quinolone-resistance rapidly emerged especially among methicillin-resistant strains, thus decreasing their use in treatment of staphylococcal infections (Hopper 2002).

Fluoroquinolones exert their antimicrobial effect by inhibiting the activity of type II topoisomerases, DNA gyrase (encoded by the *gyrA* and *gyrB* genes) and DNA topoisomerase IV [encoded by the *parC* (*grlA* in *S. aureus*) and *parE* (*grlB* in *S. aureus*) genes] (Yoon, Lee *et al.* 2010). DNA gyrase is responsible for the introduction of negative superhelical twists into bacterial DNA, which is essential for the instigation of DNA replication, as well as aid the binding of initiation proteins (Hopper 2002). Topoisomerase IV is essential in ensuring the separation of daughter cells after DNA replication by removing the interlinking chromosomes (Hopper 2002).

Resistance to fluoroquinolones is achieved by chromosomal mutations of topoisomerase IV or DNA gyrase as well as the action of a multidrug efflux pump (Ng, Trucksis *et al.* 1996; Hopper 2002). Alterations in amino acid sequences in vital regions of the enzyme-DNA complex (quinolone resistance determining region [QRDR]) consequently result in the reduction of quinolone affinity for both targets thus conferring resistance (Ng, Trucksis *et al.* 1996; Hopper 2002). Both single and multiple mutations in amino acid sequence play a role fluoroquinolone resistance. Nonetheless, complex amino acid mutations are essential for fluoroquinolone-resistance to occur (Ng, Trucksis *et al.* 1996; Hopper 2002).

2.2.4.6 *Vancomycin resistance*

Following the increase in MRSA infections, the use of vancomycin to treat these infections subsequently increased, thereby influencing the emergence of vancomycin-resistant staphylococci (Kirst, Thompson *et al.* 1998). The first report of vancomycin-resistant staphylococci was from a clinical isolate of a *Staphylococcus hemolyticus* strain (Schwalbe, Stapleton *et al.* 1987) and this was later followed by reports of vancomycin-intermediate-resistant *S. aureus* (VISA), in 1997 in Japan (Hiramatsu, Aritaka *et al.* 1997a). More cases of VISA were then reported in other countries (Hiramatsu, Hanaki *et al.* 1997b; Smith, Pearson *et al.* 1999). Reports of vancomycin-resistant *S. aureus* infections raised a tremendous amount of concern as this demonstrated complete bacterial resistance to vancomycin as well as an unusual mode of distribution (Hageman, Pegues *et al.* 2001; Ward, Johnson *et al.* 2001). It has been established that VISA result from *S. aureus* parent strains that are susceptible to vancomycin due to persistent infection (Sieradzki, Roberts *et al.* 1999; Smith, Pearson *et al.* 1999; Moore, Perdreau-Remington *et al.* 2003). Intermediate resistance to vancomycin is thought to occur

through sequential point mutations of major staphylococcal regulatory genes (Mwangi, Wu *et al.* 2007; Cui, Neoh *et al.* 2009; Cui, Li *et al.* 2009).

Resistance to glycopeptides is mediated via the acquisition of the *vanA* gene. This gene codes for an enzyme that produces an alternative peptidoglycan to which vancomycin will not bind (Showsh, De Boever *et al.* 2001). It has been suggested that alterations in peptidoglycan biosynthesis appears to play a role in lowering vancomycin susceptibility (Hiramatsu, Hanaki *et al.* 1997). A change in peptidoglycan biosynthesis also leads to the reduction of cross-linkages formed between peptidoglycan strands, consequently leading to more D-Ala-D-Ala residues being revealed (Hanaki, Kuwahara-Arai *et al.* 1998; Hanaki, Labischinski *et al.* 1998a; Hanaki, Labischinski *et al.* 1998b). The distorted cross-linking is as a result of a reduction in the levels of L-glutamine available for amidation of D-glutamate in the pentapeptide bridge (Walsh and Howe 2002). The increase in the amount of D-Ala-D-Ala residues results in the binding and trapping of vancomycin which further prevents drug molecules from reaching their target sites, thus rendering the bacteria vancomycin-resistant (Sieradzki, Roberts *et al.* 1999; Avison, Bennett *et al.* 2002).

2.2.5 The effect of medicinal plants on *S. aureus*

The use of medicinal plants in the treatment of various diseases has been documented and several studies have been conducted and are still being conducted on the effect of medicinal plants on microbes. Results from a study conducted to evaluate the antimicrobial potential of four selected plants namely *Aloe secundiflora*, *Bulbine frutescens*, *Tagetes minuta* and *Vernonia lasiopus* against *S. aureus* revealed that they all exhibited antimicrobial activity against *S. aureus*. These results further indicated that these plant extracts could be effective against other Gram

positive pathogens (Rachuonyo, Ogola *et al.* 2016). Another study carried out to assess the effect of methanolic extracts of 12 medicinal plants traditionally used for medicinal purposes in Cameroon against 11 *S. aureus* clinical isolates demonstrated that 4 out of the 12 plant extracts exhibited the greatest antimicrobial activity against the *S. aureus* isolates. These medicinal plants were *Dacryodes edulis* (showed considerable antimicrobial activity against all the isolates), *Occimum gratissimum* (showed considerable activity against 9 out of 11 isolates), *Commelina erecta* and *Spilanthes filicaulis* (both showed considerable activity against 6 out of 11 isolates) (Sama Fonkeng, Mouokeu *et al.* 2015). Further evaluation of another set of plant species against *S. aureus* revealed that they possessed antimicrobial activity against this bacterium. The medicinal plants that showed the greatest antimicrobial activity were *Salvia officinalis, Eucalyptus globulus, Coleus forskohlii, Coptis chinensis, Turnera diffusa*, and *Larrea tridentata* which had MIC values between 60 to 300 µg/ml. The results also showed that these extracts reduced bacterial replication by 10^6 fold (Snowden, Harrington *et al.* 2014).

2.3 Drug resistance in *Candida* species and use of medicinal plants

2.3.1 Introduction

A large number of fungal infections worldwide are caused by yeasts known as *Candida* (Manolakaki, Velmahos *et al.* 2010). *Candida* species are usually found as normal flora in humans particularly in areas such as the skin, digestive tract and as well as on the genitals. They become pathogenic once they enter the bloodstream when the mucosal membrane is injured or when the immune system is weakened (Kourkoumpetis, Velmahos *et al.* 2011). Most of these infections are caused by *Candida albicans* species which cause candidiasis. It has been observed that administrating broad spectrum antibiotics contributes to increased yeast infections, enhances

the growth of *Candida* species in the gastrointestinal tract and enables them to penetrate the gastrointestinal mucosa (Kennedy, Volz *et al.* 1987). In addition to the use of broad spectrum antibiotics, using medical devices such as intravenous catheters, undergoing invasive procedures, administering cytotoxic chemotherapeutic agents and receiving organ transplants give rise to increased *Candida* infections (Ortega, Marco *et al.* 2011). The ability of *Candida* species to cause invasive infections is due to specific virulence factors which include evading the host immune system and ability to form biofilms on medical devices and on tissue surfaces. *Candida* species also produce hydrolytic enzymes that damage the host tissues and play a role in disease causation. Examples of these enzymes include haemolysin, proteases and phospholipases (Silva, Negri *et al.* 2011).

Candida species form huge, round, white or cream colored colonies when grown on agar medium at room temperature. As they grown, they produce a yeast smell. *C. albicans* has the ability to break down glucose and maltose into acids and gases but cannot break down lactose. This enables it to be differentiated from other *Candida* species. The rapid growth of *Candida* species, such as *C. albicans*, can result in infections like oropharyngeal candidiasis (thrush) as well as vulvovaginal candidiasis (vaginal candidiasis). Oral thrush is normally seen in old people that wear dentures (Darwazeh, Lamey *et al.* 1990). In patients with a weakened immune system, systemic diseases like abscesses, thrombophlebitis (swelling of the vein walls resulting from blood clots) and endocarditis (inflammation of the inner lining of the heart chambers) and osteomyelitis may develop (Pappas 2006). *Candida* species can also cause infections such as nail fungus, athlete's foot, vaginal yeast infections, jock itch (an infection of the groin) and diaper rash. Vaginal yeast infections are frequently associated with both healthy individuals and those with a weakened immune system whereas oral *Candida* infections are mostly common in

individuals with a weakened immune system. Common symptoms of vulvovaginal candidiasis include vaginal itching, white vaginal discharge, pain during sex or when urinating and redness and swelling of the vulva (Fidel 2002). *C. albicans* is usually present in the mouth of adults and children. However, infection can result in infants, the elderly and immunocompromised individuals. Oral *Candida* infections cause a burden on the healthcare sector as they are seen in both HIV-infected and AIDS patients. It is believed that 90% of people living with AIDS will develop oral thrush. Oral thrush present as white sores in the mouth, tongue, cheeks and gums. It is also associated with pain when a person attempts to swallow (Saunus, Kazoullis *et al.* 2008). *Candida* infections are also commonly associated with patients suffering from cancer, renal failure, neutropenia and diabetes mellitus. Infection with *Candida* species can also occur as a result of abdominal surgery (Pfaller and Diekema 2007).

2.3.2 Epidemiology of *Candida* species

It has been established that there are over 150 *Candida* species and approximately 15 are known to cause infections in humans. These disease causing *Candida* species include *Candida albicans*, *Candida glabrata*, *Candida tropicalis*, *Candida parapsilosis*, *Candida krusei*, *Candida guilliermondii*, *Candida lusitaniae*, *Candida dubliniensis*, *Candida pelliculosa*, *Candida kefyr*, *Candida lipolytica*, *Candida famata*, *Candida inconspicua*, *Candida rugosa*, and *Candida norvegensis*. However, the frequently isolated pathogens are *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, and *C. krusei*. These have been found in 95% of patients with known *Candida* infections (Diekema, Arbefeville *et al.* 2012). A large number of bloodstream *Candida* infections are caused by *C. albicans* which is predominately present in the upper and lower respiratory tracts, gastrointestinal tract, genitourinary system and on the skin (Pfaller and Diekema 2007). *C. albicans* infections are associated with increased medical costs, prolonged

hospital stay and high death rates which are major health problems (Lai, Wang *et al.* 2012). However, it has been observed that infections arising from other *Candida* species are on the rise. These infections are based on the age of patients, period and state of hospital environment as well as other diseases the patient may have (Yapar 2014). These non-*albicans* species include *C. glabrata*, *C. parapsilosis*, *C. tropicalis* and *C. krusei*. A study conducted in major medical facilities in North America revealed that despite *C. albicans* being the main isolate obtained from patients, *C. glabrata* was the second most commonly isolated non-*albicans* species. The high incidence rates may be attributed to the increased use of broad spectrum antibiotics, weakened immune systems and an increase in age (Horn, Neofytos *et al.* 2009). As such, *C. glabrata* infections are frequent in elderly patients and patients with neoplasm. *C. parapsilosis* is associated with candidemia in newborn babies and adults where it accounts for 30% and 10-15% of cases, respectively. Since it is present on the skin, it causes infections in patients using catheters which may result in outbreaks. *C. tropicalis* infections are common in patients with leukemia and neutropenia. *C. krusei* infections are seen in patients receiving hematopoietic stem cells as well as leukemia patients with low neutrophil levels (Pappas 2006; Pfaller and Diekema 2007).

A study conducted on isolates obtained from South African patients revealed that the percentage of *Candida* species isolated were 81% *C. albicans*, 10% *C. glabrata*, 4% *C. parapsilosis*, 3% *C. krusei* and 1% *C. tropicalis* (Pfaller and Diekema 2004). Another study carried out in Bloemfontein, South Africa demonstrated that the percentage of the isolated *Candida* species were 42% *C. albicans*, 26% *C. tropicalis*, 20% *C. parapsilosis* and 7% *C. glabrata* (Badenhorst, Botha *et al.* 1991). The two studies collected isolated from both children and adults. Results from a third hospital-based study conducted in Soweto, South Africa (between distinct time points) in

1990-2007 revealed that in 1990, the percent distribution of *Candida* species was 62% *C. albicans* and 23% *C. tropicalis*. Minimal differences in percentage distribution were observed between 1998 and 2002. The percent distribution between 2005 and 2007 was 46% *C. albicans*, 25% *C. parapsilosis* and 23% *C. glabrata*. The incidence rates of these species were attributed to a number of factors such as abdominal surgery which accounted for 43% of infections, HIV (19%), trauma (16%), diabetes mellitus (12%) and cancer (8%) (Kreusch and Karstaedt 2013). In Europe, the *Candida* species that cause candidemia are *C. albicans* which accounts for over 50% of infections, *C. glabrata* and *C. parapsilosis* (14% each), *C. tropicalis* (7%) and *C. krusei* (2%) (Tortorano, Kibbler *et al.* 2006). In Chile, the incidence of non-*albicans* species is on the rise. The frequently isolated species was *C. parapsilosis* with *C. tropicalis* and *C. glabrata* being the second most isolated species. These species were responsive to amphotericin B treatment whereas 50% of *C. glabrata* species did not respond to fluconazole treatment (Ajenjo, Aquevedo *et al.* 2011). In Brazil, *C. albicans* caused 40.9% of infections with *C. tropicalis* causing 20.9%, *C. parapsilosis* causing 20.5% and *C. glabrata* causing 4.9% of infections (Nucci, Queiroz-Telles *et al.* 2010). A study conducted in USA, particularly Atlanta and Baltimore, between 2008 and 2011 showed that *Candida* infections occurred in 13.3 per 100 000 people in Atlanta with 26.2 per 100 000 people in Baltimore (Cleveland, Farley *et al.* 2012; Lockhart, Iqbal *et al.* 2012).

2.3.3 Treatment of *Candida* infections and classification of antifungal agents

Fungal infections pose tremendous healthcare challenges worldwide and account for approximately 1.5 million deaths annually (Brown, Denning *et al.* 2012; Pianalto and Alspaugh 2016). *Candida* species are normally associated with infections of the mucosal membrane. Developing antifungal drugs is not as easy as developing antibacterial drugs. This is because fungi are eukaryotic in nature and therefore possess target sites that are similar to those found in

humans. As a result, toxicity may arise which might be an unwanted side effect of antifungal treatment (Roemer and Krysan 2014; Denning and Bromley 2015). There are four main groups of antifungal drugs that are used to treat fungal infections and these include azoles, polyenes, echinocandins and pyrimidine analogs. They can be administered through the oral, topical or intravenous route (Denning and Bromley 2015). However, antifungal resistance has prompted the development of new antifungal agents that aim at targeting fungal proteins, lipids and cell wall synthesis in order for them to exert their action (Perfect 2016). Antifungal agents are categorized into various groups based on their mode of action as follows:

2.3.3.1 *Inhibitors of ergosterol biosynthesis*

Ergosterol is the main constituent found in the cell walls of fungi and plays a role in ensuring that the fluidity and structure of the cell membrane is maintained. It also enables membrane-bound enzymes to function properly (Campoy and Adrio 2016).

2.3.3.1.1 *Azoles*

This is the main class of antifungal agents and approximately 20 drugs are available on the market. These can either be applied to the skin or administered in the treatment of more serious infections. They function by inhibiting the action of a cytochrome P450-dependent enzyme, 14α-lanosterol demethylase (CYP51), responsible for the biosynthesis of ergosterol from lanosterol thereby affecting the growth and replication cycle of fungal cells (Sheehan, Hitchcock *et al.* 1999; Carrillo-Munoz, Giusiano *et al.* 2006). Azoles can be subdivided into two groups namely imidazoles and triazoles. Imidazoles include clotrimazole, miconazole, and ketoconazole. Miconazole and clotrimazole were the earliest drugs to be included in this class but oral absorption was impossible. For this reason, ketoconazole was developed in 1984 which was

easily absorbed when administered orally (Nozawa 1985; Shukla, Singh *et al.* 2016). Nonetheless, the complications associated with this class of azoles such as high toxicity, relentless side effects and several drug interactions led to the introduction of triazoles to replace the imidazole class (Pardasani 2000; Pappas, Rex *et al.* 2004).

Itraconazole and fluconazole were the earliest triazoles to be introduced and were more effective options, had reduced toxicity and were effectively absorbed orally when used to treat systemic infections in comparison to the imidazoles (Pardasani 2000; Pappas, Rex *et al.* 2004). Unfortunately, more fungal species are increasingly becoming resistant to the azole drugs and this is attributed to them being fungistatic as opposed to being fungicidal (Shukla, Singh *et al.* 2016). Voriconazole and posaconazole are also triazoles that are used to treat fungal infections and were introduced due to the limitations arising from the use of the other triazole drugs. Their use was endorsed by the Food and Drug Administration (FDA) in 2002 for voriconazole and 2006 for posaconazole (Zaragoza and Pemán 2008; George, Jose *et al.* 2009). In 2014, the FDA approved another drug called Efinaconazole to be applied topically to treat fungal infections caused by various fungal species including *Candida* (Peyton, Gallagher *et al.* 2015).

2.3.3.1.2 *Allylamines*

Allylamines are antifungal agents that are fungicidal in nature and also function in inhibiting the production of ergosterol. They inhibit the action of the enzyme squalene epoxidase (ERG1) which facilitates the generation of 2,3-squalene epoxide from squalene leading to the buildup of squalene. This buildup enhances permeability thus causing alterations in the integrity of cellular components (Vincent 2000; Denning and Hope 2010). This group comprises terbinafine and naftifine (Abdel-Kader and Muharram 2017). Terbinafine is extensively used to treat nail

infections and is effective against infections caused by *Aspergillus*, *Fusarium* as well as other filamentous fungi (Ghannoum 1997; Vincent 2000). Naftifine is used in the treatment of infections caused by *Trichophyton*, *Epidermophyton*, *Microsporum* and *Candida* species. It has been shown to be fungistatic against *Candida* species (Ghannoum, Isham *et al.* 2013).

2.3.3.2 *Fungal cell membrane disruptors*

Disruption of the cell membrane by antifungal agents is achieved by their interaction with ergosterol (a steroid present in fungi). Polyenes are antifungal agents with the ability to interact with ergosterol. They have an amphiphilic structure which enables them to attach to the lipid bilayer and consequently produce complexes with ergosterol thus creating poles in the membrane and making it permeable. The structure of ergosterol is similar to that of cholesterol found in mammalian cell membranes. Therefore, antifungal agents that target ergosterol may have a damaging effect on mammalian cells and tissues as they cause the cellular components to leak out subsequently result in cell death (Hossain and Ghannoum 2000; Hossain and Ghannoum 2001; Andes 2003). Initially, polyenes were the drugs of choice for the treatment of fungal infections. They are more effective in the treatment of a large number of fungal infections compared to other antifungal drugs and they are fungicidal. Examples of polyenes include nystatin, natamycin and amphotericin B (Lalitha, Kumar *et al.* 2008; Sklenár, Scigel *et al.* 2013).

Nystatin and natamycin are used in the treatment of infection caused by *Cryptococcus*, *Candida*, *Aspergillus* and *Fusarium*. Nystatin is administered when treating cutaneous, vaginal and esophageal candidiasis. Natamycin is incorporated when treating fungal keratosis or corneal infections (Zotchev 2003). Amphotericin B is used in the treatment of the majority of infections caused by yeasts and filamentous fungi such as *Candida*, *Aspergillus*, *Fusarium*, *Mucor*,

Scedosporium and *Cryptococcus* (Laniado-Laborín and Cabrales-Vargas 2009). Polyenes are able to bind to cholesterol which results in increased toxicity accompanied by several side effects (Lemke, Kiderlen *et al.* 2005). Nystatin and natamycin are applied topically because they are not easily absorbed in the gut and they are associated with high toxicity. Amphotericin B is reserved for treating severe infections, such as systemic infections, with the potential to cause death. It is administered intravenously because it is not easily absorbed through the gastrointestinal tract and this can be harmful to the host cell membrane as it can bind to cholesterol. However, it can cause damage to the liver and kidneys resulting in serious side effects (Odds, Brown *et al.* 2003).

2.3.3.3 *Inhibitors of fungal cell wall synthesis*

Glucans are polysaccharides made up of $_{D}$-glucose units that are linked by β-(1,3) or β-(1,6)-glucan connections. They are normally found in cells walls of yeasts and fungi (Lorand and Kocsis 2007). The cell wall is predominately made up of β-(1,3)-$_{D}$-Glucan and is the attachment point or structure for chitins and glycoproteins. It also plays an important role in ensuring that the cell wall is strong and stable. Echinocandins are antifungal agents that target the cell wall by inhibiting the synthesis of β-glucan. The drugs that belong to this group include caspofungin, micafungin and anidulafungin (Sucher, Chahine *et al.* 2009; Mukherjee, Sheehan *et al.* 2011). These drugs exert their function by inhibiting the action of β -(1,3)-$_{D}$-glucan synthase leading to the an alteration in the structure of the cell wall which results in cell death (Eschenauer, DePestel *et al.* 2007). Absorption of these drugs in the gastrointestinal tract is quite poor due to the fact that they have high molecular weights. They exhibit minimal toxicity as they target components that are not found in mammalian cells and they also do not normally interact with other drugs. Unfortunately, their half-life is short and this affects their use in the hospital environment as they have to be given to patients once every day through intravenous injections (Petrikkos and Skiada

2007; Pfaller, Messer *et al.* 2016). Caspofungin, micafungin and anidulafungin have been shown to be effective in the treatment of *Candida*, demonstrating fungicidal activities, as well as in the treatment of infections caused by *Aspergillus*, where they demonstrate fungistatic activities. Micafungin is given through intravenous injection and is administered to patients with candidemia, acute disseminated candidiasis, *Candida* peritonitis, abscesses and esophageal candidiasis. Anidulafungin is also administered intravenously to patients with fungal infections in the abdomen or stomach, blood and esophagus. It is very efficient in the treatment of *Candida* infections that have become resistant to fluconazole. Caspofungin is administered as a parenteral injection and is used to treat invasive candidiasis and invasive aspergillosis (Arevalo, Carillo-Munoz *et al.* 2003; Akins 2005; Gershkovich, Wasan *et al.* 2009).

2.3.3.4 *Inhibitors of sphingolipids biosynthesis*

Sphingolipids are found in large numbers in the cell membranes of eukaryotes. They have numerous functions in fungal cells and they also aid in enabling the fungi become pathogenic. Sphingolipids play a role in maintaining the structural integrity of the fungal cell membrane as well as the cell signaling pathway (Rollin-Pinheiro, Singh *et al.* 2016). Studies have shown that inhibiting enzymes responsible for the synthesis of sphingolipids makes the fungi less virulent. Aureobasidin A is an example of a sphingolipid biosynthesis inhibitor and can be used in the treatment of *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *C. albicans*, *C. glabrata*, *A. nidulans*, *A. niger* and *C. neoformans* (Zhong, Jeffries *et al*. 2000).

2.3.3.5 *Nucleic acid synthesis inhibitors*

Flucytosine (5FC) is an example of an antifungal agent that inhibits the synthesis of nucleic acids by hindering the metabolism of pyrimidines and prevents the synthesis of DNA, RNA and

proteins. For instance, fungal cells contain ATPases that facilitate the entry of pyrimidines into the cell by active transport. As a result, because flucytosine is a pyrimidine, it gets into the cell via these ATPases. Inside the cell, it is converted to 5-fluorouracil (5-FU) by a fungal enzyme called cytosine deaminase. 5-FU is then integrated into RNA resulting in defective RNA synthesis (Onishi, Meinz *et al.* 2000). DNA synthesis in fungi can be inhibited when 5-FU is converted to 5-FdUMP (5-fluorodeoxyuridine monophosphate) which prevents the action of thymidylate synthase (Onishi, Meinz *et al.* 2000; Arevalo, Carillo-Munoz *et al.* 2003). 5-FU is administered orally and is effective in the treatment of fungal urinary tract infections (UTIs) and can be used to treat certain strains of *Candida* and *Cryptococcus*. However, fungal cells are able to develop resistance to this drug. Resistance occurs either at the point at which the drug is transported into the cell or at the cytosine deaminase step. This is the reason why 5-FU is given in combination with amphotericin B, itraconazole or fluconazole. These combinations can be used for the treatment of *Candida* infections (Zhanel, Karlowsky *et al.* 1997).

2.3.3.6 *Protein biosynthesis inhibitors*

Drugs that target protein synthesis include cispentacin and icofungipen. Cispentacin exerts its function by inhibiting the action of isoleucyl tRNA synthetase. Icofungipen is a synthetic derivative of cispentacin. These drugs have demonstrated high antifungal activity when used to treat infections caused by *C. albicans* (Konishi, Nishio *et al.* 1989; Petraitis, Petraitiene *et al.* 2004). Another drug that is classified as a protein synthesis inhibitor is sordarin. This drug functions by inhibiting the action of the fungal translation elongation factor 2 (EF2). EF2 is a protein that plays a role in protein synthesis by facilitating the ribosomal translocation process (Herreros, Martinez *et al.* 1998). Sordarin is highly specific and studies are being conducted so as to develop derivatives of this compound (Campoy and Adrio 2016).

2.3.4 Mechanisms involved in antifungal resistance

Despite the potent nature of some antifungal agents, resistance can still occur due to the ability of fungi to evade the mechanisms of action of these antifungal agents. This is due to their natural ability to develop features and strategies that allow them to thrive and grow despite drug availability. Most of the resistance mechanisms occur at a molecular level (Peman, Canton *et al.* 2009; Sanglard 2016). In order for microorganisms to evade the action of the antifungal agents, three main resistance methods are employed (Sanglard 2016). The first method employed is reducing the concentration of the drug required to effectively treat the infection and eradicate the fungi. The second method is modifying the target sites to which the drug binds and the last method is altering the metabolic processes in order to redirect the actions of the antifungal drug (Campoy and Adrio 2016).

2.3.4.1 *Reduction of effective drug concentration*

2.3.4.1.1 *Drug efflux pumps*

Drug concentrations within the cells are reduced by means of efflux pumps. This method relies on the enhanced activity of these efflux pumps to reduce drug concentrations. These efflux pumps are transporters that confer resistance to a number of fungi and belong to the ATP-binding cassette (ABC) and major facilitator superfamily (MFS) families. Examples of drug efflux transporters include CDR1 and CDR2 which are responsible for the azole resistance observed in *C. albicans*. An increase in the regulation of these transporters increases drug efflux thereby decreasing the amount of the drug within the cells (Mishra, Prasad *et al.* 2007; Sardi, Almeida *et al.* 2011). Additional ABC transporters that confer resistance to other fungi are CgCDR1, CgCDR2, CgSNQ2 that enable *C. glabrata* to become resistant to azoles. A MFS

transporter called MDR1 plays a role in the resistance seen in clinical isolates of *C. albicans* and *C. dubliniensis* to azoles. Increased expression of MDR1 causes an increase in the removal of azoles from the cells (Mogavero, Tavanti *et al.* 2011). Fluconazole resistance seen in *C. albicans* is due to a MFS transporter called FLU1 (Calabrese, Bille *et al.* 2000). An increase in the regulation of either ABC or MFS transporters is attributed to specific mechanisms employed by the drug resistant fungi. One such mechanism is the presence of point mutations in the regulators of the expression of drug efflux transporters. These point mutations are referred to as gain-of-function mutations (GOF) and they lead to up-regulation of transporters in drug resistant fungi (Vandeputte, Ferrari *et al.* 2012). This can be seen in the case of *C. albicans* where fluconazole resistance arises from GOF mutations in a transcription factor called Upc2p (Dunkel, Liu *et al.* 2008). In addition to point mutations, up-regulation of genes such as, *ERG1*, *ERG3*, *ERG7*, *ERG9*, *ERG11* or *ERG25*, that code for enzymes involved in sterol biosynthesis, can also cause a reduction in drug concentration and are responsible for conferring azole resistance to fungal species (Henry and al. 2000; Romani 2004).

2.3.4.1.2 *Biofilm formation*

Drug concentrations can further be reduced due to the ability of some fungal species to produce biofilms. Fungi such as *Candida*, *Aspergillus*, *Cryptococcus*, *Trichosporon*, *Coccidioides* and *Pneumocystis* form biofilms that enable them to become resistant to drugs such as azoles, polyenes and pyrimidine analogs (Sardi, Scorzoni *et al.* 2013; Desai, Mitchell *et al.* 2014). Biofilms are complex structures that consist of polysaccharides, carbohydrates, proteins and are formed by cells to prevent drugs from reaching the cells and exerting their effects. The biofilms also contain signaling molecules (Bonhomme and d'Enfert 2013; Mitchell, Zarnowski *et al.* 2015). The ability of fungal species to form biofilms enables them to become pathogenic and

studies have shown that most of the infections caused by *C. albicans* are as a result of the production of biofilms (Ramage, Saville *et al.* 2005; Martinez and Fries 2010). Furthermore, other studies have indicated that *C. glabrata*, *C. parapsilosis*, and *C. tropicalis* have the ability to also produce biofilms (Silva, Henriques *et al.* 2009). Biofilms can grow and thrive on both living and non-living surfaces, such as medical devices, making this a major healthcare problem (Hawser and Douglas 1994). Biofilm formation on medical devices, particularly vascular and urinary catheters, is the main cause of infections and deaths among patients in hospitals accounting for 90% of infections. Catheter-related biofilms can result in bloodstream infections which can occur in one out of every 100 patients admitted (DiDone, Oga *et al.* 2011). Other medical devices on which fungi, such as *Candida*, can grow include joint prostheses, cardiac valves, artificial vascular bypass devices, pacemakers, ventricular assist devices and central nervous system shunts. This can lead to life threatening conditions (Byers, Chapman *et al.* 1992). Death rates associated with device-related infections can reach 30%. Infections associated with *Candid*a species that are able to form biofilms are difficult to treat as they do not respond to most of the antifungal agents. Unfortunately, surgical procedures are employed to remove infected devices which are then replaced (Viudes, Peman *et al.* 2002; Finkel and Mitchell 2011). Amphotericin B and echinocandins are the only antifungal agents that have been used in the treatment of infections caused by *C. albicans* biofilms (Kuhn, George *et al.* 2002). Nonetheless, despite treatment with these antifungal agents, the *Candida* biofilms are tremendously difficult to completely eliminate from the body (Montejo 2011).

2.3.4.2 *Modification of target sites*

Drug resistance can also develop through modifications of target sites. Resistance to azoles and echinocandins can occur through this type of mechanism. For instance, mutations in the *ERG11*

gene (a gene that codes for 14-α sterol demethylase) found in *C. albicans* reduces its sensitivity to azole drugs resulting in resistance. A large number of point mutations result in a reduction in the sensitivity of fungi to fluconazole and posaconazole (Sanglard and Coste 2015). Fungal species become resistant to echinocandins due to point mutations in either *FSK1* or *FSK2* genes which code for the enzyme (1,3)-β-$_D$-glucan synthase enzyme (Vandeputte, Ferrari *et al.* 2012; Sanglard 2016). These mutations lead to decreased effectiveness of these drugs against (1,3)-β-$_D$-glucan synthase (Wiederhold 2016). Point mutations in the *FUR1* gene are the main causes of resistance seen in pyrimidine analogs such as fluzytosine (5FC). Mutations in this gene result in fungi becoming completely resistant to 5FC and 5FU (Vandeputte, Ferrari *et al.* 2012). Point mutations in genes coding for the enzyme cytosine deaminase are the main causes of 5FC resistance in a large number of *Candida* species (Vandeputte, Pineau *et al.* 2011).

2.3.4.3 *Modification of metabolic processes or pathways*

Modifications in the metabolic processes or pathways are another way in which fungal species become resistant to antifungal agents. These modifications lead to a reduction in function. This can be seen in cases where inhibition of the *ERG3* gene results in the modification of the final step of ergosterol biosynthesis. This modification prevents the generation of toxic methylated sterols from 5FU thus facilitating azole resistance (Sanglard 2016). In addition, mutations in genes such as *ERG3*, *ERG6*, *ERG24* and *ERG2* also result in a reduction in the presence of ergosterol in the plasma membrane. In some cases, ergosterol can be completely absent due to these mutations (Vincent, Lancaster *et al.* 2013). Reduced expression of *FUR1* results in fungal strains being less susceptible to 5FC. In *C. glabrata* strains, down-regulation of this gene makes them completely resistant to 5FC (Vandeputte, Ferrari *et al.* 2012). Currently, fungal species are becoming resistant to more than one class of antifungal agents. This creates problems in the

healthcare sector as treating these multidrug resistant pathogens becomes increasingly difficult (Sanglard 2016). The resistance profiles observed in *Candida* species are as a result of point or simultaneous mutations in genes such as *ERG2*, *ERG3*, *ERG5* and *ERG11* which lead to azole and amphotericin B resistance (Vincent, Lancaster *et al.* 2013; Sanglard 2016). Reports have also indicated multidrug resistance to echinocandins and azoles resulting from mutations in the *FSK2* gene and changes in the regulation of ABC transporters (Sanglard 2016). Multidrug resistance has been observed in species such as *C. albicans*, *C. glabrata* and *C. lusitaniae*, with *C. glabrata* showing the greatest amount of resistance especially to drugs such as fluconazole, voriconazole and echinocandins (Denning and Bromley 2015; Jensen, Astvad *et al.* 2015).

2.3.5 The effect of medicinal plants on *Candida* species

Numerous studies have been carried out on medicinal plants to evaluate their effect on fungal species including *Candida*. Evaluation of the effect of *Euphorbia hirta* L. leaf extract on *C. albicans* revealed that it resulted in lysis as well as disintegration of the yeast cells after treatment as seen using transmission electron microscopy (TEM). Furthermore, this extract also showed a fungicidal effect against *C. albicans* (Rajeh, Zuraini *et al.* 2010; Basma, Zuraini *et al.* 2011). Another study carried out to assess the effect of *Pogostemon parviflorus* Benth. methanolic leaf extracts against *C. albicans*, C. *glabrata*, *C. tropicalis* and *C. dubliensis* demonstrated that this extract inhibited the growth of these *Candida* species (Najafi and Sadeghi-Nejad 2011). Evaluation of the *in vitro* and *in vivo* antifungal activities of *Sapindus saponaria* L. extracts against *C. albicans* and *C. glabrata* isolates also showed some promising results. Results of the evaluation of the *in vitro* activities of the extracts revealed that they had inhibitory and fungicidal effects against the *Candida* strains. Evaluation of the *in vivo* activities revealed that the extracts were effective against *C. albicans* and *C. glabrata* isolates that were either

susceptible or resistant to azole drugs (Dota, Faria *et al.* 2010). The study of the anti-*Candida* activity of *Zataria multiflora* Boiss aqueous, ethanolic and methanolic extracts revealed that the ethanolic and methanolic extracts exhibited greater activity against the *Candida* species whereas the aqueous extract was not as effective as the other two extracts (Saleem, Nazli *et al.* 2004). Synergistic effects of plant species and antifungal drugs against *Candida* species have also been shown. A study conducted to assess the effect of *Ficus lyrata* L. extract and nystatin on *C. albicans* isolates showed that the extract had a greater effect at lower concentrations compared to nystatin. In addition, the MIC of nystatin alone was 36 mg/ml but reduced to 0.05 mg/ml when combined with the extract thus showing a synergistic effect (Bidarigh, Khoshkholgh Pahlaviani *et al.* 2011).

2.4 Drug resistance in cancer and use of medicinal plants

2.4.1 Introduction

Cancer is defined as a set of diseases associated with irregular or uncontrolled cell growth. The cells can either form tumors within tissues or they can result in what is termed as liquid cancer when the affected area is blood or the bone marrow (Nussbaumera, Bonnabrya *et al.* 2011). These abnormal cells have the potential of spreading to other parts of the body causing major complications and high mortality rates. According to the International Agency on Cancer (IAC), 12.4 million cancer cases and 7.6 million deaths were reported worldwide in 2008. It is projected that cancer incidence rates are expected to rise to 27 million with more than 11 million cancer related deaths by 2030 worldwide (Li, Chen *et al.* 2012). In 2012, about 14 million new cancer cases were reported with 8.2 million cancer associated deaths. These numbers increased slightly in 2013 with 14.9 million new cases being reported while the cancer-related deaths remained the

same at 8.2 million (Fitzmaurice, Dicker *et al.* 2015). Unfortunately, these numbers are anticipated to increase over time (Wang, Yang *et al.* 2015). The high incidence and mortality rates create a problem for the healthcare systems worldwide (Li, Chen *et al.* 2012).

The main type of cancer associated with women worldwide is breast cancer. It has been reported that 23% of cancer cases are as a result of breast cancer and it accounts for 14% of cancer-associated deaths (Jemal, Bray *et al.* 2011). Breast cancer incidence is higher in Western countries compared to Asia. However, approximately 50% of breast cancer cases and 60% of deaths are linked to developing countries (Jemal, Bray *et al.* 2011). Breast cancer is detected in over 1.2 million women worldwide each year. Women between the ages of 14-30 and those over the age of 40 have an increased risk of developing breast cancer as a result of environmental factors or late menopause (Singha, Ngcoyaa *et al.* 2016). The major forms of cancer treatment are surgery, chemotherapy, and/or radiotherapy (Shewach and Kuchta 2009). However, these treatment options have some disadvantages. For example, even though chemotherapeutic agents target tumor cells and minimize their proliferation, they are associated with numerous unwanted side effects (Nussbaumera, Bonnabrya *et al.* 2011). These side effects include gastrointestinal tract lesions, hair loss, and bone marrow suppression. The side effects normally arise due to the action of the chemotherapeutic agents on both healthy and cancerous cells (Nussbaumera, Bonnabrya *et al.* 2011).

Apart from the numerous side effects, resistance to the chemotherapeutic agents can also develop. Even though chemotherapy is essential in cancer treatment, resistance develops as a result of dose-limiting toxicities (Wang, Yang *et al.* 2015). This drug resistance can either be inherited or acquired. Inherited drug resistance occurs when specific drugs fail to treat the cancer and acquired drug resistance occurs when the cancer cells becomes unresponsive to

chemotherapeutic agents that initially worked in the treatment of that specific type of cancer (Wang, Yang *et al.* 2015). The need to find ways to combat cancer resistance has caused researchers to find alternative treatment options in the fight against cancer. The use of natural compounds is one way in which drug resistance can be alleviated. Drugs developed from natural compounds, such as plants, are believed to be more effective because they have an effect on several target sites, are associated with fewer side effects and can be used to treat multiple types of cancer (Newman, Cragg *et al.* 2003).

2.4.2 Global breast cancer incidence rates

The high mortality rates seen in females worldwide are as a result of breast cancer. According to GLOBOCAN 2012, approximately 1.7 million breast cancer cases and 521 900 deaths were reported in 2012 (Ferlay, Soerjomataram *et al.* 2012). Breast cancer prevalence is high in Western Europe and the United States whereas the prevalence is much lower in African and Asia. In Africa, breast cancer is the major type of cancer frequently detected in women (Ferlay, Shin *et al.* 2010) and has an age standardized rate (ASR) of 28.0 cases per 100 000. This ASR is less than that seen in more developed countries which is 66.4 cases per 100 000 (Ferlay, Shin *et al.* 2010). The prevalence of breast cancer in African women is higher among younger women that have not yet reached menopause. This trend is different from that seen in developed countries (Holcombe, Weedon *et al.* 1999). This variation in ASR rates is due to the fact that African women possess characteristics that protect them from developing breast cancer compared to their counterparts in developed countries. Furthermore, the lower ASR rates are attributed to African women beginning their menstruation at a later age, giving birth to their first child at a much lower age and them normally having more than four children who are allowed to breastfeed for more than 6 months (Huo, Adebamowo *et al.* 2008; Awatef, Olfa *et al.* 2010).

It has been shown that there are differences in mortality rates among countries. It is reported that the Unites States has the highest number of breast cancer associated deaths especially among black women. The increase in the prevalence of breast cancer in HICs reveals that facilities to screen breast are easily accessible and that there are increased chances of developing breast cancer in HIC probably due to the lifestyle and environments factors (Althuis, Dozier *et al.* 2005). The risk factors that increase the chances of developing breast cancer include increase in weight over the age of 18, excess body weight, the use of menopausal hormone therapy (MHT), lack of exercise, alcohol intake and reproductive and hormone problems (Colditz, Baer *et al.* 2006; Chlebowski, Manson *et al.* 2013). However, it has been shown that breastfeeding reduces the chances of developing breast cancer (Colditz, Baer *et al.* 2006). Breast cancer prevalence in LMICs is rising and is believed to be attributed to the changes in reproductive health and advances in breast cancer knowledge and screening facilities (Althuis, Dozier *et al.* 2005; Colditz, Sellers *et al.* 2006). Despite the incidence rates being high in HICs, the number of breast cancer related deaths have been reducing in these countries mainly due to early diagnosis of breast cancer and enhanced treatment options (Althuis, Dozier *et al.* 2005). However, the same trend is not seen in LMIC like Latin America, the Caribbean and parts of Asia. The mortality rates in these countries continue to rise. This is attributed to the various risk factors and the unavailability of facilities that can easily detect and treat breast cancer (Bray, Jemal *et al.* 2012; Chatenoud, Bertuccio *et al.* 2014; Youlden, Cramb *et al.* 2014).

2.4.3 Breast cancer in South Africa

Breast cancer is a disease that is acknowledged as being cause by mutations to various genes or alleles. The different subtypes have been identified using a number of techniques such as histology, immuno- histochemical markers and geno-transcriptomics (Curtis, Shah *et al.* 2012;

Anderson, Rosenberg *et al.* 2014). These techniques have revealed significant diverse subtypes that contribute to variations in aetiology, prognosis and treatment. In order to determine sub-groups that are more susceptible to breast cancer as well as the specific disease causation patterns, it is vital to analyze the profile of the age-specific incidence rate curves and also assess the age at which women are normally diagnosed with breast cancer (Armitage and Doll 1954).

Breast cancer statistics obtained from studies conducted in USA showed that there are two distinct categories with regard to age distribution and onset of disease. Some women develop breast cancer close to 50 years of age while others develop breast cancer when they are close to 70 years (Anderson, Rosenberg *et al.* 2014). Data pertaining to the age at which breast cancer is commonly diagnosed in sub-Saharan Africa populations is lacking (Dickens, Pfeiffer *et al.* 2016). As a result, studies have to be conducted to assess the age-frequency of breast cancer diagnosis. South Africa affords an exclusive location for analyzing the age-frequency of breast cancer diagnosis. This is due to the fact that South Africa is multi-racial and contains a national cancer registry with electronic data on the hormonal and human epidermal growth factor receptor 2 (HER2) receptors that are normally assessed when diagnosing women with breast cancer all over the country (Herd, Francies *et al.* 2015).

In South Africa, there is no well-structured program intended for the screening of breast cancer in the general population. Breast cancer screening is limited to a small population group with the money to access private health care. A study was carried out to determine the receptor distribution in more than 10 000 women in South Africa (Dickens, Duarte *et al.* 2014). The findings of this study were incorporated in another study to investigate the breast cancer sub-populations in black and white South African women and the age at which breast cancer develops (Dickens, Pfeiffer *et al.* 2016). The study conducted in 2016 revealed that there was a

constant trend in the age at which women developed (early and late onset) breast cancer in the breast cancer sub-population of black and white women in South Africa. These distinct groups were similar to those seen in Europe and the US (Anderson and Matsuno 2006; Anderson, Rosenberg *et al.* 2013). Furthermore, results of the analysis of the age-frequency distribution of breast cancer development showed that South African black women are diagnosed with breast cancer at a mean age of 53 years whilst South African white women are diagnosed at a mean age of 58 years (Dickens, Pfeiffer *et al.* 2016).

Another study carried out to investigate breast and cervical cancer screening and associated factors among older adult women in South Africa revealed that of the entire sample of women, only 24.3% had previously had a PAP smear test done and only 15.5% had previously gone for a mammogram. This study also revealed that women that went for breast cancer screening were younger, highly educated, were from a White, Colored or Indian/Asian background, wealthy and had health insurance. A similar trend was observed for those women that went for cervical cancer screening (Peltzer and Phaswana-Mafuya 2014). Without proper cancer screening methods, detection of breast cancer in elderly women will be delayed which will subsequently increase mortality rates as the cancer would have reached an advanced stage by the time it is diagnosed (Peltzer and Phaswana-Mafuya 2014).

2.4.4 Classification of anticancer drugs for the treatment of breast and other types of cancer

One way in which various forms of cancer can be treated is by the use of single or multiple chemotherapeutic agents. Treatment is given to either get rid of the cancer cells or prevent cancer cell growth. However, cytotoxic agents cause a wide range of side effects which include

gastrointestinal tract lesions, hair loss, nausea, and bone marrow suppression. In addition, cancer cells can become resistant to these chemotherapeutic agents. Side effects arise due to the fact that these anticancer agents are not specific as they destroy both cancerous cells and normal cells (Nussbaumera, Bonnabrya *et al.* 2011). Chemotherapeutic agents have been used since 1940 with the first being nitrogen mustards (very potent alkylating agents) and antimetabolites. As a result, more chemotherapeutic drugs have been developed since then (Shewach and Kuchta 2009). Classification of anticancer agents is done based on their mode of action. Anticancer agents can be classified as DNA-interactive agents, antimetabolites, antitubulin agents, molecular targeting agents, hormones and monoclonal antibodies. Biological agents can also be used in the treatment of cancer (Nussbaumera, Bonnabrya *et al.* 2011).

2.4.4.1 *DNA interactive agents*

DNA interactive agents are the major group of anticancer agents and use a wide range of modes of action. Classes of drugs found in this group include alkylating agents, cross-linking agents, intercalating agents, topoisomerase inhibitors and DNA cleaving agents (Nussbaumera, Bonnabrya *et al.* 2011).

2.4.4.1.1 *Alkylating agents*

These have been used to treat cancer for many years and are capable of alkylating numerous molecules such as proteins, RNA and DNA. Alkylating drugs possesses an alkyl group that covalently binds to DNA thereby preventing accurate and proper DNA replication. This capability is what enables them to have anti-cancer properties (Lind 2008). Since DNA is composed of two strands, the alkylating drugs can either form intra-strand cross-links by binding twice to one DNA strand or form inter-strand cross-links by binding once to both strands. These

cross-links interfere with DNA replication causing breaks in the DNA strands as the cell attempts to replicate the DNA. This then ultimately results in programmed cell death referred to as apoptosis (Damia and D'Incalci 1998; Siddik 2005). These chemotherapeutic drugs are not dependent on particular stages of the cell cycle and are therefore referred to as cell-cycle independent drugs. Therefore, the outcome of treatment depends on the dosage of drug used (Malhotra and Perry 2003). Examples of alkylating agents include dacarbazine, temozolomide, procarbazine and ecteinascidin-743 (Nussbaumera, Bonnabrya *et al.* 2011).

2.4.4.1.2 *Cross-linking agents*

Cross-linking agents also exert their action by attaching to the DNA strands forming either intra-strand or inter-strand DNA cross-links. The main types of drugs that belong to this group include platinum complexes (such as cisplatin, carboplatin, oxaliplatin) (Crombag, Joerger *et al.* 2016), nitrogen mustards (e.g., cyclophosphamide, ifosfamide) (Salman, Swinden *et al.* 2016) and nitrosurea compounds (Nussbaumera, Bonnabrya *et al.* 2011).

2.4.4.1.3 *Intercalating agents*

2.4.4.1.3.1 Anthracyclines

This group comprises antitumor antibiotics that have a structure made up of a planar anthraquinone nucleus linked to an amino-containing sugar. Examples of drugs that belong to this group include doxorubicin, daunorubicin, and aclarubicin which are isolated from *Streptomyces peucetiusor* or *Streptomyces galilaeus* (Volkova and Russell 2011). Epirubicin and idarubicin also belong to this class and are regarded as semisynthetic analogues (Nussbaumera, Bonnabrya *et al.* 2011). Doxorubicin is extensively administered to treat various forms of cancer which include lymphomas, leukemia as well as numerous solid tumours such as breast cancer.

Side effects associated with the use of doxorubicin are vomiting, nausea, myelosuppression, mucositis, cardiotoxicity caused by dose buildup and alopecia (Nussbaumera, Bonnabrya *et al.* 2011; Volkova and Russell 2011)..

2.4.4.1.3.2 Mitoxantrone and actinomycin-D

These drugs also exert their effect by DNA intercalation (introducing molecules between the DNA strands), producing extremely reactive free radicals that damage cellular components as well as inhibit the action of topoisomerases (Minotti, Menna *et al.* 2004). Mitoxantrone is used to treat cancers such as adult nonlymphocytic leukaemia, non-Hodgkin's lymphoma and breast whereas actinomycin-D is employed in the treatment of AIDS-related Kaposi's sarcoma, certain testicular sarcomas and paediatric cancers (Nussbaumera, Bonnabrya *et al.* 2011).

2.4.4.1.4 *Topoisomerase inhibitors*

This class of chemotherapeutic agents has an effect on topoisomerase I and topoisomerase II enzymes thereby interfering with their function. For DNA replication or transcription to occur, unwinding of the DNA double-strand helix has to take place. However, as DNA begins to unwind, the adjoining wound DNA coils up tightly forming supercoils. Topoisomerase enzymes function to alleviate the tension created by this supercoiling. Topoisomerase inhibitors prevent the action of topoisomerase I and II thus impeding the replication and transcription processes (Lodish, Berk *et al.* 2000; Goodsell 2002). Examples of topoisomerase I inhibitors include irinotecan and topotecan. These are semi-synthetic drugs derived from camptothecin (a natural product obtained from the bark of a Chinese ornamental tree called *Camptotheca acuminata*) (Malhotra and Perry 2003). Examples of topoisomerase II inhibitors include etoposide, doxorubicin, mitoxantrone, teniposide novobiocin, merbarone, and aclarubicin (Nitiss 2009).

2.4.4.1.5 *DNA cleaving agents*

Bleomycin is a glycopeptide obtained from *Streptomyces verticillus*. Its mode of action also includes intercalating DNA and production of free radicals. This is facilitated by the ability of bleomycin to bind to a metal ion and is subsequently chemically reduced which leads to it reacting with oxygen (Dorr 1992; Airley 2009). The ability of bleomycin to accumulate in squamous cells makes it appropriate for use in the treatment of head and neck tumours as well as testicular carcinomas and Hodgkin's disease (Nussbaumera, Bonnabrya *et al.* 2011).

2.4.4.2 *Antimetabolites*

This group consists of purine and pyrimidine analogues. Examples of purine analogues include azathioprine, mercaptopurine, thioguanine, cladribine, clofarabine and fludarabine (Grem and Keith 2005). Examples of pyrimidine analogues consist of drugs such as 5-Fluorouracil (5-FU), tegafur, capecitabine, cytarabine, gemcitabine and azacitidine (Grem and Keith 2005). 5-FU is extensively used to treat breast tumours as well as cancers that are confined to the gastrointestinal tract such as advanced colorectal cancer (Nussbaumera, Bonnabrya *et al.* 2011).

2.4.4.3 *Antitubulin agents*

These chemotherapeutic drugs are obtained from plants and inhibit cell division by interfering with microtubule function. Microtubules (comprising α-tubulin and β-tubulin proteins) are essential for cell division (Rowinsky and Donehower 1991) and are constantly being assembled and disassembled. Anti-microtubule drugs can be classified into two major groups, vinca alkaloids and taxanes. Vinca alkaloids disrupt microtubule formation while taxanes inhibit the disassembly of microtubules, consequently preventing completion of mitosis. Apoptosis occurs

as a result of the cancer cells not being able to complete mitosis which leads to cell cycle arrest (Lind 2008; Yue, Liu *et al.* 2010).

Vincristine and vinblastine are naturally occurring vinca alkaloids. When these drugs proved effective in the treatment of lung and breast solid tumors, acute leukemia and lymphomas (Nussbaumera, Bonnabrya *et al.* 2011), semi-synthetic vinca alkaloids such as vinorelbine, vindesine, and vinflunine were manufactured (Yue, Liu *et al.* 2010). Taxanes consist of drugs that are both natural and semi-synthetic. Examples include paclitaxel and docetaxel (Yue, Liu *et al.* 2010). Paclitaxel and docetaxel are employed in the treatment of advanced or metastatic non-small-cell lung cancer as well as metastatic breast cancer when first-line treatment fails (Nussbaumera, Bonnabrya *et al.* 2011).

Podophyllotoxin is another drug in this class that is mainly obtained from American mayapple (*Podophyllum peltatum*) and Himalayan mayapple (*Podophyllum hexandrum* or *Podophyllum emodi*). The mode of action of this drug is the same as that of vinca alkaloids. It prevents microtubules from forming by attaching to tubulin. Two additional drugs manufactured from podophyllotoxin include etoposide and teniposide (Damayanthi and Lown 1998).

2.4.4.4 *Adjuvant hormonal therapy*

Close to 60-70% of breast cancers are sensitive to hormone therapy and classification is based on them being either estrogen receptor positive (ER+) or progesterone receptor positive (PR+). Women with ER+ or PR+ breast cancer are normally treated using either tamoxifen or an aromatase inhibitor (for post-menopausal women) and treatment can last up to 10 years (Burstein, Temin *et al.* 2014). However, it has been shown that prolonged tamoxifen can lead to stroke, pulmonary embolism and incidence of uterine cancer (Ribnikar, Sousa *et al.* 2017).

2.4.4.5 *Immunotherapy (targeted therapy)*

Immunotherapy is one of many cancer treatment options that aid the immune system combat cancer. This type of therapy is used in the management of cancer by incorporating therapeutic agents synthesized from living organisms (Spellman and Tang 2016). Immunotherapy has proved to be effective in the treatment of cancers such as breast, melanoma and non-small cell lung cancer. It utilizes therapies such as monoclonal antibodies, bispecific antibodies, immune checkpoint inhibition, vaccinations and antibody-drug conjugates (Spellman and Tang 2016). Unlike other types of solid tumors such as melanomas which are considered to be immunogenic, breast cancer was believed not to cause an immune response. Nevertheless, it has been shown that enhancing the activity of the immune system in patients with breasts cancer decreased tumor growth (Spellman and Tang 2016). A number of therapies incorporating immune system components have been developed that target breast cancer cells which include targeted monoclonal antibodies to tumor receptors (HER-2). These types of therapies can be used with chemotherapy, endocrine therapy or antibody-drug conjugates. However, obtaining the most effective combination of these treatment options for individual patients and subtypes of cancers is a problem (Spellman and Tang 2016).

2.4.4.5.1 *Targeted monoclonal antibodies*

This type of cancer treatment relies on monoclonal antibodies to inhibit the function of tumor-specific proteins as well pathways that play a role in the growth and development of tumors. They exert their functions by inhibiting angiogenesis, initiating apoptosis and blocking growth factor receptors (Mimeault and Batra 2010). The FDA recommended the use of monoclonal antibodies (MABs) such as cetuximab, trastuzumab and bevacizumab to treat different types of

cancers (Hurvitz, Hu *et al.* 2013). Cetuximab and trastuzumab function by binding to growth factors such as epidermal growth factor receptor (EGFR) and HER2/neu proteins which leads to hampering of cancer cell growth (Markovic and Chung 2012). In addition to hampering cell growth, these two drugs can also prevent the cancer from spreading particularly in colon and head and neck cancers. It has been shown that when EGFR is highly expressed, the condition of the patient becomes worse (Hirsch, Varella-Garcia *et al.* 2009).

Trastuzumab is used to treat invasive HER2/neu positive breast cancer and its mechanism of action is the same as that of cetuximab. It enhances immune response by activating natural killer (NK) cells which have the ability to cause cell lysis. Trastuzumab is also able to reduce the ability of the cancer cells to carry out angiogenesis (Hubalek, Brunner *et al.* 2010). Regrettably, patients receiving trastuzumab develop either primary or secondary resistance following its use (Spellman and Tang 2016). The use of more than one MAB (bispecific antibodies) has proved to be effective in the treatment of cancer. One such example is the use of a combination of trastuzumab and pertuzumab in the treatment of treat breast cancer (McDonagh, Huhalov *et al.* 2012). Clinical trials conducted to incorporate pertuzumab, a new HER2 MAB capable of enhancing the mode of action of trastuzumab, in the treatment of trastuzumab-resistant breast cancer showed that the activity of trastuzumab was improved when combined with pertuzumab (Gianni, Pienkowski *et al.* 2012; Swain, Kim *et al.* 2013).

Bevacizumab is employed in the treatment of a wide range of cancers such as some forms of lung cancers, renal cancers, ovarian cancers, and glioblastoma multiforme of the brain. Its mode of action is the inhibition of angiogenesis through its attachment to vascular endothelial growth factor A (VEGF-A) (Ellis 2006; Hudis 2007; Lambrechts, Lenz *et al.* 2013). These types of targeted treatment options are considered to be more effective in treating cancers that have

developed resistance to conventional chemotherapeutic agents. In addition, they are not as toxic as the standard chemotherapeutic agents (Ong, Das *et al.* 2012). Unfortunately, the use of immunotherapy has its challenges which are attributed to the differences in the immune system profile of patients and varieties in the types of tumor encountered. Immune system profiles differ among patients because of genetics, lifestyle, epigenetics, nutrition and the environment (Agur and Vuk-Pavlovic 2012). In addition to this, some forms of immunotherapy can only treat a small number of patients. Thus for the treatment to be effective, immunotherapy has to be tailored to cater for individual patients (Wayteck, Breckpot *et al.* 2014).

Antibodies can also be conjugated to chemotherapeutic drugs and used in the treatment of cancer. An example of this is T-DM1 which is an antibody-drug conjugate comprising trastuzumab and emtansine. Once this drug reaches the target site, which is the tumor location, emtansine exerts its function by damaging the microtubules leading to cell death and can be used in the treatment of breast cancer (Arteaga, Sliwkowski *et al.* 2012; Verma, Miles *et al.* 2012). Another antibody-drug conjugate that is employed in the treatment of breast cancer is MM-302. The antibody is conjugated to liposomal doxorubicin (Spellman and Tang 2016).

2.4.4.6 *Small molecule drugs (targeted therapy)*

Apart from the use of MABs, small molecule drugs are also employed in the treatment of various forms of cancer because they are small enough to enter inside the cancer cells. A large number of these small-molecule drugs inhibit the action of tyrosine kinase (Sutandyo 2016). Tyrosine kinase is an enzyme that plays a role in the transfer of a phosphate group from adenosine 3/tri phosphates (ATP) to a specific protein. Once activated, this enzyme can lead to increased cell proliferation, initiation of apoptosis, initiation of angiogenesis and metastasis and

well as resistance to other forms of treatment (Krause and Van Etten 2005). In cancer cells, disruptions of the regulation of tyrosine kinase as well as mutations of specific genes enable the tyrosine receptor to be highly expressed. This increased expression can be seen in HER2 positive breast cancer (Krause and Van Etten 2005). Small molecule drugs inhibit the action of tyrosine kinase and can be classified as epidermal growth factor receptor (EGFR) or vascular endothelial growth factor (VEGF) (Sutandyo 2016).

2.4.4.6.1 *EGFR inhibitors*

The small molecules that target the EGFR and hence regarded as EGFR inhibitors include gefitinib, erlotinib, lapatinib, neratinib, afatinib and canertinib. Their mode of action is to block EGFR from being phosphorylated thus leading to an inhibition of protein signaling. Gefitinib is incorporated in the treatment of non-small cell lung cancer in the Unites States. It targets the cyclin-dependent kinases and causes an inhibition of their activities (Haber, Bell *et al.* 2005) Erlotinib (known as Tarceva on the market) is a drug that has the same mode of action as gefitinib (Katzel, Fanucchi *et al.* 2009). Lapatinib causes an inhibition of pathways such as EGFR, HER2, and Akt. It can be used for the treatment of a wide range of cancers and the rate of resistance is lower that other anti-EGFR agents (Verma, Miles *et al.* 2012). Neratinib is a drug with the same mode of action as lapatinib and targets EGFR- HER4 or EGFR- HER2 (Hubalek, Brunner *et al.* 2010). Afatinib is another drug that is classified as an EGFR inhibitor which targets HER2 and EGFR kinases. Not only is afatinib effective against EGFR mutations, it can also be used to treat cancers resistant to erlotinib or gefitinib treatment (Minkovsky and Berezov 2009). Results obtained from clinical trials indicate that it is capable of preventing the growth of a wide range of cancers such as metastatic breast cancer, metastatic gastric carcinoma and advanced hepatocellular carcinoma (Li, Zhao *et al.* 2010). Canertinib is used in the treatment of a

number of cancers because of its extensive effects on cancer cells. Nonetheless, some EGFR mutations do not respond to treatment with EGFR inhibitors (Kancha, von Bubnoff *et al.* 2009). Furthermore, EGFRs are also found on normal epithelial cells of the skin and mucosa and inhibiting them could result in side effects affecting the skin and gastrointestinal tract (Agero, Dusza *et al.* 2006).

2.4.5 VEGF inhibitors

Members of the VEGF family include VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placenta growth factor (PlGF). Three receptors are also included in this family and these are VEGF receptor (VEGFR) 1, 2 and 3 (Hicklin and Ellis 2005). All malignant tumors express VEGF-A and VEGFR-2. VEGFs exert their effects on endothelial cells and stimulate vascular permeability which leads to fibrin being deposited into the extracellular matrix. This then leads to cell proliferation and migration. *In vitro* studies demonstrated that in addition to the production of endothelial cells, new blood vessels were also formed once VEGF interacted with the specific receptors (Agero, Dusza *et al.* 2006; Sitohy, Nagy *et al.* 2012). An example of a drug found in this class is sorafenib which functions by inhibiting targets such as VEGFR 1-3, PDGF-h receptor serine threonine kinase and Raf-1. The action of sorafenib on these pathways prevents cell proliferation and tumorigenesis and has shown to be effective in the treatment of hepatocellular and renal cell carcinomas (Rini 2007).

2.4.6 Mechanisms of drug resistance in cancer

Drug resistance develops when cancer cells no longer respond to the effects of chemotherapeutic agents. Initially, drug resistance was thought to occur when bacteria were no longer susceptible to particular antibiotics. However, it has been found that the same principle applies to diseases

such as cancer. Drug resistance in cancers occurs through various processes such as drug inactivation, DNA mutations, drug inhibition and degradation, drug efflux, DNA damage repair, drug target alteration, cell death inhibition and the epithelial-mesenchymal transition (EMT) (Housman, Byler *et al.* 2014). Figure 2.1 outlines the different drug resistance mechanisms.

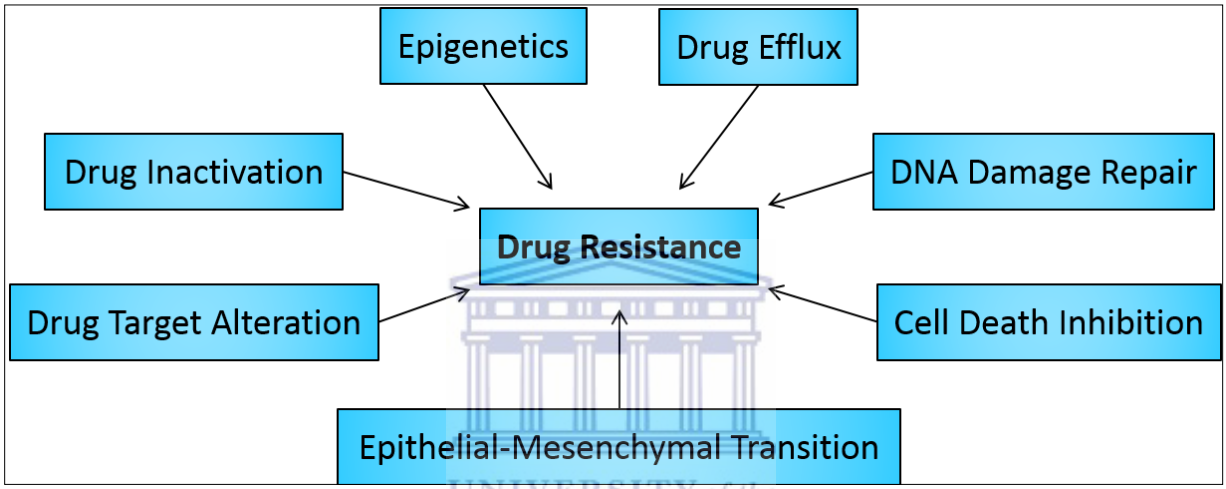

Figure 2.1: Categories of cancer drug resistance mechanisms. These mechanisms can enable or promote direct or indirect drug resistance in human cancer cells. They can act independently or in combination and through various signal transduction pathways.

2.4.6.1 *Drug inactivation*

For most anticancer drugs to effectively function in the body, they have to be activated. This activation process requires the interaction of drug compounds with molecules and proteins in the body. This interaction subsequently causes alteration, breakdown or intercalation of the drug with molecules and proteins thus resulting in drug activation. Nonetheless, drug resistance can arise when cancer cells inhibit the drugs from effectively being activated (Housman, Byler *et al.* 2014).

2.4.6.2 *Alteration of drug targets*

The effectiveness of a drug is determined by the target site to which it binds. If these targets are modified or changed in any way, as seen in mutations or when there are changes to their expression levels, then the effectiveness of the drug is affected. Drug resistance in cancers develops when the drug targets are modified. This can be seen in the case where cancer cells develop resistance to topoisomerase II inhibitors as a result of mutated topoisomerase II genes (Zwelling, Hinds *et al.* 1989; Hinds, Deisseroth *et al.* 1991; Stavrovskaya 2000). Drug resistance can also develop to drugs that target the human epidermal growth factor receptor 2 (HER2). This is a type of receptor tyrosine kinase that plays a role in the normal growth of cells. The expression levels of HER2 are very high in about 30% breast cancer patients (Holohan, Van Schaeybroeck *et al.* 2013). Cancer cells become resistant to HER2 inhibitors following prolonged use of these drugs (Slamon, Godolphin *et al.* 1989; Slamon, Leyland-Jones *et al.* 2001).

Furthermore, drug resistance can develop as a result of disruptions in the drug activation processes. This is evident in cases where HER2-positive breast cancer tumors are treated with trastuzumab. Trastuzumab has been shown to be effective against breast cancer when administered with chemotherapy. Unfortunately, trastuzumab resistance occurs in patients that originally respond to treatment despite continued treatment. In other cases, it has been observed that trastuzumab is less effective when administered on its own (Berns, Horlings *et al.* 2007; Dieras, Vincent-Salomon *et al.* 2007). Tomoxifen resistance has been shown in breast cancer cells. Tomoxifen resistance occurs when ER signaling interacts with other signaling pathways involved in the growth of breast cancer cells. For instance, when the growth factor receptor signaling activity (such as HER2) is high in tumors, tamoxifen administration will cause an

increase in tumor growth due to an alteration in its normal function (Shou, Massarweh *et al.* 2004).

2.4.6.3 *Drug efflux*

The majority of research conducted on cancer resistance focuses on drug efflux. Drug efflux is made possible through the action of ATP-binding cassette (ABC) transporter family proteins. These proteins are located on the membrane (transmembrane proteins) of cells and enable the movement of a wide range of substances into and out of the cell. Despite the ABC transporters being numerous (approximately 49 known members), they are categorized based on the presence of the transmembrane domain (Chang and Roth 2001). The transmembrane domain facilitates the expulsion of substances from inside the cell via the alteration in structure that occurs once the substance binds to this domain. This ensures that toxins do not build up within the cell (Sauna and Ambudkar 2001). The expression levels of these proteins are high in the epithelium found in the liver and intestines where they prevent the accumulation of drugs and other dangerous compounds (Borst and Elferink 2002; Housman, Byler *et al.* 2014). Cancer drug resistance is made possible through the action of three ABC transport proteins namely multidrug resistance protein 1 (MDR1), multidrug resistance-associated protein 1 (MRP1), and breast cancer resistance protein (BCRP). The transporters are able to remove xenobiotics, including vinca alkaloids, epipodophyllotoxins, anthracyclines, taxanes, and kinase inhibitors from inside the cancer cell (Housman, Byler *et al.* 2014). MDR1 is not usually expressed in tissues such as breast, lung and prostate and drug resistance in these tissues is attributed to MRP1 and BCRP expression.

2.4.6.4 *DNA damage repair*

Repairing damage done to DNA is essential in the development of drug resistance. When chemotherapeutic agents cause damage to DNA, DNA damage response (DDR) processes enable this damage to be repaired. In platinum resistance, drugs like cisplatin bring about DNA damage by forming cross-links which result in apoptosis. Nevertheless, this damage can be repaired by removing the damaged DNA and through homologous recombination (Selvakumaran, Pisarcik *et al.* 2003; Olaussen, Dunant *et al.* 2006; Bonanno, Favaretto *et al.* 2014). As a result, for a drug to be effective, this DNA damage repair system must malfunction in some way to prevent the damaged DNA from being repaired. Disrupting the regulation and function of DDR genes and the processes involved in DNA damage repair through mutations and epigenetic silencing are implemented in numerous cancers (Esteller 2000; Goode, Ulrich *et al.* 2002; Curtin 2012).

2.4.6.5 *Cell death inhibition*

Apoptosis and autophagy are two processes that play a vital role in cell death. Apoptosis is carried out by two well known pathways: the intrinsic pathway in the mitochondria facilitated by B-cell lymphoma 2 (BCL-2) family proteins (i.e. caspase-9 and Akt) as well as the extrinsic pathway facilitated by receptors on the surface of cells that are involved in death (Housman, Byler *et al.* 2014). The merging of these two pathways occurs once caspase-3 is activated thus resulting in apoptosis. In numerous types of cancers, the expression levels of Akt (BCL-2 family protein) as well as other antiapoptotic proteins are high. Drug trials being carried out on drugs such as BCL-2 family inhibitors, histone deacetylase inhibitors (HDACi), protease inhibitors and kinase inhibitors demonstrate that they can be effectively utilized in cancer treatment (Housman, Byler *et al.* 2014). However, long term use of current BCL-2 family protein inhibitors results in

resistance despite them being able to induce apoptosis in cancerous cells (Mataga, Rosenthal *et al.* 2012; Holohan, Van Schaeybroeck *et al.* 2013; Sarkar and Faller 2013).

2.4.6.6 *Epithelial-Mesenchymal Transition and Metastasis*

Solid tumors have the ability to spread (become metastatic) as a result of processes that enable them to transition from epithelial cells to mesenchymal cells which is referred to as epithelial to mesenchymal transition (EMT). As the tumor spreads, new blood vessels form around it in a process known as angiogenesis. EMT is also incorporated in cancer drug resistance and studies are being conducted to target this mechanism in drug development (Shang, Cai *et al.* 2013). Current studies show that cancer stem cells, also called progenitor cells, are involved in the development of metastatic cancer cells. This may give an explanation as to why cancer may reappear in different areas of the body despite the cancer being successfully treated (Chaffer, Brueckmann *et al.* 2011; Byler, Goldgar *et al.* 2014a; Byler and Sarkar 2014b). A number of aspects are utilized in EMT for drug resistance to occur. Nonetheless, they rely on the metastatic grade of the tumor (degree of differentiation and EMT). This can be seen in ERBB2 (HER2) positive breast cancer, where β1 integrins levels are highly expressed in tumors leading to increased resistant traits to antibody inhibitors like transtuzumab (Lenisak, Xu *et al.* 2009).

2.4.6.7 *Epigenetics and cancer resistance*

Epigenetic alterations play an essential role in the development of cancer resistance. The two major kinds of epigenetic modifications are DNA methylation as well as histone modification through acetylation or methylation. DNA methylation involves the attachment of methyl groups to cytosines at CG-dinucleotides located on CpG islands, mainly found in upstream gene promoter regions but methylation may take place anywhere on the genome. On the other hand,

histone alterations change chromatin structure and arrangement. Current studies indicate that epigenetic modifications like histone methylation and acetylation contribute to drug resistance (Housman, Byler *et al.* 2014). Furthermore, it is believed that DNA methylation is linked to the ability of cancer cells to acquire multidrug resistance. Experiments carried out indicated that when the *MDR1* promoter in cancer cell lines was demethylated, the cancer cells attained multidrug resistant traits (Kantharidis, El-Osta *et al.* 1997). Epigenetic modifications also play a role in repairing damaged DNA (Plumb, Strathdee *et al.* 2000). Epigenetic modifications have also been shown to enable human breast cells become resistant to drugs. For instance, MDA-MB-231 breast cancer cells become resistant to methotrexate by means of a natural flaw in drug uptake and their inability to express reduced folate carriers (RFC) (Housman, Byler *et al.* 2014).

2.4.7 Effect of medicinal plants on cancer cell lines

A number of studies have been conducted to assess the potential anticancer properties of medicinal plants. A study carried out to evaluate the anticancer activity of *Eclipta alba* against human breast cancer cell lines (MCF-7 and MDA-MB-231) demonstrated that the extract caused apoptosis and the disruption of the mitochondrial membrane potential in addition to DNA damage. The results also revealed that the extract inhibited the growth of both cell lines in a dose-dependent fashion (Yadav, Arya *et al.* 2017). Another study that assessed the anticancer activity of a medicinal plant, *Chamerion angustifolium* L., against breast cancer cell lines (MCF-7, MDA-MB-468 and MDA-MB-231) revealed that the extract inhibited the growth of all the cell lines. The results also showed that the extract caused cell death as seen by the lack of proliferation at high concentrations particularly with the MCF-7 cells (Maruška, Ugenskienė *et al.* 2017). A study evaluating the effect of the crude extract of *Piper cubeba* on breast cancer (MCF-7, MDA-MB-468 and MDA-MB-231) and normal breast (MCF-12A) cell lines revealed

that its fractions had a cytotoxic effect on the breast cancer cell lines resulting in apoptosis and DNA fragmentation. However, this was not the case with the normal breast cells as the fractions did not cause apoptosis or DNA fragmentation (Graidist, Martla *et al.* 2015). Another medicinal plant, *Mangifera indica* L., has also been evaluated for its potential anticancer properties. Its ethanolic extract was tested on breast cancer (MCF-7 and MDA-MB-231) and normal breast (MCF-10A) cells. The results showed that it was very toxic to the MCF-7 and MDA-MB-231cells with minimal toxicity to the MCF-10A cells. Furthermore, the extract seemed to demonstrate cytotoxicity against both estrogen positive and estrogen negative breast cancer cells (Abdullah, Mohammed *et al.* 2014).

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3 CHAPTER 3: Evaluation of the potential toxicity of *Galenia africana* using acute oral, acute dermal, skin sensitization and skin irritation studies

3.1 Abstract

Background: Medicinal plants are used by a large proportion of the global population as complementary and alternative medicines. However, little is known about their toxicity. Sensitization to chemicals is a severe condition that arises as a result of reactive molecules. *G. africana* has been used to treat wounds, coughs and skin diseases and is used in cosmetic formulations such as lotions and shampoos.

Objective/Purpose: To assess the toxicity profile of *G. africana* in animal and reconstructed human *epidermis* models using the acute oral, acute dermal, dermal sensitization and skin irritation toxicity studies.

Methods: The acute oral and acute dermal toxicity potential of *G. africana* was analyzed after a single administration of 300 mg/kg and 2000 mg/kg for acute oral toxicity and 2000 mg/kg for acute dermal toxicity. Sprague-Dawley rats were used for both the acute oral and acute dermal toxicity studies. In the dermal sensitization study, CBA/Ca mice were treated with *G. africana* concentrations of 25% (50 mg/ml), 50% (100 mg/ml) and 100% (200 mg/ml) respectively. The vehicle of choice was dimethylformamide which was also included as a control. The *in vitro* skin irritation potential of *G. africana* was assessed using the EpiSkin® irritation test. The irritation potential of *G. africana* (concentrate) and *G. africana* (in-use dilution) extracts were assessed using the EpiSkin® reconstituted human *epidermis* models.

Results: The results of the acute oral and acute dermal toxicity studies revealed that the median lethal dosage (LD_{50}) for *G. africana* extract in Sprague-Dawley rats was considered to exceed 2000 mg/kg. In the dermal sensitization study, the stimulation index (SI) values for the mice treated with the *G. africana* extract at concentrations of 25% (50 mg/ml), 50% (100 mg/ml) and 100% (200 mg/ml), when compared to the control group, were 1.3, 0.9 and 1.3, respectively. The open application of the extract at the various concentrations did not result in a SI of ≥ 3 in any group. Hence, it did not elicit a hypersensitivity response. Results from the EpiSkin® irritation test showed that both the *G. africana* (concentrate) and *G. africana* (in-use dilution) extracts were non-irritant to the EpiSkin® reconstituted human *epidermis* models.

Conclusion: These findings demonstrate that the toxicity profile of *G. africana* is acceptable for its intended use and can subsequently be used in the pharmacology and cosmetic industries.

3.2 Introduction

Medicinal plants have been used over the years in the treatment of various ailments (Lee 2013). Evidence has shown that the use of these medicinal plants and plant-derived medicine dates back to prehistoric times (Aboelsoud 2010). They have been used in the Indian, Chinese, Greek and African cultures, and are still being used (Ifeoma and Oluwakanyinsola 2013). Medicinal plants possess a wide range of beneficial properties that enable them to be used for the treatment of illnesses (Fankam, Kuiate *et al.* 2014). They are potential sources of vitamins, dietary fiber and minerals. They also contain various phytochemicals such as flavonoids that have numerous health benefits (Sarker and Nahar 2004; Radji, Kurniati *et al.* 2015). Herbal medicine research and its market have increased over the years. This can be attributed to the advent of drug resistance, and the need for alternative treatment methods (Oluwatuyi, Kaatz *et al.* 2004).

Galenia africana (*G. africana*), commonly known as "kraalbos", is predominantly found in the Namaqualand region as well as the Western and Southern Karoo. Ethnobotanical surveys have shown that it has been used in the treatment of various ailments such as coughs, wounds, skin infections, eye infections and venereal sores (Watt and Breyer-Brandwijk 1962; Mativandlela, Muthivhi *et al.* 2009). *G. africana* plant extract is being commercialized for use as a synergistic adjuvant in pesticide formulations and plant growth stimulant in agrochemical applications (Klaasen and Vries 2009; Klaasen 2014). Additional interest in the commercialization of this indigenous South African plant is the invasive nature of this pioneer shrub that could provide income for small-holder farmers that have access to the *G. africana* biomass. Watt and Breyer-Brandwijk also noted that *G. africana* was used by indigenous tribes of South Africa as a component of various cosmetic products such as lotions, decoctions and wound dressings (Watt and Breyer-Brandwijk 1962). It is for this reason that *G. africana* is currently being incorporated in shampoo, lotion, soap and hand wash formulations. The antifungal and antimycobacterial activity of *G. africana* extracts and flavonoid fractions have also been reported (Vries, El Bitar *et al.* 2005; Mativandlela, Meyer *et al.* 2008; Ticha, Klaasen *et al.* 2015). Toxicity data for *G. africana* extracts was identified as a requirement for the registration of agrochemical products. Environmental toxicity (Pool, Klaasen *et al.* 2009a) and *in vitro* immunotoxicity (Pool, Klaasen *et al.* 2009b) of *G. africana* have been reported. Regulatory bodies such as the Organization for Economic Co-operation and Development (OECD) published guidelines that help govern the toxicological assessment of botanical products. These guidelines are aimed at establishing the safety and toxicity levels of various botanical and chemical products (OECD 2010). One of the first tests assesses the potential of compounds to stimulate skin sensitization, thus causing allergic contact dermatitis (ACD) (Goebel, Aeby *et al.* 2012; OECD 2012). The principle of skin

sensitization tests is based on the utilization of animal model studies, which rely on the penetration of the test compound into the upper layer of the skin and subsequent interaction with underlying cells (Reisinger, Hoffmann *et al.* 2015). The effect of the sensitizing compound on the skin is evaluated based on events occurring in the animal models as a result of their immune response mechanisms (Ainscough, Frank Gerberick *et al.* 2013). With the potential commercial product applications of *G. africana* extract, it was essential to carry out skin sensitization tests.

Toxicity studies can be classified in three groups based on the extent of drug exposure, namely acute, sub-acute and chronic toxicological studies. Initial exposure/contact to a single quantity of a substance is referred to as acute toxicity. In addition, corrosion, irritancy and sensitization (topical or local toxicity) tests are incorporated to assess the effect of substances on the skin or eyes (Rajalakshmi, Jayachitra *et al.* 2014). Toxicological studies are essential in evaluating the safety and potential side effects of medicinal plants. This is important because it has been shown that they are capable of causing severe side effects when consumed or used (Menegati, de Lima *et al.* 2016).

With the potential application of *G. africana* in the agricultural and medical sectors, its safety had to be established. The aim of this study was to evaluate the toxicological profile of *G. africana* ethanolic extract in animal and reconstructed human skin models.

3.3 Materials and Methods

3.3.1 Preparation of *G. africana* extract

The *G. africana* extract (Batch No. Ga-1-1 (06/06/08) was commercially prepared by Afriplex Pty (Ltd), South Africa, for The University of the Western Cape (UWC), Bellville, Cape Town. The plant material was collected from Komaggas farmers, Komaggas (29.7987 S, 17.4825 E), Namaqua District, Northern Cape Province. The plant was dried for several weeks to maintain the bioactivity and then passed through a hammer mill to produce a powdery material (approximately 2-3 mm). The milled plant material was mixed with 80% ethanol, in the maceration process, to produce a yellow-green to brown-green 20% (w/v) extract with a pH of 6.9. This was then used as a stock solution. The identity, strength, purity and composition, or other characteristics which appropriately defined the batch from which the test item for this study was drawn, were determined by UWC, South Africa. Stability of the test item and method of fabrication were documented by Afriplex Pty (Ltd) and the certificate of analysis supplied (see Appendix I). The certificate of analysis outlined the composition and physical and chemical properties of the extract. After the data was reviewed by the Study Director at Charles River Laboratory, Edinburgh, United Kingdom (UK) on 27th February 2009, the *G. africana* extract was approved for use in the study. Two containers, each holding approximately 1 L of the extract, were received under ambient conditions at Charles River.

This stock solution was used either undiluted or diluted in an appropriate vehicle for the studies described in the following sections. *G. africana* doses and concentrations used in these studies were expressed as the ammonium salt and adjusted for the strength of the stock solution. Chemical analysis of *G. africana* revealed that it contained total solids of 1.7% m/m and a

specific gravity of 0.8611. The stock solution was demonstrated to be stable for at least 48 months at ambient room temperature in the dark. Dose formulations were generally prepared on the day of use. A DMA 38 Density Meter (No. 005260) was used to record the *G. africana* density of 0.883 g/ml and this value was used in preparing the dose formulations. Formulation analysis with regard to concentration, homogeneity and stability was done by Charles River Laboratories. Furthermore, the plant name has been checked with http://www.theplantlist.org.

3.3.2 Testing facilities and regulatory compliance

The studies described in the following sections were performed by Charles River Laboratories. All animal experiments were conducted in the P39 room of the toxicology accommodation at Charles River. Chemical analysis of the extract was performed at Afriplex (Pty) Ltd. All the studies were performed according to OECD Guidelines for the Testing of Chemicals and Good Laboratory Practices (see Appendix II). Study protocols were approved by the testing facilities' Institutional Animal Care and Use Committees. Animal husbandry and use were in accordance with applicable local and international regulations and guidelines. All animals were supplied by Charles River UK Limited, Test Facility Study No. 515938 (Kent, UK). The animals were allowed to acclimatize to the toxicology housing at these laboratories for at least 7-8 days before dosing.

3.3.3 Acute oral toxicity study

The acute oral toxicity test of *G. africana* extract was evaluated in mice according to the procedures outlined by the OECD guideline No. 423 (OECD 2001). It was conducted to assess the adverse effects which may result within a short time period following a single oral

administration of *G. africana* extract. Oral administration was selected as it is a potential route of accidental human exposure and it allows for hazard classification.

3.3.3.1 *Animals and management*

Nine female (nulliparous and non-pregnant) rats of the Sprague-Dawley Crl:CD (SD) strain were used. The animals were supplied by Charles River UK Limited (Kent, UK). They were received in a batch that was 6-7 weeks old and that weighed 165-177 g at dispatch.

3.3.3.2 *Acclimatization, dose group allocation and animal identification*

Animals were allowed to acclimatize to the toxicology accommodation at these laboratories for at least 14 days before dosing. No formal randomization protocol was followed when allocating the animals to the groups. On the day of arrival, the animals were removed from their transport box in a random fashion and assigned to dose groups by placing them in cages labeled with at least the study number, animal number and group number. Each animal received a unique ear mark, through manual punch, which identified it individually within the study and which corresponded to that particular animal's number.

3.3.3.3 *Room environment*

The animals were housed in groups of three in polypropylene cages (dimensions 61 x 43.5 x 24 cm) with stainless steel grid tops and solid bottoms. Wood shavings were provided by Datesand (Manchester, UK) and used as bedding. Wooden chew sticks were placed in each cage and they were supplied by Estap OÜ, Harjumaa, Estonia). The environment was also monitored throughout the day and recordings made every 15 min. Standard conditions were maintained from the time the animals arrived until the end of the observation period. These included an

average of temperature 21°C, average daily humidity of 36% and 83% (minimum and maximum respectively), a 12 h light/dark cycle (7:00-19:00) and minimum ventilation of 15 air changes per h. Each cage was also provided with a water bottle and food hopper.

3.3.3.4 *Room sanitation, diet and water*

Cages, cage racks and water bottles were changed weekly during the study period. In addition, the floor was cleaned daily with 0.5% Tego 2000 disinfectant solution (Th. Goldschmidt and Company Limited, Middlesex, UK). The walls and ceiling were cleaned weekly. Rat and Mouse No. 1 Maintenance Diet (Special Diets Services Limited, Essex, UK) and water taken from the public supply (Scottish Water, Edinburgh, Midlothian, UK) were available *ad libitum*, except for a period of overnight food deprivation before dosing. *Ad libitum* feeding was resumed as soon as possible after dosing. Each batch of the diet was analyzed routinely by the supplier for numerous nutritional components as well as chemical and microbiological contaminants. Water quality was assessed and certificate of analysis for dissolved materials, heavy metals, pesticide residues, pH, nitrates and nitrites were periodically supplied.

3.3.3.5 *Experimental design*

Dose groups

Nine female rats were identified, allocated to dose groups and treated as follows (Table 3.1):

Table 3.1: Dosage and dose volume of *G. africana* administered to animals in different groups.

Group	Dosage (mg/kg)	Dose volume (ml/kg)	Animals
1	300	0.34	1-3
2	2000	2.27	4-6
3	2000	2.27	7-9

Justification of route and dosage

Oral administration was selected for this study as it a potential route that humans can be accidentally exposed to test substances. It also allows for hazard classification of test substances. Due to the fact that the study director's review of *G. africana* revealed that low toxicity was likely, a starting dose of 300 mg/kg was thought to be appropriate and it was administered to three female rats (Group 1). Following the administration of 300 mg/kg, no adverse signs were observed after 48 h, hence, another group of three female rats (Group 2) was included that received a dosage of 2000 mg/kg, which is the next dosage stated in the regulatory guideline for this study (OECD No. 423). There were no adverse signs in these animals either and so to complete the study a further three females (Group 3) received a dosage of 2000 mg/kg.

Route and duration of treatment

Each animal received a single oral administration by means of a gavage tube fitted to a graduated syringe. Treatment was followed by a 14 day observation period. The groups were dosed at least 2 days apart. The dose was calculated on the basis of the animals' body weights on the day of dosing.

3.3.3.6 *Observations*

All animals were checked for viability early in the morning and again as late as possible on each day. Animals were also observed for reaction to treatment at least 5 times on the day of dosing (Day 1) and once daily thereafter, until Day 15. The body weights of each individual animal were recorded on Day 1 as well as on Days 8 and 15.

3.3.3.7 *Terminal studies*

All animals were subjected to a gross necropsy at the end of the observation period on Day 15. Animals were euthanized by exposure to an increasing concentration of carbon dioxide and major blood vessels were cut to exsanguinate. The necropsy consisted of an examination of the cranial, thoracic and abdominal organs and tissues *in situ*. Carcasses were discarded after this procedure.

3.3.3.8 *Statistical analysis and electronic data capture software used*

No formal statistical analysis was carried out. Test item/control formulation: Dispense version 7.0.3.7 was used to electronically capture the data.

3.3.4 Acute dermal toxicity study

The acute dermal toxicity test was conducted in accordance with the OECD guideline No. 402 for chemical testing (OECD 1987; OECD 2015a). It was conducted to assess the adverse effects that may occur within a short time period following a single dermal administration of *G. africana* extract. Dermal administration was chosen as it is a potential route of accidental human exposure and allows hazard classification to be evaluated.

3.3.4.1 *Animals and management*

All animal experiments were conducted in the P39 room of the toxicology accommodation at Charles River. Five male and five female (nulliparous and non-pregnant) rats of the Sprague Dawley Crl:CD(SD) strain, obtained in a batch, were used. All animals were supplied by Charles River UK Limited, Test Facility Study No. 515938 (Kent, UK). They were approximately 8 weeks old and weighed 291-301 g (males) and 182-190 g (female) on dispatch.

3.3.4.2 *Acclimatization, dose group allocation and animal identification*

Animals were allowed to acclimatize to the toxicology housing at these laboratories for at least 7 days before dosing. No formal randomization procedure was utilized. On arrival the animals were removed from their transport box in a random fashion and assigned to dose groups by placing them in cages labeled with at least study number, animal number and group number. Each animal received a unique ear mark, by manual punch, which identified it individually within the study and which corresponded to that animal's number.

3.3.4.3 *Room environment*

The animals were housed individually in cages (dimensions 61 x 43.5 x 24 cm) with steel grid tops and solid bottoms. Wood shavings were provided by Datesand (Manchester, UK) and used as bedding. Wood shavings and wooden chew sticks were placed in the cages and supplied by the same companies outlined in the acute oral toxicity study. The bedding and chew sticks did not contain any extra substances in adequate amounts to influence the outcome of the study. Every cage contained a water bottle and food hopper. The environment was monitored throughout the day and recordings made every 15 min. The animals were maintained under the same standard conditions outlined in the acute oral toxicity study. Each cage was also provided with a water bottle and food hopper.

3.3.4.4 *Room sanitation, diet and water*

Cages, cage racks and water bottles were changed weekly during the study. The floor was cleaned everyday with 05% ‘Tego 200’disinfectant solution. The walls and ceiling were cleaned weekly. Diet and water was supplied as outlined in the acute oral toxicity study *ad libitum*. Each batch of the diet was analyzed routinely by the supplier for numerous nutritional components as

well as chemical and microbiological contaminants. Water quality was assessed and certificate of analysis for dissolved materials, heavy metals, pesticide residues, pH, nitrates and nitrites were periodically supplied.

3.3.4.5 *Experimental design*

Dose group, justification of route and dosage

Five animals per sex were identified and allocated to the dose group. The treatment was as follows: Group number 1, dosage of 2000 mg/mg and dosage volume of 2.3 mg/kg. Five animals per sex (n=5) were identified and allocated to the dose group, *i.e.* males (1-5) and females (6-10).

Dermal administration was selected for this study due to the possibility of accidental human exposure via this route. The dosage applied was 2000 mg/kg based on OECD guideline 402 which states that a limited test at one dose level should be 2000 mg/kg body weight for this type of study.

3.3.4.6 *Route and duration of treatment*

G. africana was applied topically in a single 24 h application under occlusion. 24 h prior to applying *G. africana*, the hair was clipped from the dorsal area of the trunk of each rat (approximately 6 cm x 7 cm). Care was taken to prevent grazing the skin. On the day of treatment, the appropriate amount of *G. africana* was applied topically onto the dorsal skin and spread uniformly over as much of the clipped area of the trunk as possible. *G. africana* was then covered by a gauze patch (approximately 5 cm x 6 cm) and semi-occlusive tape (‘Micropore’, 3M Medical-Surgical Division, USA) and secured in place with a strip of non-irritating occlusive tape (‘Sleek’, Smith and Nephew Medical Limited, USA) wound around the trunk.

The administered doses were calculated on the basis of the body weights of the animals on the day of dosing and the dose volume was based on the density of the test item (0.883 g/ml). The males were treated first and the females were treated one week later. On the basis that the test item covered approximately 80% of the patch and that the mean surface areas of the rats were approximately 425 cm^2 (males) and 338 cm^2 (females), the following calculations were made:

Table 3.2: Mean dose volumes of *G. africana* application based on body surface areas.

Sex	Mean dose (ml)	Body surface covered (%)	Dosage (mg/ cm^2)
Males	0.78	6	29
Females	0.55	7	20

Table 3.2 outlines the mean does volumes applied to the body surface of the animals. After a contact period of approximately 24 h, the patches were removed, the dosing sites were demarcated and the skin was wiped with water. This was followed by a 14 day observation period.

3.3.4.7 *Observations*

All animals were checked daily for viability early in the morning and again as late as possible. The animals were also inspected for reaction to treatment six times on the day of dosing (Day 1) and everyday afterwards until they were killed on Day 15. Each animal's body weight was assessed and recorded on Day 1 and again on Days 8 and 15.

3.3.4.8 *Terminal studies*

All animals were subjected to a gross necropsy at the end of the observation period on Day 15. Animals were euthanized by exposure to increasing concentrations of carbon dioxide and major

blood vessels were severed to exsanguinate them. The necropsy consisted of examination of the cranial, thoracic and abdominal organs and tissues *in situ*. Lesions were recorded in descriptive terms. Abnormal tissues were collected and preserved in 10% neutral buffered formalin. Carcasses were discarded following this process.

3.3.4.9 *Statistical analysis and electronic data capture software used*

No formal statistical analysis was carried out. Dose formulation: Dispense version 7.0.3.7 was used to electronically capture the data.

3.3.5 Skin sensitization test using the local lymph node assay (LLNA)

The local lymph node assay was conducted in accordance with the OECD test guideline No. 429 (OECD 2010). This study examined the delayed contact hypersensitivity potential of *G. africana* in CBA/Ca mice.

3.3.5.1 *G. africana and control formulations*

A pre-dose formulation trial was carried out that revealed that the preferred vehicle, acetone: olive oil (4:1 v/v) did not produce a formulation that was appropriate for dosing. However, results from the trial showed that dimethylformamide was a more suitable vehicle and was thus selected to be used in this study.

G. africana dose formulations were prepared volumetrically on the day of dosing. The required amount of *G. africana* was weighed and the necessary quantity of dimethylformamide was added until the graduated mark was reached (this was only done for Group 2 and 3 formulations). The *G. africana*/dimethylformamide mixture was then manually mixed using a spatula until a visibly homogenous formulation was obtained. A suitable amount of *G. africana* extract was

administered to animals in Group 4 as supplied (100% undiluted state). Group 1 (control group) animals were administered with an appropriate amount of dimethylformamide. On Day 1 of the main phase, the formulations that were prepared had pH values ranging from 4.5 to 5.5.

3.3.5.2 *Radioisotope*

The radioisotope used was [*methyl*-3H] thymidine (batch No. 335) obtained from GE Healthcare UK Limited (Buckinghamshire, England, UK). This was stored in the refrigerator, away from light, in the radiochemistry laboratory at Charles River. The supplier indicated that the purity of this chemical was 98.7%.

3.3.5.3 *Animals and management*

Twenty-two female (nulliparous and non-pregnant) mice of the CBA/Ca strain were used for this assay. All animals were supplied by Charles River UK Limited (Kent, UK) to Charles River International Laboratory (Edinburgh, UK). They were 7-8 weeks old and weighed 16-20 g on dispatch.

3.3.5.4 *Acclimatization, dose group allocation and animal identification*

The animals were allowed to acclimatize to the toxicology housing at these laboratories for at least 8 days prior to the start of dosing. There was no formal randomization procedure carried out when allocating the animals to the dose groups. On arrival, the animals were randomly removed from their transport box and allocated to dose groups by placing them in cages labeled with at least study number, animal number and group number. Each animal received a subcutaneous implant which individually identified it within the study and which corresponded to that animal's number.

3.3.5.5 *Room environment*

The animals were accommodated in cages (dimensions 36.5 x 20.7 x 14 cm) and were placed in them in groups of 2 or 3. The cages had a stainless steel grid top, a water bottle and were incorporated with a food hopper. The cages were provided with wood shavings, used as bedding, and nesting material ('Nestlets') both supplied by Datesand Limited (Manchester, UK). In addition, wooden chew sticks were placed in every cage (Estap OÜ, Harjumaa, Estonia). Analysis of the bedding, nesting material and chew sticks was done and it revealed that there was no contamination. The environment in which the animals were housed was monitored throughout the day and recordings were made every 15 min. The standard conditions were; average daily temperatures of 19-21°C, average daily relative humidity 54-70%, lighting 12 h light/dark cycle (7:00-19:00) and ventilation minimum of 15 air changes per h.

3.3.5.6 *Room sanitation, diet and water*

Cages, cage racks and water bottles were changed weekly during the study period. The floor was cleaned daily with same disinfectant used in the other studies (0.5% Tego 2000). The wall and the ceiling were cleaned weekly. Diet and water were available *ad libitum* throughout the study as outlined in the acute oral and dermal toxicity studies. The supplier conducted routine analysis of each diet batch to assess the nutritional components as well as any chemical and microbiological contaminants. The water was analyzed for dissolved materials, heavy metals, pesticide residues, pH, nitrates and nitrites. The analysis of the diet and water revealed that there was no contamination.

3.3.5.7 *Experimental design*

Dose groups

In order to determine whether *G. africana* extract had any toxic effects, the preliminary and main phases were conducted.

Preliminary phase

In this phase of the study, two female mice were identified, allocated to the dose group and treated. This group (Group 5) consisted of animals 21 and 22 that received a *G. africana* treatment of 100% (undiluted extract).

Main phase

Following the preliminary phase, test concentrations were selected for the main phase and five animals per group were identified, allocated to dose groups and treated as follows as outlined in Table 3.3: Group 1 received dimethylformamide, Group 2 received 25% (50 mg/ml) extract, Group 3 received 50% (100 mg/ml) extract and Group 4 received 100% (200 mg/ml) extract.

Table 3.3: Treatment of animals with the vehicle and formulations of *G. africana*.

Group	Treatment	Formulation concentration (%)	Animal
1	Dimethylformamide	0	1-5
2	*G. africana* extract	25	6-10
3	*G. africana* extract	50	11-15
4	*G. africana* extract	100	16-20

Positive control

The local lymph node assay was regularly checked, at the laboratories, in order to validate this test method. Hexyl cinnamicaldehyde was used as a positive control at concentrations of 5%, 10% and 25% because it was considered to be a sensitizer in CBA/Ca mice.

Justification of dose group

At the time of the study, there was no data relating to the potential of *G. africana* components to cause local irritation. Investigations conducted by the study director showed that ethanol had an oral LD_{50} value of 3450 mg/kg in mice. A preliminary study was conducted in order to find out whether the undiluted extract could result in local irritation or systemic toxicity.

Results from the preliminary study revealed that there were no signs of local irritation or systemic toxicity and the body weight of the animals was not affected. As a result, dose concentrations of 25%, 50% and 100% were selected as suitable non-toxic doses to be administered in the main phase. This concentration series is outlined in the OECD guideline No. 49.

Route and duration of treatment and collection of lymph nodes

Preliminary phase

For 3 consecutive days (Days 1-3), an open application of 25 µl of undiluted *G. africana* extract was administered to the dorsum of each ear of the animals. The animals did not receive treatment on Days 4 and 5.

Main phase

In this phase, for 3 consecutive days (Day 1-3), animals received an open application of 25 µl of the appropriate formulation onto the dorsum of each ear. Similar to the preliminary phase, there was no treatment on days 4 and 5. On day 6, each animal received an intravenous injection (250 µl) of phosphate buffered saline (PBS) containing approximately 20 µCi of [*methyl*-3H] thymidine (GE Healthcare UK Limited, England, UK) into the lateral tail vein. Approximately 5 h following intravenous administration, all animals were killed by exposure to increasing concentrations of carbon dioxide and the major blood vessels were severed to exsanguinate the animals. Each pair of draining auricular lymph nodes was collected from each animal and the animals were then discarded. A single cell suspension of lymph node cells from each paired sample was prepared by gentle disaggregation through 200 µm mesh stainless steel gauze. The lymph nodes cells were then washed twice in excess of PBS and precipitated with 5% trichloroacetic acid at 2-8°C for approximately 19 h. The pellets were re-suspended in 200 µl 'Solvable' and transferred to vials containing 10 ml scintillation fluid (Aquasafe 500 plus liquid, Zinsser Analytic, Maidenhead, UK). Incorporation of titrated thymidine was measured by β-scintillation counting and was expressed as disintegrations per minute (DPM).

3.3.5.8 *Observations*

All animals were checked for viability early in the morning and again as late as possible daily. For both the preliminary and main phases, the body weight of each individual animal was recorded on Day 1 (before the first dose) and on Day 6 (before the thymidine injection).

Daily observations

Preliminary phase

Animals were inspected for any signs of reaction to treatment on each day of dosing. Observations were performed pre-dose, immediately post dose and then a minimum of 3 more times within 4 h of dosing. Thereafter observations were performed once daily until Day 6. The animals were killed by cervical dislocation on Day 14 and then later discarded.

Main phase

Just like the preliminary phase, animals in this phase were also examined for reaction to treatment. On each day of dosing, the observations were performed prior to dosing, immediately after dosing and approximately 1, 2, 3 and 4 h after dosing. From then on, animals were monitored once daily until they were killed on Day 6 (day of thymidine injection).

3.3.5.9 *Calculation of results*

The results were corrected for background radiation and expressed as stimulation index (SI). The SI was obtained by dividing the mean DPM obtained from each group by the mean DPM of the control group.

$$SI = \text{Mean group DPM} \div \text{Mean control DPM}$$

The SI for the control group would consequently be 1. A positive response was indicated by an SI value of ≥ 3 accompanied by consideration of dose-response and, where applicable, statistical significance. Since none of the groups produced SI values ≥ 3, it was not possible to calculate the estimated concentration of *G. africana* that would produce a 3-fold increase in draining lymph node cell proliferation (i.e. the EC3 value). No formal statistical analysis was performed.

3.3.6 SkinEthic EpiSkin® skin irritation assay

The SkinEthic EpiSkin® irritation assay was conducted in accordance with OECD 439 (OECD 2015b). This skin irritation assay measures the skin irritation potential of a test item by assessing the cytotoxic effects after a specific exposure period and recovery time. The endpoint of this assay is the estimation of the cell viability by evaluating the cell viability by means of the cells' ability to reduce the MTT to its formazan metabolite via mitochondrial reductase. Irritant materials are classified as those that are able to reduce cell viability below a threshold of 50% of the negative control value.

3.3.6.1 *Chemicals and reagents*

EpiSkin® kits containing tissue culture units and assay medium/maintenance medium were supplied by Episkin SNC (Lyon, France). Eugenol was supplied by Sigma Aldrich, Germany. Dulbecco's phosphate buffered saline (PBS), sodium dodecyl sulphate, methylthiazoldiphenyl-tetrazolium bromide (MTT), isopropanol and hydrochloric acid (fuming) were obtained from Sigma-Aldrich Inc. (St Louis, MO, USA). All other materials were provided by Charles River (Edinburg) and were of analytical grade where possible.

3.3.6.2 *Justification of the Episkin® skin irritation test system*

Evaluation of skin irritation potential of test items normally involves the use of laboratory animals as outlined by the OECD guidelines for toxicity studies. However, there is concern pertaining to pain and suffering experienced by the animals when these studies are conducted and this issue has been addressed in animal protection legislation (Louhimies 2002). Article 23 of Council Directive 86/609/EEC states that:

"The Commission and Member States should encourage research into the development and validation of alternative techniques which could provide the same level of information as that obtained in experiments using animals but which involve fewer animals or which entail painful procedures, and shall take other steps as they consider appropriate to encourage research in this field. The Commission and Member States shall monitor trends in experimental methods" (Louhimies 2002).

This is the reason why this skin irritation study was conducted in order to replace, reduce and refine animal use while ensuring that this assay does not affect the integrity of the test or the results produced (Louhimies 2002). Furthermore, the European Centre for the Validation of Alternative Methods (ECVAM) sanctioned the used of the Episkin® skin irritation assay in a validation statement released in April 2007. ECVAM describes the test as demonstrating evidence of being a reliable and relevant standalone test for predicting skin irritation through the assessment of MTT reduction. The skin irritation tests can also be used, according to ECVAM, to categorize test substances as being irritants or non-irritants to the skin (Hartung 2007).

3.3.6.3 *Episkin® Reconstructed Human Epidermis*

The EpiSkin® reconstituted human epidermis (RhE) models were obtained from Episkin SNC, Lyon, France. EpiSkin® technical data, safety sheet and certificate of analysis were provided (see Appendix III). The RhE models were generated by culturing adult human keratinocytes on a collagen base in conditions that allow their terminal differentiation and the reconstruction of an *epidermis* containing a functional horny layer (*stratum corneum*). The human keratinocytes are collected from mammary or abdominal samples obtained from healthy consenting donors during plastic surgery. HIV 1 and 2, HEP B and HEP C tests are conducted on the donors. The

bacteriological and fungal sterility of the cells is also verified. During the emersion process (10 days), the *epidermis* differentiates and a horny layer is produced. The reproducibility of each batch is assessed by means of histological analysis taking into consideration the general organization, the stratification of the epidermis, the nucleation of the basal layer and the size of the intercellular spaces, adhesion of the basal layer to the support and the quality of the granular cells and horny layer. Every single criterion is scored out of 4 with a maximum score of 28. The minimum score for the criterion to be accepted as a model is 19.5. The reproducibility of the response of each EpiSkin® batch is tested against a reference irritant which is SDS. The IC_{50} of the surfactant is measured by the MTT assay after 18 h of contact. The minimum score for the acceptability of the model is 1.5 mg/ml.

3.3.6.4 *Episkin® units and pre-incubation*

Upon arrival of the EpiSkin® kits at Charles River, examination of their quality was done. The pH and temperature indicators were both in acceptable ranges. The units were placed in a 12 well microtitre plate with each individual unit being transferred to a single well containing 2 ml maintenance medium pre-warmed in a 37°C water bath. Before dosing, all units were incubated for 24 h at 37ºC in a humidified atmosphere with 5% CO_2.

3.3.6.5 *Formulation of In-Use Dilution*

To prepare the in-use dilution (1%, v/v), the concentrate *G. africana* extract (0.1 ml) was added to water (9.9 ml) and mixed by vortexing. The concentrated extract contains plant extract in ethanol at a concentration of 20% (w/v). Therefore, the concentration of the plant extract in the in-use dilution was 0.2% (w/v).

3.3.6.6 *MTT direct reduction by G. africana*

Prior to conducting the irritation assay, *G. africana* was tested to determine if it was capable of reducing MTT to its formazan metabolite. Since the assay depends on the ability of viable skin cells to reduce MTT to formazan, any reduction by *G. africana* would interfere with the integrity of the results. 10 µl of *G. africana* was added to 2 ml MTT (approximately 0.3 mg/ml) in PBS in a glass universal vial (three replicates) and incubated at 37°C in a humidified atmosphere with 5% CO_2 for approximately 3 h. Formazan production was assessed by visual inspection. Three replicates of the positive control (eugenol, 10 µl) and negative control (sterile, ultra-pure water, 10 µl) were tested in parallel to demonstrate the efficacy of the MTT solution.

3.3.6.7 *G. africana application and terminal exposure procedures (15 min post-dose)*

Concentrated *G. africana* extract and the in-use dilution were each applied onto the exposed surface of three viable EpiSkin® reconstructed human *epidermis* units using a Gilson positive displacement pipette set to deliver 10 µl. The test item was gently spread over the entire exposed skin surface using the tip of the pipette, taking care to avoid damaging the *epidermis*. The surface area of the EpiSkin® was 0.38 cm^2, therefore, the application rate was 26.3 µl/cm^2. After exposure to the *G. africana* or positive and negative controls for 15 min, the EpiSkin® units were washed by rinsing with Dulbecco's phosphate buffered saline (25 ml). After washing, all skin units were blotted dry with tissue paper, transferred to fresh maintenance medium and incubated for 42 h at 37°C in a humidified atmosphere with 5% CO_2.

3.3.6.8 *MTT test*

After the recovery period, all EpiSkin® units were transferred to a new 12 well microtiter plate containing MTT solution (2 ml per well, 0.3 mg/ml) in assay medium. Each unit was tapped

gently on tissue paper to remove residual moisture before transferring to the MTT solution. The skin units were then incubated for 3h at 37°C in a humidified atmosphere with 5% CO_2. After incubation, each skin unit was removed from the MTT solution and gently tapped dry on tissue paper to remove any excess moisture. The exposed skin was then completely removed from the unit using a specially designed biopsy punch. The *epidermis* was separated from the collagen matrix and both layers added to an appropriately labeled microfuge tube containing acidic isopropanol (500 µl). The tubes were capped and the contents mixed by vortexing. The biopsies were completely immersed in the solvent. Samples were then stored at about 4°C protected from light.

3.3.6.9 *Absorbance Measurement and calculation of results*

After 69 h storage, the samples were removed from the refrigerator and mixed by vortexing to ensure a homogenous mixture. Two aliquots (200 µl) were then added to a 96 well flat bottom microtiter plate for each sample, ensuring that no solid material was removed from the sample tube. Plates were analyzed using an MRX plate reader at a wavelength measurement of 550 nm. Absorbance values were calculated against the background acidified isopropanol sample contained on the plate.

Optical Density (OD)

OD Treated Tissue (TT) $= OD_{TT\ raw} - OD_{blank\ mean}$

OD Negative Control (NC) $= OD_{NC\ raw} - OD_{blank\ mean}$

The corrected mean OD Negative Control corresponds to 100% viability

OD Positive Control (PC) $= OD_{PC\ raw} - OD_{blank\ mean}$

Individual Viabilities (%)

% Positive Control 1 = [OD_{PC1} / Mean OD_{NC}] x 100

% Positive Control 2 = [OD_{PC2} / Mean OD_{NC}] x 100

% Positive Control 3 = [OD_{PC3} / Mean OD_{NC}] x 100

% Treated Tissue 1 = [OD_{TT1} / Mean OD_{NC}] x 100

% Treated Tissue 2 = [OD_{TT2} / Mean OD_{NC}] x 100

% Treated Tissue 3 = [OD_{TT3} / Mean OD_{NC}] x 100

Mean Viabilities (%) and Relative viability (%)

Mean Positive Control % = (%PC1 + %PC2+ %PC3) / 3

Mean Treated Tissue % = (%TT1 + %TT2 + %TT3) / 3

% Viability = [OD_{TT} / OD_{NC}] x 100

Acceptance Criteria

The assay was deemed acceptable if the following occurred:

The mean OD value of the 3 negative control tissues was ≥ 0.6 and the standard deviation value (SD) of the % viability was ≤ 18. The mean % viability of the 3 positive control tissues was ≤ 30% and the SD was ≤ 18. The mean % viability SD of the 3 treated tissues was ≤ 18.

Interpretation of skin irritancy

According to the GHS category 2, a test chemical is considered to be irritant if the tissue viability after exposure and post-treatment incubation is less than or equal (≤) to 50%. However, classification can differ depending on country/regional regulatory requirements. In this case, the test chemical can be considered as "no category" if the tissue viability after exposure and post-treatment incubation is greater than (>) 50%.

3.4 Results

3.4.1 Acute oral toxicity study

There were no unscheduled deaths among animals receiving *G. africana* extract at either the 300 mg/kg or 2000 mg/kg dosage. There were no adverse signs of reaction to treatment recorded at either the 300 mg/kg or 2000 mg/kg dosage (Table 3.4). Body weight gain was considered to be acceptable for rats of this age and strain (Figure 3.1). Macroscopic examination on Day 15 revealed no abnormalities in any animal (Table 3.5).

Table 3.4: Individual daily observations of the effect of *G. africana* on the animals in Group 1 (300 mg/kg) and Groups 2 and 3 (2000 mg/kg).

		Time after dosing on day 1 (h)					
Group	Sign	At dosing	¼-1 ¼	2-2 ½	3-4	4¼ -5 ½	Day 2-15
1-3	No abnormalities detected (NAD)	1-9	1-9	1-9	1-9	1-9	1-9

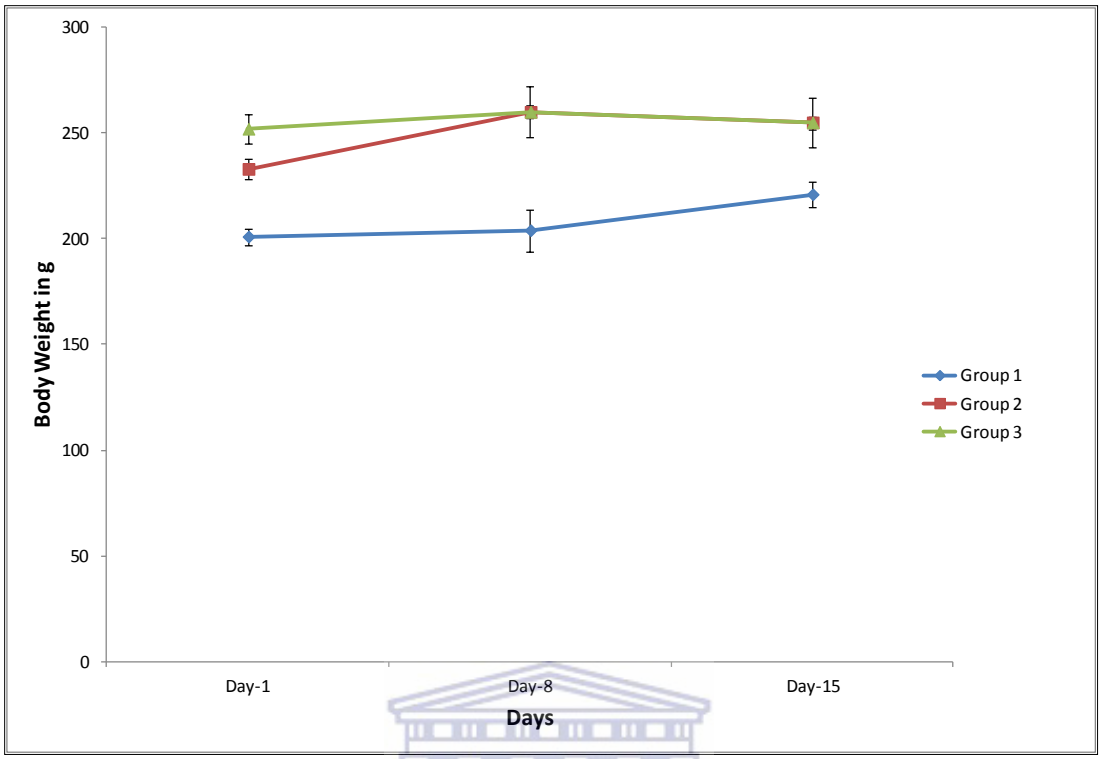

Figure 3.1: Trends in mean body weight gain of animals treated with *G. africana* extract at concentrations of 300 mg/kg (Group 1) and 2000 mg/kg (Groups 2 and 3) within a 15 day period.

Table 3.5: Individual necropsy findings of the effect of *G. africana* on the animals in Group 1 (300 mg/kg) and Groups 2 and 3 (2000 mg/kg).

Group	Animal	Individual finding	Day of death
1	1	No abnormalities detected (NAD)	15
	2	NAD	
	3	NAD	
2	4	NAD	15
	5	NAD	
	6	NAD	
3	7	NAD	15
	8	NAD	
	9	NAD	

3.4.2 Acute dermal toxicity study

There were no unscheduled deaths during the observation period. There were no systemic signs of toxicity recorded in any animal at any observation time-point (Table 3.6). Body weight gain was considered to be acceptable for rats of this age and strain (Figure 3.2).

Table 3.6: Individual daily observations of the effect of *G. africana* (2000 mg/kg) on the animals.

Group/Sex	Sign	Day(s) of Study	
		1-7	**8-15**
Male	No abnormalities detected (NAD)	1-5	1-5
Female	NAD	6-10	6-10

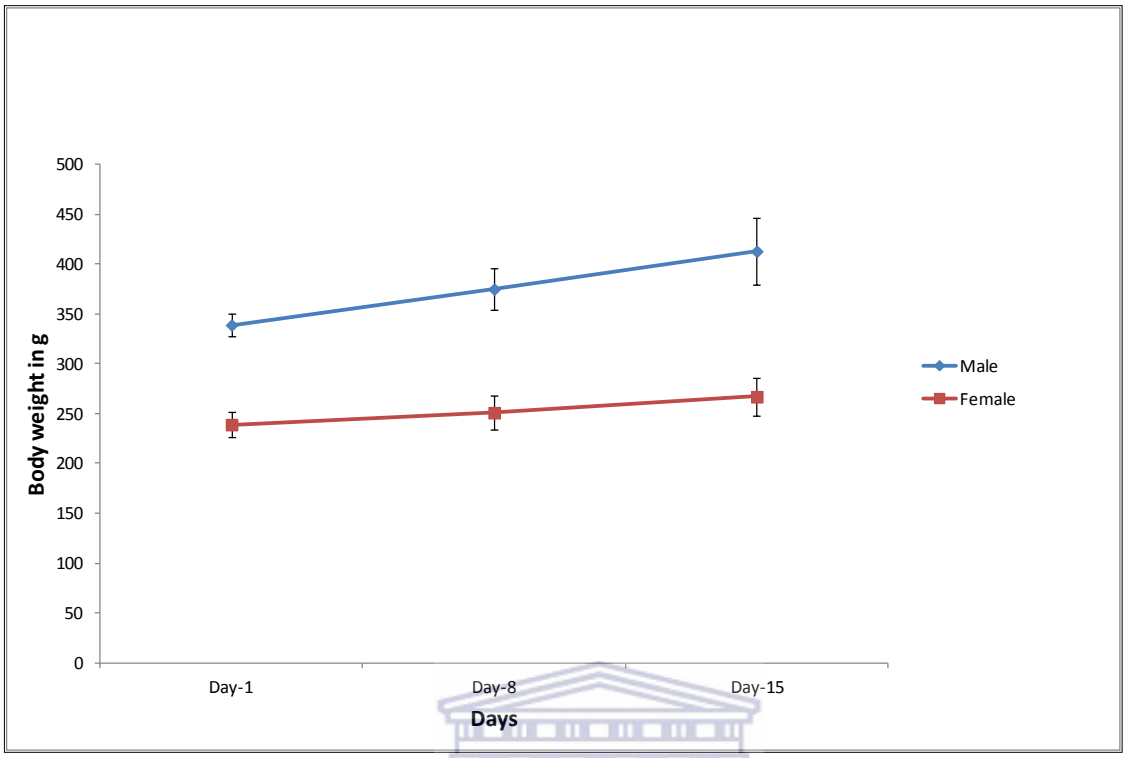

Figure 3.2: Trends in mean body weight gain of male and female rats after a single dose application of *G. africana* extract (2000 mg/kg) within a 15 day period.

Necropsy of the females on Day 15 revealed no macroscopic abnormalities. Among males, enlargement of the mandibular lymph nodes was recorded in 4 of the 5 animals. Other macroscopic abnormalities included dark and/or pale foci in the lungs, which affected 3 males, and intestinal abnormalities such as pale appearance and/or abnormal contents of the jejunum, which was recorded in 2 males. In addition there were single instances of reddening of the lungs, prominence of Peyer's patch, speckling of the thymus and distension of the ileum. The necropsy findings seen in males are commonly observed background findings in rats of this strain and are not considered to be a result of dermal application of *G. africana* extract (Table 3.7).

Table 3.7: Individual necropsy findings of the effect of *G. africana* (200 mg/kg) on the animals.

Sex	Animal	Individual Finding
Male	1	Mandibular lymph nodes: enlarged (14x8x2 mm) Lungs: reddened, all lobes many dark foci, all lobes (red, pinpoint appearance) many pale loci, all lobes (cream, ≤3 mm diameter)
	2	Mandibular lymph nodes: enlarged (11x7x2 mm) Intestines: jejunum- pale Peyer's patch- prominent Thymus: specked, right lobe Lungs: dark focus, left lobe (red, 3 mm diameter)
	3	Mandibular lymph nodes: enlarged (14x8x2 mm)
	4	Intestines: jejunum- pale, with abnormal (white) contents Lungs: 3 dark foci, left lobe (red, 1 mm diameter)
	5	Mandibular lymph nodes: enlarged (11x6x2 mm) Intestines: ileum-distended by contents (6 mm)
Female	6-10	No abnormalities detected

3.4.3 Local lymph node assay

Results were corrected for background radiation and expressed as SI. This was obtained by dividing the mean DPM obtained from each group by the mean DPM of the control group. The SI for the control group, therefore, is one. A positive response is indicated by an SI ≥ 3, together with consideration of dose-response and, where appropriate, statistical significance. As there was no stimulation index ≥ 3 recorded for any group it was not possible to calculate the estimated concentration of test item that would produce a 3-fold increase in draining lymph node cell proliferation (the EC3 value). No systemic signs and no signs of local irritation were noted in either animal receiving undiluted *G. africana* extract. Body weight gain was considered to be acceptable for mice of this age and strain. Based on these findings, concentrations of 25% (50 mg/ml), 50% (100 mg/ml) and 100% (200 mg/ml) (*i.e.* undiluted) *G. africana* extract were selected for testing in the main phase. No systemic signs were noted in any animal during the

observation period. There was no effect of treatment on body weight (Figure 3.3). Although body weight losses were recorded in 4 of the 20 mice, they were restricted to animals treated with either the 25% (50 mg/ml) or 50% (100 mg/ml) concentrations. There were no body weight losses among control mice or among mice treated with undiluted *G. africana* extract. The stimulation indices for mice treated with the *G. africana* extract at concentrations of 25% (50 mg/ml), 50% (100 mg/ml) and 100% (200 mg/ml) were 1.3, 0.9 and 1.3, respectively (Table 3.8).

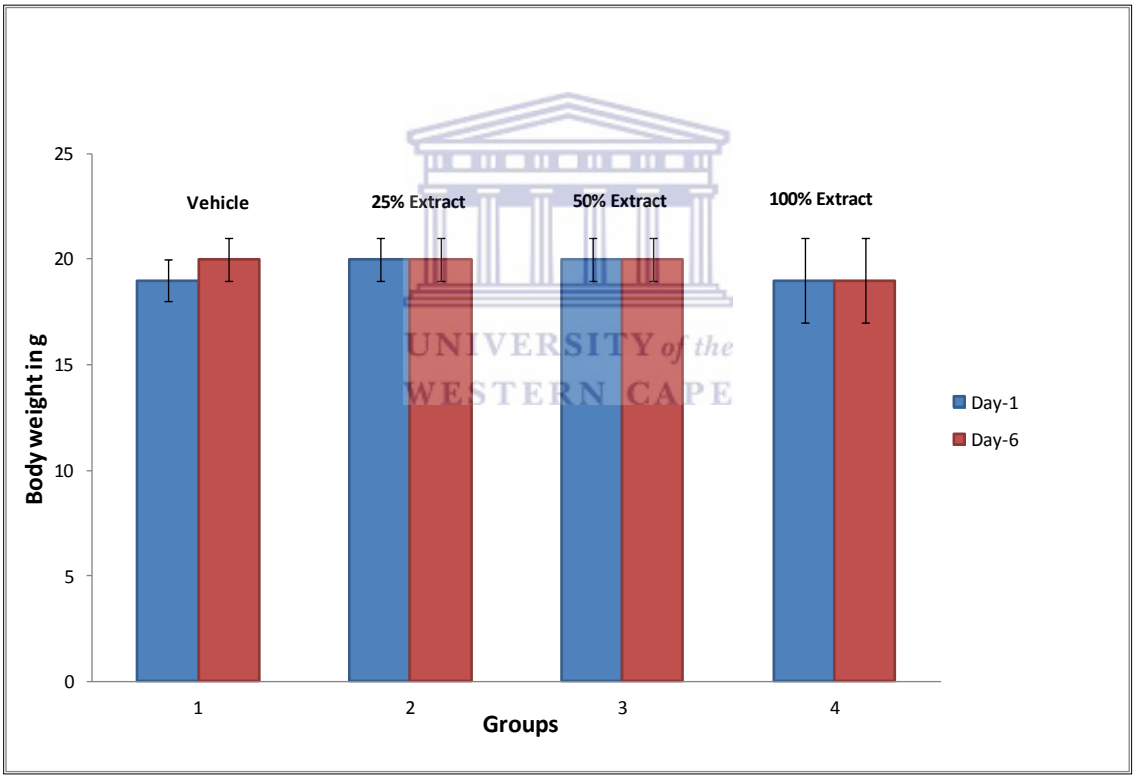

Figure 3.3: Trends in mean body weight gain of female mice after an open application of *G. africana* extract (25, 50 and 100%) and vehicle (dimethylformamide) within a 6 day period.

Table 3.8: Overall assessment of individual and group mean disintegration per minute counts (DPM) and SI values obtained after treatment with dimethylformamide (vehicle) and *G. africana* concentrations.

Group	Concentration (%)	Animal	DPM	Group Mean DPM	Stimulation Index
1	vehicle	1	-[a]	1456	1
		2	1760		
		3	1906		
		4	338		
		5	1821		
2	25	6	4269	1922	1.3
		7	653		
		8	1169		
		9	681		
		10	2840		
3	50	11	1235	1240	0.9
		12	-[a]		
		13	-[a]		
		14	1783		
		15	703		
4	100	16	1095	1829	1.3
		17	1542		
		18	2318		
		19	2260		
		20	1932		

[a]Suspensions for lymph node cells from animals 1, 12 and 13 were spilled during processing. Therefore, DPM counts from these animals are not presented in the Table.

3.4.4 SkinEthic EpiSkin® irritation assay

A graphical representation of the SkinEthic EpiSkin® irritation assay results are shown in Figures 3.4 and 3.5.

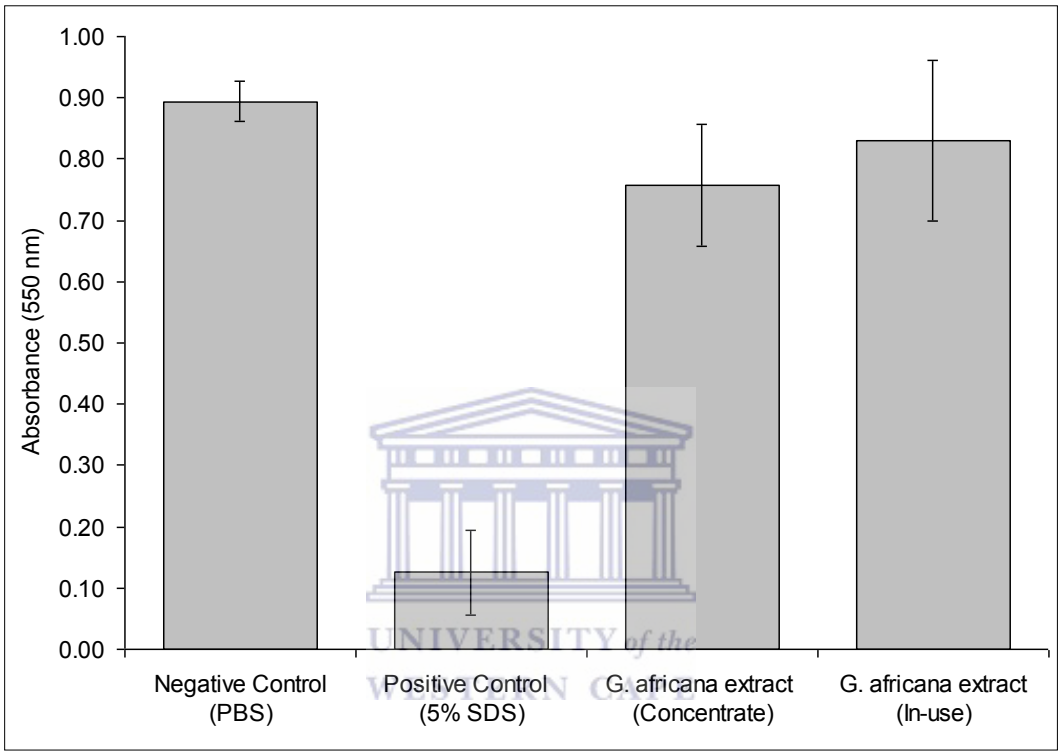

Figure 3.4: Optical density of EpiSkin® MTT test samples (mean and SD, n=6). However, mean and SD were calculated from n=4 test samples for positive and *G. africana* extract (concentrate).

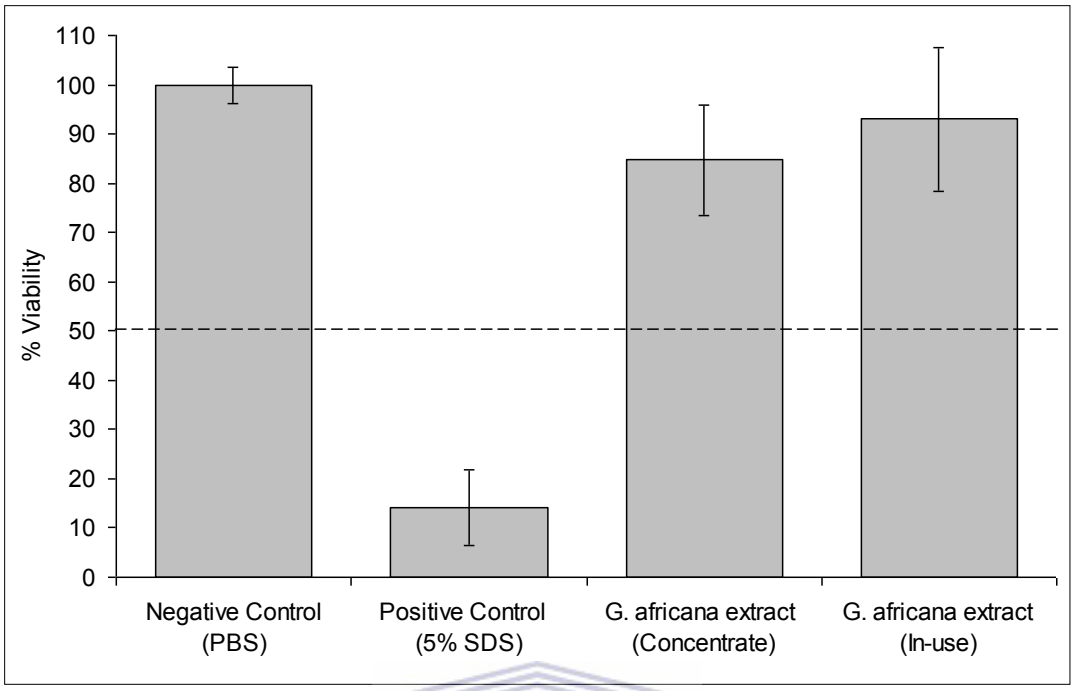

Figure 3.5: Relative cell viability of EpiSkin ® cultures treated with *G. africana* extract after 42 h recovery time (mean and SD, n=6). However, mean and SD were calculated from n=4 samples for positive and *G. africana* extract (concentrate).

----------Threshold value for positive response (50% of the negative control viability).

The results of the direct reduction test are shown in Table 3.9. The test was scored by visual assessment of the formation of the purple-colored formazan. The positive control (eugenol) reduced the MTT solution to formazan almost immediately, generating a dark purple color before incubation. The negative control (sterile ultra-pure water) and test items (*G. africana* concentrated extract and in-use dilution) did not reduce MTT to formazan after approximately 3 h incubation.

Table 3.9: Evaluation of direct reduction of MTT by *G. africana* extract.

Test Item	Replicate ID	Volume	MTT Solution Volume	Color change observed
G. africana extract Concentrate	Rep 1	10 µl	2 ml	No color change after 3 h incubation
	Rep 2	10 µl		No color change after 3 h incubation
	Rep 3	10 µl		No color change after 3 h incubation
G. africana extract Dilution (1%, v/v)	Rep 1	10 µl	2 ml	No color change after 3 h incubation
	Rep 2	10 µl		No color change after 3 h incubation
	Rep 3	10 µl		No color change after 3 h incubation
Sterile Water (Negative Control)	Rep 1	10 µl	2 ml	No color change after 3 h incubation
	Rep 2	10 µl		No color change after 3 h incubation
	Rep 3	10 µl		No color change after 3 h incubation
Eugenol (Positive Control)	Rep 1	10 µl	2 ml	Immediate dark blue/purple color change
	Rep 2	10 µl		Immediate dark blue/purple color change
	Rep 3	10 µl		Immediate dark blue/purple color change

The results of the EpiSkin® irritation assay with *G. africana* concentrated extract and in-use dilution are shown in Tables 3.10 and 3.11. Table 3.10 shows the mean corrected absorbance (OD) values for the positive and negative controls and for the test item. Table 3.11 shows the relative viabilities of the EpiSkin® cultures as a percentage of the mean negative control value. The negative control results were within the acceptance criteria defined in the ECVAM validation SOP. The positive control results were within the acceptance criteria defined in the ECVAM validation SOP. The results of the assay were similar for both viable EpiSkin® units

dosed with concentrated *G. africana* extract. Exposure to concentrated *G. africana* extract resulted in a mean EpiSkin® viability of 84.75% ± 11.26% of the negative control value. The results of the assay were similar for all viable EpiSkin® units dosed with *G. africana* extract (in-use dilution). Exposure to *G. africana* extract (in-use dilution) resulted in a mean EpiSkin® viability of 92.97% ± 14.63% of the negative control value.

Table 3.10: Optical density (OD) of EpiSkin® MTT test samples.

Test Item	Replicate ID	Corrected OD (550 nm)	Mean	SD	CV (%)
G. africana extract Concentrate	Rep 1	0.798	0.757	0.101	13.28
		0.880			
	Rep 2	0.664			
		0.687			
	Rep 3	*0.117*[*]			
		0.121[*]			
G. africana extract Dilution (1%, v/v)	Rep 1	0.688	0.831	0.131	15.73
		0.747			
	Rep 2	0.960			
		1.019			
	Rep 3	0.756			
		0.814			
5% SDS Solution (Positive Control)	Rep 1	*0.321*[*]	0.126	0.064	55.02
		0.278[*]			
	Rep 2	0.185			
		0.187			
	Rep 3	0.069			
		0.063			
PBS Solution (Negative Control)	Rep 1	0.840	0.894	0.033	3.70
		0.904			

	Rep 2	0.900			
		0.941			
	Rep 3	0.880			
		0.896			

*Data was rejected from the mean and SD calculations. See section 3.4.4.1.

Table 3.11: Percentage viability of EpiSkin® cultures treated with *G. africana* extract after 42 h recovery time.

Test Item	**Replicate ID**	**Viability after 42 h (%)**	**Mean (%)**	**SD (%)**	**CV (%)**
G. africana extract Concentrate	Rep 1	89.31	84.75	11.26	13.28
		98.49			
	Rep 2	74.31			
		76.89			
	Rep 3	*13.09*[*]			
		15.54[*]			
G. africana extract Dilution (1%, v/v)	Rep 1	77.00	92.97	14.63	15.73
		83.60			
	Rep 2	107.44			
		114.05			
	Rep 3	84.61			
		91.10			
5% SDS Solution (Positive Control)	Rep 1	*35.93*[*]	14.10	7.76	55.02
		31.11[*]			
	Rep 2	20.71			
		20.93			
	Rep 3	7.72			
		7.05			
PBS Solution (Negative Control)	Rep 1	94.01	100.00	3.70	3.70
		101.18			
	Rep 2	100.73			
		105.32			
	Rep 3	98.49			
		100.28			

*Data was rejected from the mean and SD calculations. See section 3.4.4.1.

3.4.4.1 *Sample Rejection*

Due to inadvertent excision of the reconstructed skin tissue from the collagen matrix, it was necessary to reject the results from one of the EpiSkin® units dosed with *G. africana* extract (concentrate). The optical density and cell viability were therefore calculated from 2 EpiSkin® units. It was also necessary to reject the results from one of the EpiSkin® units dosed with the positive control. This was due to incomplete spreading of the SDS over the surface of the EpiSkin®.

3.5 Discussion

Botanicals are attractive alternatives to synthetic agrochemicals because they are said to pose little threat to human health and to the environment (Isman 2006). In spite of the scale of scientific data documenting the activity of plant extracts, only a handful, such as neem oil, essential oils, pyrethrum and rotenone are used commercially on agricultural products because of increasingly stringent regulatory requirements (Isman 2006). Toxicity testing is vital as it facilitates the evaluation of the possible harmful side effects attributed to these products. Because a number of substances provoke allergic contact dermatitis in humans, assessment of safety by carrying out skin sensitization tests is crucial. Furthermore, it is imperative to evaluate the short or long term effects these products might have as well as the potential course of human exposure (Bras, Gumilar *et al.* 2011). For this reason, this study evaluated the effects of acute exposure of the ethanolic extract of *G. africana* in mice, rats and EpiSkin® reconstituted human epidermis. The local lymph node assay (LLNA) was utilized to assess skin sensitivity of *G. africana*. At the time of the study, no data pertaining to the potential of *G. africana* components to cause local

irritation in mice, rats or skin was available. This study was performed in accordance with the OECD guidelines and Principles of Good Laboratory Practice.

This study showed that at the highest (undiluted) concentration (2000 mg/ml) the extract had no toxic effects as demonstrated by the local lymph node assay. In addition, it did not elicit a hypersensitivity reaction in the mice. No concentration resulted in a stimulation index of ≥ 3 in any group. Consequently, it was considered that the test item does not have the potential to cause sensitization. In the acute oral and acute dermal toxicity studies, the median lethal oral dosage (LD_{50}) of *G. africana* extract was considered to exceed 2000 mg/kg. Evaluation of the effect of the extract on EpiSkin® reconstituted human epidermis revealed that the *G. africana* extract (concentrate) *G. africana* extract (in-use dilution) were non-irritants. These findings will be included in the documentation for a toxicological review to register *G. africana* extracts as pesticidal adjuvants and plant growth stimulants.

Environmental toxicology studies of *G. africana* extracts have been found to have a variety of inhibitory effects (EC_{50}) against *Vibrio fischeri* (0.7 µg/ml, 30 min), *Selenastrum capricornatum* (100 µg/ml, 72 h), *Daphnia pulex* (30 µg/ml, 48 h) and *Poecilia reticulata* (20 µg/ml 96 h) (Pool et al., 2009 a). The minimum inhibitory concentrations (MIC) of plants against various fungal pathogens (*Alternaria, Botrytis, Cylindrocarpon, Eutypa, Fusarium*) ranged from 25 to 100 mg/ml (Vries et al., 2005). The MIC value for *Mycobacterium smegmatis* was 0.78 mg/ml (Mativandlela, Meyer *et al.* 2008). An immune toxicity study of *G. africana* extract found that concentrations of 19 to 500 µg/ml modulate pro-inflammatory cytokines of BALB/c mouse spleenocytes (Pool, Klaasen *et al.* 2009b).

A number of toxicity studies have been carried out on plant compounds in the hope of assessing their skin sensitization potential. A study on the essential oils of western juniper oil and Port-Orford-cedar oil evaluated their potential to cause dermal toxic effects on mice and rabbits (Craig, Karchesy *et al.* 2004). Data collected in the Port-Orford-cedar oil study showed that concentrations of 0.5%, 5% and 50% did not induce a hypersensitivity reaction and hence, was not considered to be a sensitizer. However, western juniper oil extract at 50% concentration demonstrated a positive reaction of 3.33 SI (with 3.0 or greater representing a positive response). This result classified western juniper oil as a potential sensitizer (Craig, Karchesy *et al.* 2004). A study of the skin irritancy potential of *Angelica keiskei* leaf aqueous and ethanol fractions (100 mg/dose), demonstrated that these fractions did not induce acute toxicity in the skin of the animals as assessed by anatomical and pathological observations (Lee 2013). Toxicity studies on different root extracts of *Anacyclus pyrethrum DC* showed that this medicinal plant was safe to use at known dosages (Kumar and Lalitha 2013). All these studies were carried out in accordance with OECD guidelines. Acute oral toxicity studies of the methanol extract of *Mentha spicata*, a medicinal plant used for the alleviation of different classes of pain, revealed that this plant extract can be classified as non-toxic (Naidu, Ismail *et al.* 2014).

The abundance of medicinal plants and their use in traditional medicine makes them ideal choices as agrochemicals and/or pesticides (Manjula and Mamidala 2013). With the cost of conventional chemicals being high, agrochemicals can be used and made available even in rural areas. Furthermore, agrochemicals help protect the environment and preserve wildlife (Nnamonu and Ali 2013). Toxicity and safety assessment of medicinal plants already in use, including the ones not commercialized, is therefore important.

3.6 Conclusion

From this study, it can be concluded that the median lethal oral dosage (LD_{50}) of *G. africana* extract was considered to exceed 2000 mg/kg in both the acute oral and acute dermal toxicity studies. Evaluation of the effect of the extract on EpiSkin® reconstructed human epidermis revealed that the *G. africana* extract (concentrate) *G. africana* extract (in-use dilution) were non-irritants. Furthermore, open application of the extract at various concentrations did not result in a SI $\geq$ 3 in any group. Hence, it did not elicit a hypersensitivity response. These findings will be included in the documentation for a toxicological review to register *G. africana* extracts as pesticidal adjuvants and plant growth stimulants. *G. africana* has been shown to have bio-stimulating properties. Studies carried out on this medicinal plant revealed that it increased the levels and concentrations of polyphenolics, flavonoids and chlorophyll when applied. *G. africana* is applied directly (foliar application) onto filed crops, fruit trees and grass at a concentration of 0.15% (750 ml/100 L). For cosmetic formulations, *G. africana* is used at a concentration of 1% or lower (10 ml/ 250 ml).

The outcomes of this study demonstrated that the ethanolic extract of *G. africana* did not result in any *in vivo* danger. Pharmacological and biochemical investigations will be essential in elucidating the mechanism of action and will be beneficial in utilizing this plant as a therapeutic agent. Furthermore, a detailed experimental analysis of the chronic toxicities and genotoxicity are important to support these findings. Toxicity assessment of medicinal plants already in use, including the ones not yet commercialized, is important in evaluating their safety and sensitizing potential at dosages for which these formulations are being used. Clinical trials have not yet been performed on this medicinal plant.

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4 CHAPTER 4: Antimicrobial activity of *Galenia africana* alone and in combination with antimicrobial drugs against *Staphylococcus aureus*

4.1 Abstract

Background: Methicillin-resistant *Staphylococcus aureus* (MRSA) is responsible for causing severe hospital and community acquired infections. Globally, MRSA infection rates have continued to rise due to its ability to develop resistance to commonly used drugs. It is for this reason that medicinal plants are being considered as alternative sources of treatment. *G. africana* is a medicinal plant known to have antifungal and antimycobacterial properties. It has also been used in the treatment of skin infections, eye inflammations and wounds.

Objective/Purpose: The purpose of this study was to evaluate the antimicrobial activity of *G. africana* alone as well as its interaction with antimicrobial drugs against methicillin-sensitive (MSSA) and methicillin-resistant (MRSA) *Staphylococcus aureus*.

Methods: The antimicrobial activity of *G. africana* extracts and the interactions between the ethanolic extract and various antibacterial agents were investigated by the broth microdilution, checkerboard assay and the Sensititre susceptibility testing method. The minimum bactericidal concentrations (MBCs) of the extracts were also determined.

Results: The MIC of the dried extract against both MSSA and MRSA was 25 mg/ml while that of the ethanolic extract was 3.12 mg/ml for both strains. The MIC results indicated that the growth inhibitory effect of the ethanolic extract was more than that of the dried extract. In the checkerboard assay, the fractional inhibitory concentration (FIC) indices showed the ethanolic and ampicillin combination was additive (FICI of 0.64) against MSSA but indifference (FICI of

1.002) against MRSA. The results of the Sensititre susceptibility test showed that the extract increased the activity of some of the antibacterial agents. The MBC results showed that the dried extract was bacteriostatic against both MSSA and MRSA strains. The ethanolic extract was bactericidal against the MSSA and MRSA strains with MBCs of 3.125 and 6.25 mg/m,l respectively.

Conclusion: The results of this study suggest that *G. africana* could be employed for use as a natural antimicrobial agent in the treatment of MRSA. The results of the combination studies also suggest the potential use of *G. africana* extract and antibacterial drug combinations for the treatment of bacterial infections.

4.2 Introduction

Staphylococcus aureus causes a wide range of both hospital and community associated infections which include pneumonia, meningitis, bacterial arthritis, endocarditis and septicemia (Cha, Lee *et al.* 2014). In addition, *S. aureus* has been shown to cause severe and complex infections of the skin and skin-structures (Gould, David *et al.* 2012). Methicillin-resistant *Staphylococcus aureus* (MRSA) is a growing concern as it affects the global healthcare system. Not only is MRSA related to human infections, but it has been reported to be present in animals as well, thus increasing the risk of people being infected through zoonotic spread (Malm, Biernasiuk *et al.* 2005; Defres, Marwick *et al.* 2009). The increase in MRSA associated infections has resulted in extensive use of antibiotics, particularly vancomycin (Levine 2008; Moravvej, Estaji *et al.* 2013). The extensive and inappropriate use of these antibiotics has led to the development of drug resistance (Usha, Jose *et al.* 2010).

It has been reported that *S. aureus* isolates are resistant to over 20 diverse groups of antibacterial drugs (Aqil, Khan *et al.* 2005; Maltezou and Giamarellou 2006). The advent of antibiotic resistant microorganisms is a worldwide problem due to the fact that drug resistance increases mortality rates and puts a strain on the expenditure of the healthcare system (Abd El-Kalek and Eman A. Mohamed 2012). The increase in drug resistance and the increased risk of microorganisms being resistant to newly developed antimicrobial agents has prompted the need to explore the use of natural products and medicinal plants as potential therapeutic agents (Konate, Mavoungou *et al.* 2012).

Medicinal plants are used across the world for the treatment of various diseases as they possess numerous health beneficial compounds such as alkaloids, tannins, terpenoids and flavonoids (Cha, Lee *et al.* 2014). *G. africana* is a South African medicinal plant reported to have numerous health benefits. Studies have shown that it has been used for the treatment of various conditions such as skin infections, eye infections and wounds (Watt and Breyer-Brandwijk 1962). Evaluation of the antimycobacterial activity of *G. africana* revealed that it exhibited significant antimycobacterial activity against *Mycobacterium smegmatis* and *Mycobacterium tuberculosis* with MICs of 0.78 and 1.2 mg/ml, respectively (Mativandlela, Meyer *et al.* 2008).

In this study, the antimicrobial activity of *G. africana* extract against methicillin-sensitive and methicillin-resistant *Staphylococcus aureus* isolates was assessed using the broth microdilution assay. The bacteriostatic and bactericidal concentrations of the extract were also determined. The synergistic effect of the extract and several antimicrobial agents was evaluated using the checkerboard assay and Sensititre susceptibility testing method.

4.3 Materials and Methods

4.3.1 Bacterial cultures and growth conditions

The bacterial cultures used for the study were *S. aureus* strains ATCC 25923 and ATCC 33591, representing methicillin-sensitive (MSSA) and -resistant (MRSA) strains, respectively. These were obtained from the American Type Culture Collection (ATCC). Prior to sub-culturing, the isolates were maintained on Müller Hinton agar (MHA) (Sigma Aldrich, USA) and checked for contamination throughout the experiments. The bacterial strains were sub-cultured on MHA plates for 18-24 h at 37ºC. The nephelometer was used in the standardization process of the culture suspensions. Prior to standardizing the culture suspensions, the nephelometer was calibrated with the 0.5 McFarland standard (which was gently mixed, by inverting the tube containing the standard, a couple of times). 3-5 colonies were picked to prepare a suspension in 5 ml of de-ionized water and homogenized by vortexing. The suspension was then adjusted to 0.5 McFarland standard which corresponded to 1-2 x 10^8 CFU/ml. This was used for subsequent experiments. Cation Adjusted Müller Hinton broth (CAMHB) (Trek Diagnostics Systems, UK) was used in the broth microdilution assay, checkerboard assay and Sensititre susceptibility testing method.

4.3.2 Preparation of *G. africana* and ampicillin

In this study, two different types of *G. africana* extracts were incorporated to assess its effect against *S. aureus* strains. The first was a 20% (w/v) extract of air dried leaves and shoots of *G. africana* (Kraalbos) commercially prepared in 60% ethanol and then oven dried under negative pressure to form crystals (Brenn-O-Kem (Pty) Ltd, Wolseley, South Africa). In order to make up the stock solution, the dried extract was weighed out and crushed in a tube containing CAMHB

(TREK Diagnostics System, UK) in order to emulsify it. Once this was done, the tube was centrifuged and the supernatant filter sterilized into another sterile 50 ml greiner tube. This was appropriately labeled and stored at 4ºC for subsequent use. The second was a 20% (w/v) extract in 60% ethanol with a pH of about 6.9. This extract was not dried. The desired concentrations of both *G. africana* extracts were made up in CAMHB. Samples of the dried extract and neat ethanolic extract were sent to Cape Peninsula University of Technology (CPUT) (Bellville, South Africa) for analysis in order to determine their chemical composition. Once analysis was done, a certificate of analysis was supplied (see Appendix IV). Ampicillin was purchased from Sigma-Aldrich (USA) and dissolved in sterile distilled water to make the appropriate stock solution from which the desired concentrations were made. De-ionized water was purchased from TREK Diagnostics Systems, UK.

4.3.3 MIC determination using the broth microdilution assay

The MIC (minimum inhibitory concentration) of *G. africana* was determined by the broth microdilution method performed in sterile 96-well flat-bottom microtitre plates as outlined by the CLSI (CLSI 2012). Antimicrobial susceptibility testing was performed using 5 replicates for both MSSA (ATCC 25923) and MRSA (33591) strains. Culture suspensions adjusted to 0.5 McFarland standard (in de-ionized water) were used in this assay. A 1:100 dilution cell suspension was prepared, from the 0.5 McFarland standard cell suspension, that contained a final number of 5×10^5 bacterial cells per well. 100 µl of broth was then added to columns 2-11 (rows A-E) and 100 µl of the extract was added to column 2 and serially diluted (two-fold serial dilutions) until column 11 and 100 µl discarded from the wells in this column. Care was taken to ensure that no broth was added to column 12. Column 1 contained the extract at double the required concentration so as to obtain the desired concentration once the cell suspension was

added to the wells. Column 12 contained the following controls: positive control (100 µl of cell suspension and 100 µl of broth in 12 A&B); ampicillin control at a final concentration of 0.25 µg/mg (100 µl of ampicillin and 100 µl of cell suspension in 12 C); de-ionized water control (200 µl of de-ionized water in 12 D) and a broth control (200 µl of broth in 12 E). The broth and de-ionized controls were included as sterility controls. 100 µl of the cell suspension was then added to each well except the broth and de-ionized control wells. The plates were then covered with a plastic film and incubated for 24 h at 37ºC and observed for microbial growth. After the incubation period, the MIC was determined by adding 40 µl of 0.2 mg/ml *p*-iodonitrotetrazolium chloride (INT) (Sigma-Aldrich, USA) and incubating for a further 30 min at 37ºC (Eloff 1998; Mativandlela, Lall *et al.* 2006). This was used as an indicator for microbial growth. The MIC was selected as the lowest concentration that did not show any bacterial growth as indicated by a lack of color change after the incubation period (changing of color from yellow to pink if wells contained viable cells and no color change if growth was inhibited). This broth microdilution assay was performed for both the dried and ethanolic *G. africana* extracts.

4.3.4 Minimum Bactericidal Concentration (MBC) determination

The MBC was evaluated by sub-culturing aliquots from wells whose concentrations were ≥ MIC (Rabe, Mullholland *et al.* 2002). This was determined by sub-culturing a 10 µl aliquot from each negative well (wells that did not produce any color change) and from a positive well (cell suspension control well) onto drug-free MHA. The plates were incubated for 18-24 h at 37ºC. The concentration that yielded no single bacterial colony on the solid medium was taken as the MBC (Costa, Endo *et al.* 2015).

4.3.5 Checkerboard assay

The synergistic interaction between *G. africana* and ampicillin was evaluated using the checkerboard assay as previously described (Hsieh, Yu *et al.* 1993; Petersen, Labthavikul *et al.* 2006), with some modifications. This assay was employed to establish the effect that combinations of *G. africana* extract and ampicillin would have on the bacterial cell growth. In this method, the extract-ampicillin combination effect was assessed using only the ethanolic extract of *G. africana*, as it exhibited the greatest antibacterial effect in the broth microdilution assay. This method allowed for the evaluation of various combinations ranging from 4 x MIC to 1/16 x MIC for *G. africana* and ampicillin. This broad range allowed for the evaluation of antagonism and synergism. Both *G. africana* and ampicillin were tested on their own, on the same 96-well plate, for easy comparison. Two-fold serial dilutions of ampicillin were made across the X-axis (Row A: 2-10) and two-fold serial dilutions of *G. africana* extract were made across the Y-axis (Column 1: B-H). Ampicillin concentrations ranged from 0.62-16 µg/ml and *G. africana* concentrations ranged from 0.39 to 25 mg/ml. Both the extract and ampicillin were made up at double their concentrations so as to acquire the desired concentrations once the cell suspensions were added to the wells. This was done in order to ensure that the appropriate concentration was obtained once all the components were added to the wells. For example, since the starting concentration for ampicillin was 16 µg/ml and that of *G. africana* was 25 mg/ml, the necessary starting concentrations were, therefore, 32 µg/ml and 50 mg/ml for ampicillin and *G. africana*, respectively. In order to carry out the checkerboard assay, two plate panels were used for both strains. Panel 1 was used for the extract-ampicillin combination and panel 2 was used for the preparation of the ampicillin dilutions. For panel 1, 50 µl of CAMHB was added to all the wells except the wells in rows A (1-11) and B (1-11) and column 1 (A-H). Once the broth

was added, 50 µl of *G. africana* extract at a concentration of 100 mg/ml was then added to row C (2-10). Two fold serial dilutions were made, down the Y-axis, from each well in row C (2-10) down to row H (2-10). 50 µl was discarded from these last wells. 50 µl of the extract at a concentration of 100 mg/ml was the added to row B (2-10). For panel 2, 100 µl of CAMHB was added to wells in columns 3-10. 64 µg/ml of ampicillin was then added to column 3 (B-H) and two-fold serial dilutions made until column 10 (B-H). Care was taken to ensure that 100 µl of ampicillin was discarded from column 10 and not added to column 11 as this column contained the control wells. Column 2 contained only 100 µl of ampicillin (64 µg/ml) with no CAMHB added. This was done to make sure that the final concentration of ampicillin and *G. africana* in the combination assay would be the appropriate range. In addition, this plate layout also ensured that the ampicillin would be added to panel 1 (plate containing the extract) in the same way that it appeared in panel 2, as the wells were corresponding to each other in both plate panels. Once the concentration panels were established, 50 µl of ampicillin in column 2 (in panel 2) was added to column 2 (in panel 1), and the same applied to all the other concentrations. Once this was done, panel 1 now had both ampicillin and *G. africana* at double their desired starting concentration (32 µg/ml and 50 mg/ml for ampicillin and *G. africana*, respectively). 100 µl of bacterial cell suspension was then added to the wells and this resulted in starting concentrations of 16 µg/ml for ampicillin and 25 mg/ml for *G. africana*. Control wells were also included in column 11 that contained the following controls: positive control (cell suspension and broth only, wells A&B); negative control (broth only; wells C&D) and de-ionized water control (water only; wells E&F). Well A1 was also used as a positive control well. The plates were then covered with a plastic film and incubated at 37ºC for 24 h. After the incubation period, 40 µl of 0.2 mg/ml of *p*-

iodonitrotetrazolium chloride (INT) was then added and the plates incubated for another 30 min at 37ºC.

4.3.5.1 Calculating the Fractional Inhibitory Concentration Index (FICI)

The FICI was used to evaluate the interaction between ampicillin and *G. africana* in the checkerboard assy. It was determined using the fractional inhibitory concentrations (FICs) of ampicillin and *G. africana*. The FIC for both ampicillin and *G. africana* was calculated using the negative wells (wells that exhibited no growth) in the growth-no-growth interface. The FIC for a particular drug in a given well is established by dividing the concentration of that drug in that well by the control MIC of the test organism to that same drug. The FIC index of a well is obtained by adding the FICs of each of the drugs present in the well. The FICI is calculated as follows:

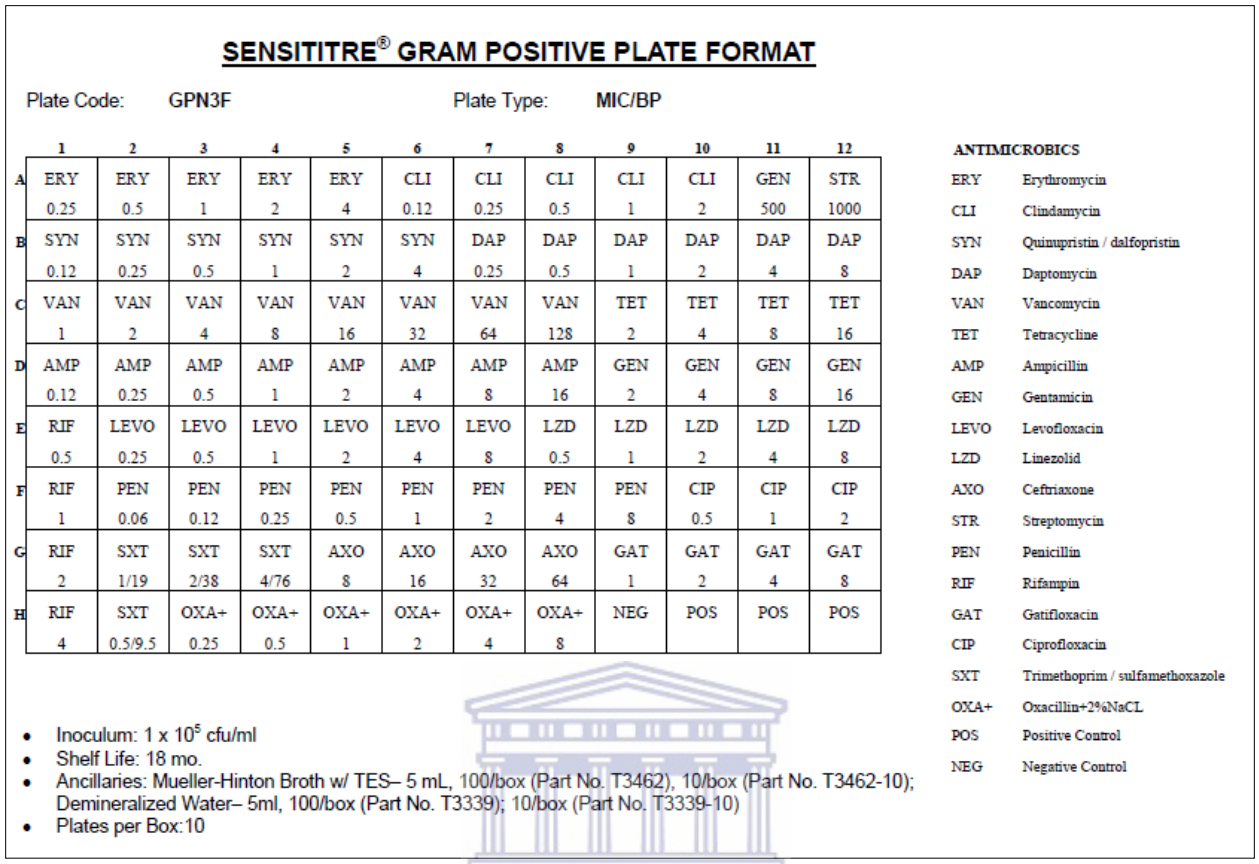
$$\text{FIC index} = \text{FICx} + \text{FICy} = \frac{(X)}{(\text{MICx})} + \frac{(Y)}{(\text{MICy})}$$

The (X) is the MIC of drug X in combination; (MIC_x) is the MIC of drug X alone; FIC_x is the fractional inhibitory concentration of drug X; and (Y), (MIC_y) and FIC_y are established in the same way for Y as they are for X (Krogstad and Moellering 1986). The results were interpreted as follows: FICI $\leq$ 0.5 was defined as synergy, 0.5 < FICI < 1 as additive, 1 < FICI < 4 as indifference and antagonism as FICI > 4 (Olajuyigbe and Afolayan 2013).

4.3.6 Sensititre susceptibility testing

The Sensititre susceptibility testing system is a type of broth microdilution method that provides qualitative and quantitative data as outlined by the manufacturer (TREK Diagnostic Systems, UK). This data establishes whether an organism is either susceptible or resistant to antimicrobial

drugs (qualitative data) and also provides the MIC (quantitative data) results. This method was performed using plates that contained different concentrations of antimicrobial agents. This particular assay allowed for the interpretation of results by either reading them manually (through visual reading of growth) or using plate readers such as the Sensititre ARIS or Sensititre Auto reader that rely on fluorescence. The Sensititre susceptibility system was performed according to the method outlined by TREK Diagnostic Systems and is normally used to select drugs of choice for the treatment of microbial infections. The format of the Sensititre Gram positive plate is shown in Figure 4.1. The Sensititre GPN3F plates (for evaluating Gram-positive bacteria) were used for this assay and they were obtained from TREK Diagnostics Systems (UK).



SENSITITRE® GRAM POSITIVE PLATE FORMAT

Plate Code: GPN3F Plate Type: MIC/BP

	1	2	3	4	5	6	7	8	9	10	11	12
A	ERY 0.25	ERY 0.5	ERY 1	ERY 2	ERY 4	CLI 0.12	CLI 0.25	CLI 0.5	CLI 1	CLI 2	GEN 500	STR 1000
B	SYN 0.12	SYN 0.25	SYN 0.5	SYN 1	SYN 2	SYN 4	DAP 0.25	DAP 0.5	DAP 1	DAP 2	DAP 4	DAP 8
C	VAN 1	VAN 2	VAN 4	VAN 8	VAN 16	VAN 32	VAN 64	VAN 128	TET 2	TET 4	TET 8	TET 16
D	AMP 0.12	AMP 0.25	AMP 0.5	AMP 1	AMP 2	AMP 4	AMP 8	AMP 16	GEN 2	GEN 4	GEN 8	GEN 16
E	RIF 0.5	LEVO 0.25	LEVO 0.5	LEVO 1	LEVO 2	LEVO 4	LEVO 8	LZD 0.5	LZD 1	LZD 2	LZD 4	LZD 8
F	RIF 1	PEN 0.06	PEN 0.12	PEN 0.25	PEN 0.5	PEN 1	PEN 2	PEN 4	PEN 8	CIP 0.5	CIP 1	CIP 2
G	RIF 2	SXT 1/19	SXT 2/38	SXT 4/76	AXO 8	AXO 16	AXO 32	AXO 64	GAT 1	GAT 2	GAT 4	GAT 8
H	RIF 4	SXT 0.5/9.5	OXA+ 0.25	OXA+ 0.5	OXA+ 1	OXA+ 2	OXA+ 4	OXA+ 8	NEG	POS	POS	POS

ANTIMICROBICS

ERY	Erythromycin
CLI	Clindamycin
SYN	Quinupristin / dalfopristin
DAP	Daptomycin
VAN	Vancomycin
TET	Tetracycline
AMP	Ampicillin
GEN	Gentamicin
LEVO	Levofloxacin
LZD	Linezolid
AXO	Ceftriaxone
STR	Streptomycin
PEN	Penicillin
RIF	Rifampin
GAT	Gatifloxacin
CIP	Ciprofloxacin
SXT	Trimethoprim / sulfamethoxazole
OXA+	Oxacillin+2%NaCL
POS	Positive Control
NEG	Negative Control

- Inoculum: 1 x 10^5 cfu/ml
- Shelf Life: 18 mo.
- Ancillaries: Mueller-Hinton Broth w/ TES– 5 mL, 100/box (Part No. T3462), 10/box (Part No. T3462-10); Demineralized Water– 5ml, 100/box (Part No. T3339); 10/box (Part No. T3339-10)
- Plates per Box:10

Figure 4.1: Sensititre Gram positive plate format.

However, in this study, this method was used to analyze the effect of the combination of *G. africana* with the various antimicrobial agents contained on the plates. For this analysis, three *G. africana* concentrations were chosen and these were 3.12 mg/ml, 6.25 mg/ml and 12.50 mg/ml (as they showed the best antimicrobial effect). 3-5 colonies of both MSSA (ATCC 25923) and MRSA (ATCC 33591) were picked from primary cultures on agar plates and emulsified in 5 ml of de-ionized water. After being emulsified, the cell suspensions were adjusted to 0.5 McFarland standard using a nephelometer. According to the protocol, in order to obtain a cell suspension of 1 x 10^5 CFU/ml (the recommended number of cells for this method), 10 µl of the cell suspension, adjusted to 0.5 McFarland standard, should be added to the tube containing 11 ml of CAMHB.

However, since *G. africana* was to be added to this tube, calculations were made to ensure that the required final *G. africana* concentrations were obtained while ensuring that the appropriate number of cells was maintained and used. Prior to adding the cell suspension to the tube containing the CAMHB, *G. africana* was added to the tube. This was done by removing an appropriated volume of broth from the tube and replacing it with the same volume of the *G. africana* extract in order to have the desired *G. africana* concentration. For example, to have a final concentration of 3.12 mg/ml, 172 µl of broth was removed and replaced with 172 µl of *G. africana* extract. Once the extract was added to the broth, 10 µl of the cell suspension was transferred into the tube containing the extract-broth mixture using a calibrated pipette. This was done for all the *G. africana* concentrations used in this method. Plates that did not contain the extract were also included in order to assess the effect of the antimicrobial agents on their own against both MSSA (ATCC 25923) and MRSA (ATCC 33591) strains. The tubes were then vortexed for 15-30 s (or inverted 8-10 times) to ensure that a homogenized mixture was obtained. 50 µl of the broth-extract suspension was then poured into a sterile seed trough and added to the plates using a multichannel pipette. The plates were then covered with an adhesive seal provided and care was taken to ensure that all wells were covered and sealed properly. The plates were then placed in a 37ºC incubator for 24 h. After the incubation period, the plates were read manually by visual reading of growth. Growth appeared as turbidity or as a deposit of cells at the bottom of the well. The MIC was recorded as the lowest concentration of antimicrobial agent alone as well as the antimicrobial-extract combination that inhibited visible growth.

4.4 Results

4.4.1 Broth microdilution

The broth microdilution method was performed using both the dried *G. africana* extract as well as the ethanolic extract. Figures 4.2 and 4.3 represent the antibacterial activity of the dried extract against MSSA (ATCC 25923) and MRSA (ATCC 33591), respectively. Figures 4.4 and 4.5 represent the antibacterial activity of the ethanolic extract. Ampicillin, at a concentration of 0.25 µg/ml, was also included as a control. The breakpoints for ampicillin, as outlined by the CLSI, are that if an organism is sensitive to ampicillin, then the MIC would be ≤ 0.25 µg/ml and if the organism was resistant to ampicillin, then the MIC would be ≥ 0.5 µg/ml.

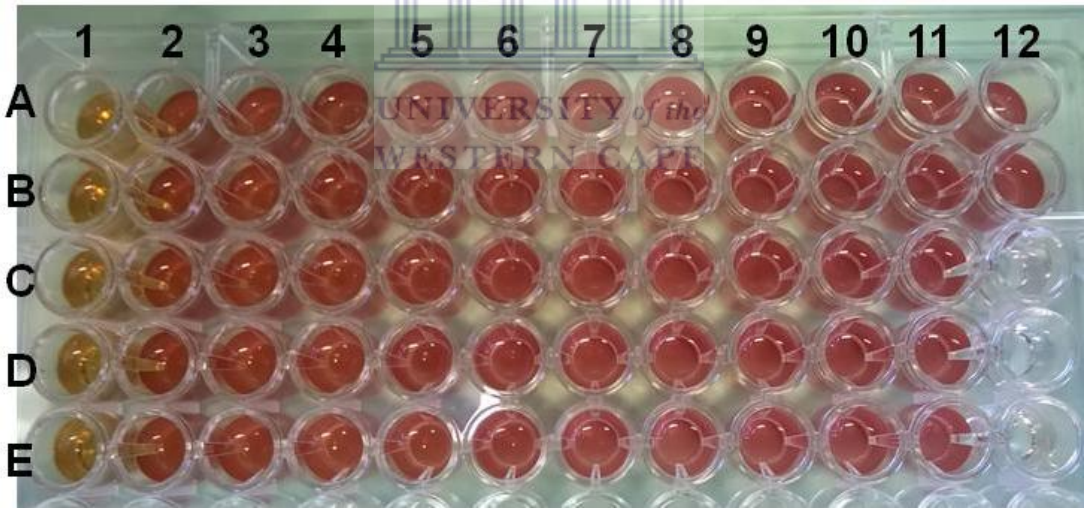


Figure 4.2: Broth microdilution results of MSSA treated with the dried *G. africana* extract (0.024 to 25 mg/ml). Columns 1-11 (A-E) represent the extract concentrations from highest to lowest. Concentrations were from highest to lowest. Column 12 contained control wells as follows: 12A&B: Positive control; 12C: Ampicillin control; 12D: De-ionized water control and 12E: Broth control.

Figure 4.2 shows the effect of the dried *G. africana* extract against MSSA (ATCC 25923). The MIC obtained after the stipulated incubation period was 25 mg/ml. The ampicillin control demonstrated that MSSA was sensitive to it as there was no visible bacterial growth in the well (well C12).

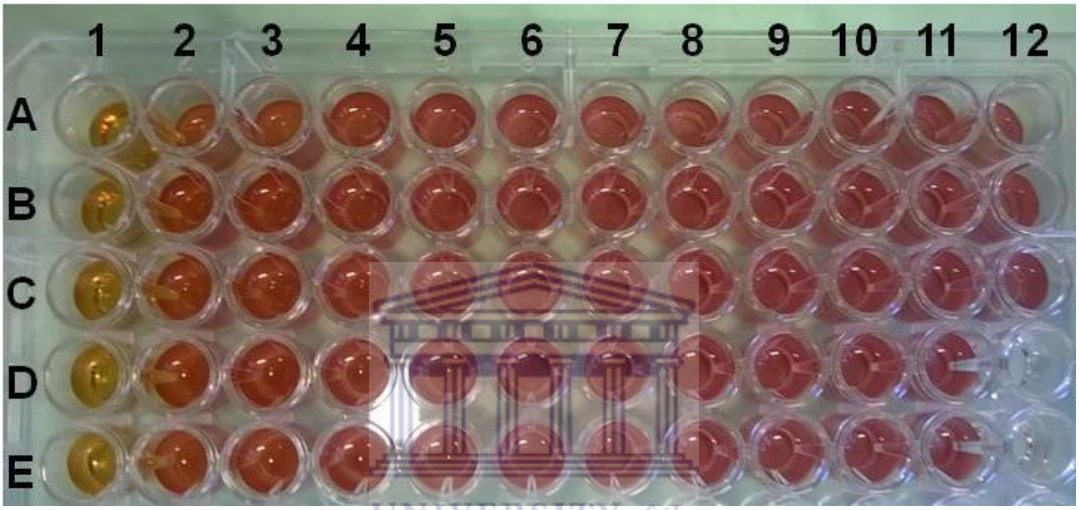


Figure 4.3: Broth microdilution results of MRSA treated with the dried *G. africana* extract (0.024 to 25 mg/ml). Columns 1-11 (A-E) represent the extract concentrations from highest to lowest. Concentrations were from highest to lowest. Column 12 contained control wells as follows: 12A&B: Positive control; 12C: Ampicillin control; 12D: De-ionized water control and 12E: Broth control.

Figure 4.3 shows the effect of the dried *G. africana* extract against MRSA (ATCC 33591). The MIC obtained after the stipulated incubation period was also 25 mg/ml, revealing that *G. africana* had the same effect on both MSSA and MRSA strains. However, the ampicillin control demonstrated that MRSA was resistant to it as there was visible growth in the well (well C12).

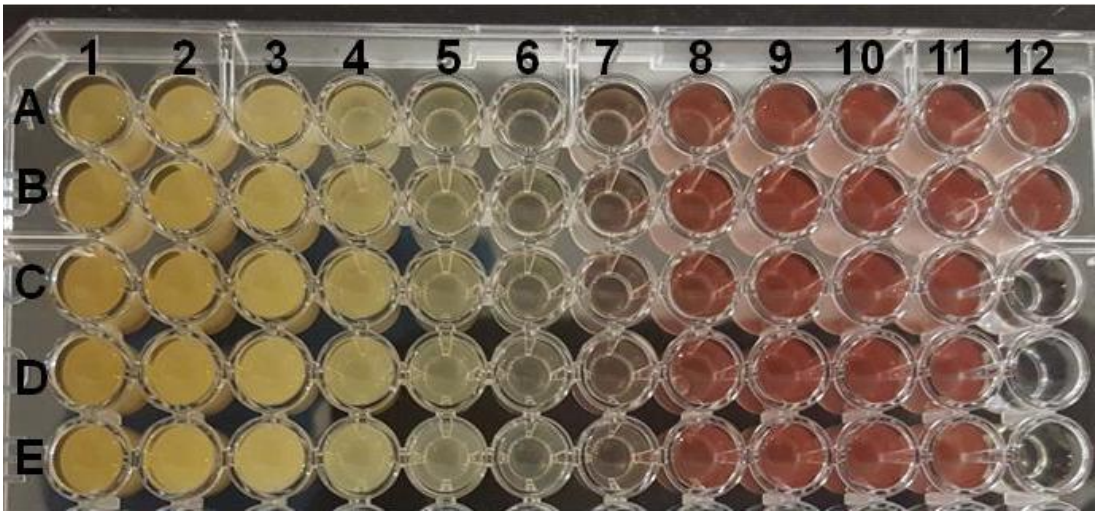


Figure 4.4: Broth microdilution results of MSSA treated with the *G. africana* ethanolic extract (0.098 to 100 mg/ml). Columns 1-11 (A-E) represent the extract concentrations from highest to lowest. Column 12 contained control wells as follows: 12A&B: Positive control; 12C: Ampicillin control; 12D: De-ionized water control and 12E: Broth control.

Figure 4.4 represents the results obtained after treating the MSSA bacterial cells with the ethanolic *G. africana* extract. It was clear to see that the antibacterial activity of the ethanolic extract was greater than that of the dried extract. The MIC obtained with the ethanolic extract was 3.12 mg/ml (which was much lower than the MIC of the dried extract). The ethanol concentration in these well was 0.94%, which was low and did not influence the outcome of the results.

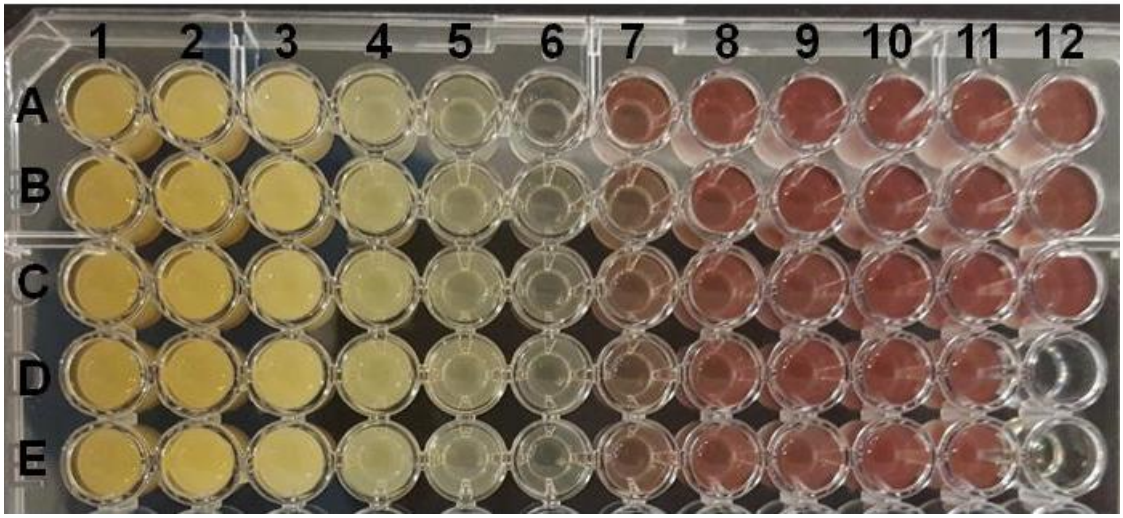


Figure 4.5: Broth microdilution results of MRSA treated with the *G. africana* ethanolic extract (0.098 to 100 mg/ml). Columns 1-11 (A-E) represent the extract concentrations from highest to lowest. Column 12 contained control wells as follows: 12A&B: Positive control; 12C: Ampicillin control; 12D: De-ionized water control and 12E: Broth control. Concentrations were from highest to lowest.

Figure 4.5 also represents the results obtained after the treatment of bacterial cells with the ethanolic *G. africana* extract, except in this case, the effect on MRSA (ATCC 33591) was evaluated. In the same way, it was clear to see that the antibacterial activity of the ethanolic extract was greater than that of the dried extract. The MIC obtained with the ethanolic extract was also 3.12 mg/ml (column 6: A-E), demonstrating that both the dried extract and ethanolic extract had the same effect on both bacterial strains. The ethanol concentration in these well was also 0.94%, which was low and did not influence the outcome of the results.

4.4.2 Minimum Bactericidal Concentration (MBC) determination

This was done in order to evaluate the effect of the dried and ethanolic extracts on the bacterial cells. Sub-culturing the negative wells (wells with no visible growth) onto MHA enabled the antibacterial effect to be evaluated.

Figure 4.6: Results of the MFC of MSSA and MRSA treated with the dried *G. africana* extract. A1 represents a *G. africana* concentration of 25 mg/ml.

The results obtained in Figure 4.6 showed that the dried extract did not kill the bacterial but instead just inhibited their growth as seen by the presence of colonies on the MHA plates for both MSSA (ATCC 25923) and MRSA (ATCC 33591). This demonstrated that the dried extract was bacteriostatic and not bactericidal.

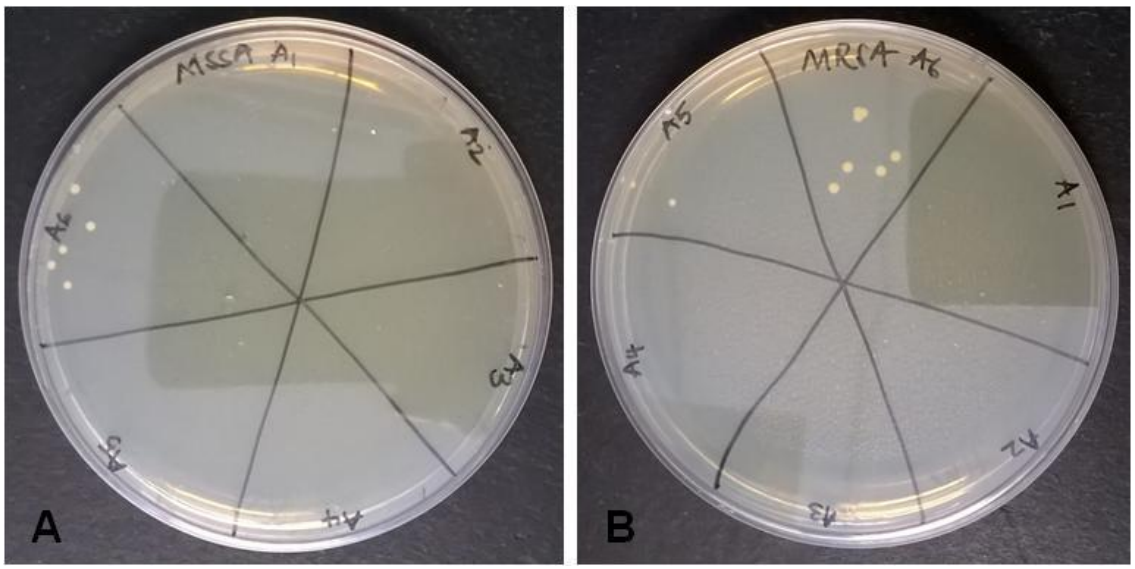


Figure 4.7: Results of the MFC of MSSA (A) and MRSA (B) treated with the *G. africana* ethanolic extract. A1-A6 represent the different *G. africana* concentrations as follows: A1: 100 mg/ml; A2: 50 mg/ml; A3: 25 mg/ml; A4: 12.5 mg/ml; A5: 6.25 mg/ml and A6: 3.12 mg/ml.

On the other hand, the ethanolic extract showed considerable killing potential (bactericidal ability) than the dried extract against both MSSA (ATCC 25923) and MRSA (ATCC 33591) (Figure 4.7). The MBC of the extract against MSSA (ATCC 25923) was 6.25 mg/ml while that of the MRSA (ATCC 33591) was 12.5 mg/ml.

4.4.3 Checkerboard assay

The checkerboard assay was used to assess the effect of the interaction between ampicillin and *G. africana* against MSSA (ATCC 25923) and MRSA (ATCC 33591). Since the ethanolic extract exhibited the greatest antibacterial activity against both strains, as seen by the lower MIC, it was selected to be used in this assay.

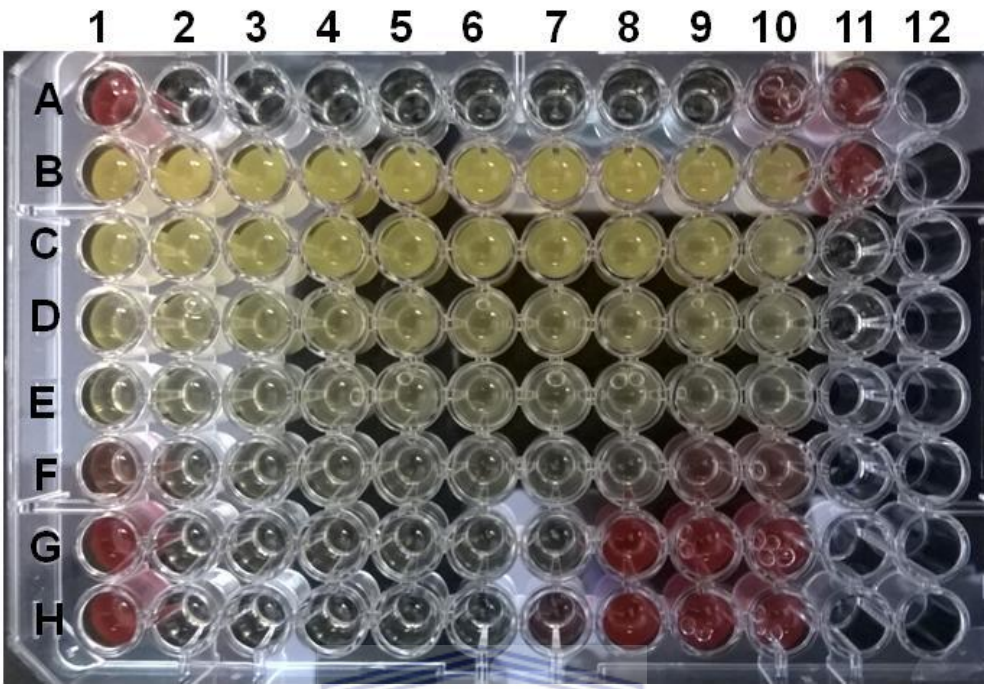


Figure 4.8: Checkerboard assay results of MSSA treated with the *G. africana* ethanolic extract (0.039 to 25 mg/ml) and ampicillin (0.062 to 16 µg/ml). Row A (A2-A10) represents ampicillin and Column 1 (B-H) represent *G. africana* concentrations. Concentrations were from highest to lowest. The remaining wells represent combinations of ampicillin and *G. africana*.

Figure 4.8 represents the checkerboard assay results obtained after the treatment of MSSA (ATCC 25923) with the ethanolic extract. Column 1 (B-H) represents the *G. africana* ethanolic extract at concentrations of 25 to 0.039 mg/ml, respectively. Row A (2-10) represents ampicillin at concentrations of 16 to 0.062 µg/ml, respectively. The MIC of ampicillin alone was 0.12 µg/ml and the MIC of ampicillin in the combination was 0.062 µg/ml. The MIC of *G. africana* alone was 3.12 mg/ml and the MIC in the combination was 0.39 mg/ml. Hence, the FIC index was calculated as follows:

$$\text{FIC index} = \frac{0.062\ \mu g/ml}{0.12\ \mu g/ml} + \frac{0.39\ mg/ml}{3.12\ mg/ml} = 0.52 + 0.12 = 0.64$$

Since the FICI obtained was 0.64, it meant that the interaction between ampicillin and *G. africana* was additive when tested against MSSA (ATCC 25923).

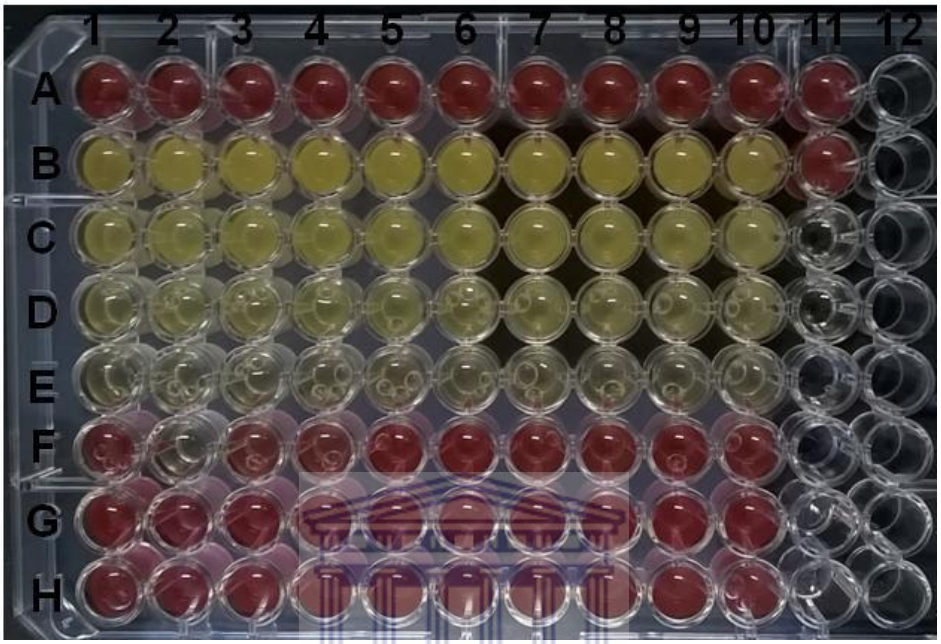


Figure 4.9: Checkerboard assay results of MRSA treated with the *G. africana* ethanolic extract (0.039 to 25 mg/ml) and ampicillin (0.062 to 16 µg/ml). Row A (A2-A10) represents ampicillin and Column 1 (B-H) represent *G. africana* concentrations. Concentrations were from highest to lowest. The remaining wells represent combinations of ampicillin and *G. africana*.

The plate layout of Figure 4.9 was similar to that of the MSSA (ATCC 25923). Column 1 (B-H) represented *G. africana* ethanolic extract at concentrations of 25 to 0.039 mg/ml, respectively and row A (2-10) represented ampicillin at concentrations of 16 to 0.062 µg/ml, respectively. However, the results obtained were different from those of MSSA (ATCC 25923). There was no MIC established for ampicillin using the range of concentrations and so for calculation sake, the next concentration in the sequence, 32 µg/ml, was used as the MIC of ampicillin alone. The MIC of ampicillin in the combination was 0.062 µg/ml. The MIC of the extract alone was 3.12 mg/ml

and the MIC of the extract in the combination was still 3.12 mg/ml. Hence, the FIC index was calculated as follows:

$$\text{FIC index} = \frac{0.062\ \mu g/ml}{32\ \mu g/ml} + \frac{3.12\ mg/ml}{3.12\ mg/ml} = 0.002 + 1 = 1.002$$

The FICI value indicated that the interaction between ampicillin and *G. africana* was classified as indifference (meaning that the result obtained was due to the effect of the most active therapeutic agent, in this case *G. africana*).

4.4.4 Sensititre susceptibility testing

The effect of the interaction between ampicillin and *G. africana* was also assessed using this susceptibility testing method. It is generally used to evaluate the susceptibility of microorganisms to various drugs. The MIC interpretive criteria used to interpret the results obtained in this testing method are summarized in Table 4.1 according to the CLSI (CLSI 2012).

Table 4.1: MIC Interpretive Criteria for *Staphylococcus aureus* species as outlined by the CLSI.

		MIC (µg/ml)		
Antibacterial drugs	**Dilution range**	**S**	**I**	**R**
Erythromycin	0.25-4	≤ 0.5	1-4	≥ 8
Clindamycin	0.12-2	≤ 0.5	1-2	≥ 4
Quinupristin/ dalfopristin	0.12-4	≤ 1	2	≥ 4
Daptomycin	0.25-8	≤ 1	–	–
Vancomycin	1-128	≤ 2	4-8	≥ 16
Tetracycline	2-16	≤ 4	8	≥ 16
Ampicillin	0.12-16	≤ 0.25	–	≥ 0.5
Gentamicin	2-16	≤ 4	8	≥ 16
Levofloxacin	0.25-8	≤ 1	2	≥ 4
Linezolid	0.5-8	≤ 4	–	≥ 8
Ceftriaxone	8-64	≤ 8	16-32	≥ 64
Penicillin	0.06-8	≤ 0.12	–	≥ 0.25
Rifampicin	0.5-4	≤ 1	2	≥ 4
Gatifloxacin	1-8	≤ 0.5	1	≥ 2
Ciprofloxacin	0.5-2	≤	–	≥

Trimethoprim/ sulfamethoxazole		≤ 2/38	–	≥ 4/76
Oxacillin + 2% NaCl	0.25-8	≤ 2	–	≥ 4

S: Susceptible; I: intermediate and R: resistant

Table 4.2: Results of the Sensititre susceptibility testing method for the different *G. africana* and antibiotic combinations against MSSA (ATCC 25923) and MRSA (ATCC 33591).

	MIC of antibacterial drug (µg/ml)							
	MSSA				**MRSA**			
Antibacterial drugs	**Drugs alone**	**Drugs + GA[a]**	**Drugs + GA[b]**	**Drugs + GA[c]**	**Drugs alone**	**Drugs + GA[a]**	**Drugs + GA[b]**	**Drugs + GA[c]**
Erythromycin	≤ 0.25	≤ 0.25	≤ 0.25	≤ 0.25	> 4	> 4	> 4	= 2
Clindamycin	**= 0.25**	**≤ 0.12**	**≤ 0.12**	**≤ 0.12**	**> 2**	> 2	**= 0.5**	**= 0.5**
Quinupristin/ dalfopristin	**= 0.5**	**= 0.25**	**= 0.25**	**≤ 0.12**	**= 1**	**= 0.5**	**= 0.5**	> 4
Daptomycin	≤ 0.25	≤ 0.25	≤ 0.25	≤ 0.25	= 0.5	= 1	=0.5	=0.5
Vancomycin	≤ 1	≤ 1	= 4	= 4	**= 2**	**≤ 1**	= 4	= 8
Tetracycline	≤ 2	≤ 2	≤ 2	≤ 2	**> 16**	> 16	**= 16**	**=16**
Ampicillin	≤ 0.12	≤ 0.12	≤ 0.12	≤ 0.12	**> 16**	**= 4**	**= 4**	**= 2**
Gentamicin	≤ 2	≤ 2	≤ 2	≤ 2	≤ 2	≤ 2	≤ 2	= 16
Gentamicin 500	≤ 500	≤ 500	≤ 500	≥ 500	≤ 500	≤ 500	≤ 500	≥ 500
Levofloxacin	≤ 0.25	≤ 0.25	≤ 0.25	≤ 0.25	≤ 0.25	= 0.5	= 1	= 1
Linezolid	**= 2**	= 2	= 2	**≤ 0.25**	**= 2**	**= 1**	= 1	= 1
Ceftriaxone	≤ 8	≤ 8	≤ 8	≤ 8	**> 64**	> 64	> 64	**≤ 8**
Streptomycin 1000	≤ 1000	≤ 1000	≤ 1000	≤ 1000	≥ 1000	≥ 1000	≤ 1000	≤ 1000
Penicillin	≤ 0.06	≤ 0.06	≤ 0.06	= 2	> 8	= 8	= 8	> 8
Rifampicin	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	= 2	= 2
Gatifloxacin	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	> 8	> 8
Ciprofloxacin								
Trimethoprim/ sulfamethoxazole	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5
Oxacillin + 2% NaCl	≤ 0.25	≤ 0.25	≤ 0.25	≤ 0.25	**> 8**	**≤ 0.25**	**≤ 0.25**	**≤ 0.25**

GA[a]- *G. africana* at 3.12 mg/ml
GA[b]- *G. africana* at 6.25 mg/ml
GA[c]- *G. africana* at 12.5 mg/ml
The numbers in bold represent the concentrations that exhibited a reduction in MIC compared to the control plates (plates that did not contain the extract).

Table 4.2 represents the results obtained after the treatment of MSSA (ATCC 25923) and MRSA (ATCC 33591) with different combinations of *G. africana* and antibiotics. The results showed that the antimicrobial agents that exhibited a decrease in their MIC, as a result of the combination, were clindamycin, quinupristin/dalfopristin and linezolid for MSSA (ATCC 25923). With regard to MRSA (ATCC 33591), the antimicrobial agents that showed a decrease in the MIC and thus increased the susceptibility of MRSA (ATCC 33591) to these drugs, as a result of the combination, were clindamycin, quinupristin/dalfopristin, vancomycin, tetracycline, ampicillin, linezolid, ceftriaxone and oxacillin + 2% NaCl. It was easier to see the trend of a reduction in MIC for MRSA (ATCC 33591) as it was less susceptible to the drugs than MSSA (ATCC 25923) as observed by the MICs. Since MSSA (ATCC 25923) was susceptible to the drugs even at the lowest concentrations, a reduction in MIC could not be observed.

4.5 Discussion

The increase in multidrug resistant microorganisms is causing tremendous strain on the health care sector and is a cause of global concern (Olajuyigbe and Afolayan 2013). This multidrug resistance results in high death rates and causes an increase in infectious diseases that may have been eliminated in the past to reappear (Konate, Mavoungou *et al.* 2012). MRSA is resistant to a wide range of therapeutic agents that are commonly used in the treatment of its infections (Bachi and Rohrer 2002). This increase in resistance has led to the need to explore alternative treatment methods to combat these drug resistant microorganisms (Barbour, Al Sharif *et al.* 2004). South African possesses a wide range of plants species that can be utilized for their health beneficial properties especially in the fight against drug resistant pathogens. More than 20 000 plant species have been identified. Medicinal plants have been used for years in the treatment of numerous diseases and their use as alternative therapeutic agents can be beneficial (Cherry 2005; Cha, Lee

et al. 2014). In this study the dried and ethanolic *G. africana* extracts were evaluated for their antibacterial activity against MSSA (ATCC 25923) and MRSA (ATCC 33591).

The broth microdilution assay was used to determine the MIC of the two extracts against the two strains. The MIC of the dried extract was much higher (25 mg/ml) than that of the ethanolic extract (3.12 mg/ml) against both strains. This suggested that the ethanolic extract had significantly greater antimicrobial activity compared to the dried extract. The drying of the ethanolic extract could have resulted in changes in the composition of the compounds found in extract resulting in reduced activity. Studies evaluating the effect of aqueous and ethanolic extracts on bacteria such as *S. aureus* have also shown that the greatest inhibitory effect on bacteria was exhibited by ethanolic extracts. For instance, a study carried out by Mehraban *et al.* (Mehraban, Dovom *et al.* 2016) showed that the ethanolic extract had a greater inhibitory effect on the growth on the bacterial as shown by the MIC values. Another study by Behbahani *et al.* (Behbahani, Yazdi *et al.* 2013) revealed that the ethanolic extract of *Eucalyptus camaldulensis* leaves were more effective against *Staphylococcus aureus* than the aqueous extract with MIC concentrations of 4 mg/ml and 8 mg/ml, respectively. Furthermore, it is suggested that plant extraction methods that use ethanol or Hydro alcohol extract more active compounds from plants compared to aqueous extraction methods. In addition, the rate at which the active compounds are extracted using these two solvents is higher and could contribute to the extraction of more compounds (Nostro, Germano *et al.* 2000). A study conducted by Costa and colleagues also showed that their ethanolic extracts were more effective than the aqueous extracts (Costa, Endo *et al.* 2015).

For medicinal plants to be categorized as antimicrobial agents, based on susceptibility tests, they ought to generate an MIC in the range of 100 to 1000 mg/ml (Simoes, Bennett *et al.* 2009). Since

both the dried and ethanolic extracts produced MICs that were significantly below the stipulated range, they could be classified as antimicrobial agents. In order to establish whether the effect of treatment would be bacteriostatic or bactericidal, the MBC was determined for both extracts against both strains. The results revealed that the ethanolic extract was bactericidal at a concentration of 6.25 mg/ml for MSSA (ATCC 25923) and a concentration of 12.5 mg/ml for MRSA (ATCC 33951). However, the dried extract was bacteriostatic as seen by the presence of colonies after sub-culturing. These results were similar to those observed by Costa and colleagues which indicated that their extracts exhibited good bacterial activity against the *S. aureus* (ATCC 25923) strain with an MBC of 62.5 µg/ml (Costa, Endo *et al.* 2015). Given that bacteria are resistant to numerous antibiotics and the rate of resistance is also on the increase, it was important to assess the effect of the combination of the ethanolic extract with antimicrobial agents that are used in the treatment of *Staphylococcal* infections.

The checkerboard assay and the Sensititre Susceptibility testing method were employed to evaluate the interaction between the combinations of *G. africana* with various antimicrobial agents. The FICI obtained after treating MSSA (ATCC 25923) with a combination of *G. africana* and ampicillin indicated an additive effect. This indicated that the activity of both *G. africana* and ampicillin increased as seen by the reduction in the MIC for both antimicrobial agents. Regarding MRSA (ATCC 33591), the FICI indicated that the result was indifference. This meant that the results observed were as a result of the antibacterial agent with the greatest effect, which was *G. africana*. These results are similar to those obtained by Haroun and Al-Kayali (Haroun and Al-Kayali 2016) who demonstrated that the interactions observed between crude plant extracts and antibiotics were synergistic, additive and indifference against *S. aureus* with FICI ranges of 0.02 to 1.5. In addition, results of this study showed that combinations of

ampicillin, amikacin and cefotaxime with plant extracts had synergistic effects on the *S. aureus* strains. Another study evaluated the synergistic antimicrobial activity of *Camellia sinensis* and *Juglans regia* against multidrug-resistant bacteria. Results revealed that the combination of *Juglans regia* and oxacillin resulted in MRSA strains resistant to oxacillin becoming susceptible to oxacillin again (Farooqui, Khan *et al.* 2015). A study carried out by Olajuyigbe and Afolayan also demonstrated that there were various interactions between the plant extracts and first line antibiotics. These interactions included synergistic, additive/indifference and antagonistic effects (Olajuyigbe and Afolayan 2012). Similar results were also seen with the Sensititre susceptibility testing method as observed by the reduction in the MICs of some of the antibiotics for both strains. Because of the advent of microorganisms that are resistant to a large numbers of antimicrobial agents, combination therapy has become common practice and it is advantageous (Rybak and McGrath 1996; Olajuyigbe and Afolayan 2012). The additive, indifference and synergistic interactions observed indicated that the activity of the combination of the extract and the antimicrobial agents were more effective than the activity of the individual antimicrobial agents.

4.6 Conclusion

The present study demonstrates the antimicrobial potential of *G. africana* extracts and provides supportive data and scientific evidence for its subsequent use for the prevention and management of bacterial infections. The increased activity of the various antibacterial agents combined with the extract demonstrate that combinations of extracts and with antibacterial drugs could be an effective way of treating bacterial infection including those caused by drug resistant bacteria. This study supplies additional information on the activity of *G. africana* and contributes to the knowledge of antimicrobial properties of plants commonly found in South Africa. However,

additional research such as exploring the mechanism of action and performing *in vivo* studies to corroborate the antimicrobial potential of the extract alone and in combination with antibacterial agents will have to be conducted.

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5 CHAPTER 5: Antifungal activity of G*alenia africana* alone and in combination with antifungal drugs against *Candida* strains

5.1 Abstract

Background: The frequency of *Candida* infections have increased, in recent years, and are causing serious public health concern. Despite the availability of numerous antifungal drugs, *Candida* species are capable of becoming resistant to them. Hence, alternative treatment options are being explored, using medicinal plants, to curb this trend of drug resistance. *Galenia africana* is a South African medicinal plant that has been used in the treatment of various ailments such as toothaches, venereal diseases and skin infections. It has also been shown to possess antifungal and antimycobacterial properties.

Objective/Purpose: The aim of this study was to evaluate the anti-*Candida* activity of an ethanolic extract of *G. africana* alone and combination with antifungal drugs against *Candida albicans* and *Candida glabrata*.

Methods: The anti-*Candida* activities of the extract alone and in combination with the drugs were evaluated using the broth microdilution assay, checkerboard assay and the Sensititre YeastOne colorimetric MIC procedure. The minimum fungicidal concentration (MFC) of the extract was also determined. The effect of the extract on the structure of the *Candida* cells was evaluated using light and scanning electron microscopy (SEM).

Results: The minimum inhibitory concentration (MIC) (6.25 mg/ml for both *Candida* strains) and the MFC (6.25 and 12.5 mg/ml for *C. albicans* and *C. glabrata*, respectively) indicated that *G. africana* possessed antifungal activity at relatively high concentrations. After combining with FLC, *G. africana* exerted a strong synergistic effect against *C. albicans* and an indifferent effect

against *C. glabrata* when interpreted by the fractional inhibitory concentration index (FICI) (0.36 and 1.002 for *C. albicans* and *C. glabrata*, respectively). The indifferent effect with *C. glabrata* suggested that the effect depended more on the concentration and activity of *G. africana*. The MFC results confirmed the fungicidal effect of the extract and microscopic analysis revealed that there was cell damage including a decrease in cell size after *G. africana* treatment.

Conclusion: Collectively, these results suggested that *G. africana* possessed antifungal activities against the *Candida* strains and a synergistic effect with FLC. Results of the Sensititre test showed that *G. africana* enhanced the activity of most of the antifungal drugs. Cell damage observed with SEM possibly contributed to this synergistic effect. This provides new information for the development of new antifungal agents using this medicinal plant.

5.2 Introduction

Fungal infection occurrence and frequency has been on the rise for a number of years now (Sardi, Scorzoni *et al.* 2013). In spite of the availability of treatment options, they contribute considerably to the high morbidity and mortality rates thus creating public health concern (Ibrahim, Hong *et al.* 2013). Candidiasis is the major cause of fungal infections especially in immune compromised and hospitalized individuals. It is especially common in people suffering from cancer, diabetes, AIDS, as well as those with severe diseases and transplant patients (Khatoon, Jahan *et al.* 2014). Candidiasis is caused by commensal fungi called *Candida* species found in the gastrointestinal tract, oral mucosal cavity, skin and vagina (Shao, Sheng *et al.* 2007). *Candida* species are capable of causing both superficial and systemic infections due to their ability to adapt to different environments. Oral candidiasis and vulvovaginal (VVC) candidiasis are common infections of *Candida* species (Yan, Hua *et al.* 2012; Ekanola, Ogunshe *et al.* 2014). Patients suffering from *Candida* infections are prone to oropharyngeal candidiasis which can

result in malnutrition and hinder the assimilation of therapeutic drugs (Sardi, Scorzoni *et al.* 2013). This can consequently lead to treatment failure and create a global health challenge.

Candida albicans is the leading cause of persistent and severe fungal infections (Horn, Neofytos *et al.* 2009). Even though *C. albicans* is the most common organism associated with severe *Candida* infections, the prevalence of infections caused by non-*albicans* species is on the rise (Sardi, Scorzoni *et al.* 2013). Some of the non-*albicans* species include *Candida glabrata*, *Candida krusei*, *Candida tropicalis* and *Candida parapsilosis* (Krcmery and Barnes 2002). A study carried out on 2019 patients in major medical centers in North America showed that a high proportion of infections were causes by non-*albicans* species. Results showed that despite *C. albicans* being the most commonly isolated species, *C. glabrata* was the second most isolated species followed by the other non-*albicans* species. This increase in the number of infections associated with non-*albicans* species could be attributed to severe immunosuppression, increased use of broad-spectrum antibiotics and an increase in the age of patients (Horn, Neofytos *et al.* 2009; Panghal, Kaushal *et al.* 2011). These factors have also played a role in the increase and spread of drug resistant organisms. Antifungal drugs that are currently on the market have proved to be less effective in the treatment of fungal infections. This is as a result of a number of factors which include decreased potency, low solubility, increased toxicity leading to uncomfortable side effects and emergence of resistant strains (Carretto, Almeida *et al.* 2010). Over the years, it has been observed that a large number of fungal species are resistant to a variety of antifungal drugs. Furthermore, this trend of drug resistance has been identified worldwide (Ingham, Boonstra *et al.* 2012). *Candida* species have the ability to form drug-resistant biofilms. This capability significantly contributes to their disease causing characteristic in humans (Rajendran, Robertson *et al.* 2010). Treatment of fungal infections with first-line

antifungal agents is proving to be futile as the organisms have become resistant to these drugs. This is especially seen in patients with other underlying medical conditions as well as those undergoing treatment with other therapeutic drugs (Ruhnke, Eigler *et al.* 1994; Ruhnke, Schmidt-Westhausen *et al.* 2000). The advent of fungal species resistant to frequently used antifungal agents and the need for treatment options with fewer side effects paves the way for the development of new antifungal agents (Hanif, Al-Maskari *et al.* 2011; Moghimipour, Sadaghi-Nejad *et al.* 2014).

Galenia africana is a medicinal plant commonly referred to as "kraalbos" (Van Wyk, De Wet *et al.* 2008). *G. africana* has numerous health benefits which include alleviation of toothaches, and treating wounds on both animals and humans (Watt and Breyer-Brandwijk 1962). It has also been used in the treatment of venereal disease, ringworms, relieving of eye inflammation and is incorporated in lotions for the treatment of skin diseases (Watt and Breyer-Brandwijk 1962). *G. africana* extracts have also demonstrated antifungal characteristics against *Botrytis cinerea* (Vries, El Bitar *et al.* 2005).

In this study, the antifungal activity of *G. africana* was assessed using disk diffusion, broth microdilution, checkerboard assay and the Sensititre susceptibility testing method. The fungicidal and fungistatic concentrations of the ethanolic extract were also determined. Furthermore, the effect of the extract on the structure of the *Candida* cells was assessed using light and scanning electron microscopy.

5.3 Materials and Methods

5.3.1 Preparation of the *G. africana* ethanolic plant extract

The plant preparation was the same as that outlined in Chapter 2. Two types of *G. africana* extracts were used in this study. The first was a 20% (w/v) extract of air dried leaves and shoots of *G. africana* (Kraalbos) commercially prepared with 60% ethanol and then oven dried under negative pressure to crystals by a company called Brenn-O-Kem (Pty) Ltd (Wolseley, South Africa). The dried *G. africana* crystals were then crushed and reconstituted in RPMI 1640 (Sigma-Aldrich, USA) media buffered with 0.165 M MOPS (Sigma-Aldrich, USA) to make a stock solution that was utilized in the subsequent experiments. The second was a 20% (w/v) extract in 60% ethanol with a pH of about 6.9. This extract was not dried and was supplied in liquid form. The desired concentrations of both *G. africana* extracts were made up in RPMI 1640 media buffered with 0.165 M MOPS. Samples of the dried extract and neat ethanolic extract were sent to Cape Peninsula University of Technology (CPUT) (Bellville, South Africa) for analysis in order to determine their chemical composition. Once analysis was done, a certificate of analysis was supplied (see Appendix IV).

5.3.2 Preparation of *C. glabrata* (ATCC 26512) and *C. albicans* (ATCC 90028) Suspensions and growth conditions

Type strains of *C. glabrata* (ATCC 26512) and *C. albicans* (ATCC 90028) were a kind gift from Prof Charlene WJ Africa from the Microbial Endogenous Infectious Studies (MEnIS) Research Laboratory at the University of the Western Cape (Bellville, South Africa). Type strains were used in this study as they served as a good reference point for studying the effect of the extract on the *Candia* species. The *Candida* strains were maintained on Sabouraud Dextrose Agar (SDA)

(Sigma-Aldrich, USA) and checked for contamination throughout the experiments. The isolates were sub-cultured on to SDA and left to incubate for 24 h at 37˚C. Sterile glass test tubes, containing 7 ml of saline, were inoculated with colonies picked from the SDA. The inoculums were then adjusted to 0.5 McFarland standard with a known concentration of approximately $3x10^8$ microorganisms per ml using a nephelometer. This is a technique used for known densities of microorganism suspensions for standardization (McFarland, 1907). In the broth microdilution assay, cell suspensions were standardized using Sabouraud Dextrose Broth (SDB) (Sigma-Aldrich, USA) instead of sterile saline. Confirmation of the two strains was done by aseptically streaking the fungal strains on Oxoid chromogenic differential medium (Thermo Scientific, UK). On this medium, *C. albicans* (ATCC 90028) grew as green colonies whereas *C. glabrata* (ATCC 26512) produced beige/brown or purple/mauve colonies.

5.3.3 Disk diffusion (Kirby-Bauer) assay

The disk diffusion assay is employed in a number of clinical microbiology laboratories for the susceptibility testing of antimicrobial agents. In recent times, the Clinical and Laboratory Standards Institute (CLSI) has published numerous standards that have been accepted and approved for the testing of bacteria and fungi (CLSI 2004; CLSI 2012). This method utilizes agar plates inoculated with an inoculum adjusted to match the turbidity of a standard (usually 0.5 McFarland standard). This assay is more valuable compared to other methods as it is simple to perform, costs less than other methods, a large number of antimicrobial agents and microorganisms can be tested and the results are easy to interpret (Sadiki and Ibnsouda 2016). As a result of this, the disk diffusion assay is normally incorporated in the screening of plant extracts, essential oils and other drugs in order to evaluate their antimicrobial potential (Das, Tiwari *et al.* 2010; Konate, Mavoungou *et al.* 2012). In this study, the disk diffusion assay was used to

evaluate the antifungal effect of *G. africana* extract against *C. albicans* (ATCC 90028) and *C. glabrata* (ATCC 26512). The extract used in this assay was prepared using crystals that were previously dried and then re-dissolved in 50% ethanol (Sigma-Aldrich, South Africa) to give a stock solution of 500 mg/ml. This solution was placed in a water bath at 37˚C to aid in dissolving the crystals with brief shaking in between until the crystals were completely dissolved. Sterile Muktell filter paper disks (9 mm) (Lasec, South Africa) were impregnated with two-fold serial dilution of the extract at concentrations of 3.91, 7.81, 15.63, 31.25, 62.5, 125 and 250 mg/ml. Control disks were also incorporated that were impregnated with 25 µg/ml fluconazole and 50% ethanol. The ethanol control (50%) disk was included as this was the solvent used to dissolve the extract. The disks were then allowed to dry at 37°C overnight before being placed on Yeast Nitrogen Base Glucose (YNBG) (Sigma-Aldrich, USA) agar plates. A sterile cotton swab was dipped into the *C. albicans* (ATCC 90028) and *C. glabrata* (ATCC 26512) inoculums (adjusted to 0.5 McFarland standard) and used to swab the YNBG agar plates. The disks infused with the extract, fluconazole and ethanol were aseptically placed on to the surface of the inoculated plates and left to incubate for 24 and 48 h at 37˚C. All experiments were performed in triplicate. Observations of antifungal sensitivity and resistance were done after 24 and 48 h of incubation by measuring zones of inhibition.

5.3.4 Broth microdilution assay

As described in Chapter 2, the broth microdilution method was used in susceptibility testing of antimicrobial agents as well as in the determination of the MIC. This method can be used in the quantitative measurement of the *in vitro* activity of bacteria and fungi. Cell suspensions of microorganisms are adjusted to 0.5 McFarland standard for use in this method. The CLSI has

standardized this broth microdilution method to test yeasts (CLSI 2002) and filamentous fungi (CLSI 2008).

The antifungal activities of both the dried and ethanolic *G. africana* extracts were determined using the broth microdilution assay. It was performed in sterile polystyrene 96-well flat bottom microtitre plates according to the CLSI (CLSI 2008). Prior to conducting the assay, the cultures were grown on Sabouraud Glucose Agar (SGA) (Sigma-Aldrich, USA) at 37°C overnight for 18-24 h. About 2-5 colonies were picked and homogenized in phosphate buffered saline (PBS) (Life Technologies, USA) and the turbidity adjusted to 0.5 McFarland standard using a nephelometer. A 1:100 cell suspension was prepared containing a final concentration of 1-5 x 10^3 CFU/ml. In the sterile 96-well plates, 100 µl of RPMI broth was added to columns 2-11. This was followed by the addition of 100 µl of the extract at a concentration of 50 mg/ml into column 2 (wells A-E) and serially diluted until column 11 (wells A-E). Column 1 (wells A-E) had the extract at a concentration of 50 mg/ml and no broth was added to the well in this column. This was done so as to ensure that once the cell suspensions were added, the final concentration of the extract in those wells would be the desired 25 mg/ml starting concentration. The concentration of the extract ranged from 0.024 to 25 mg/ml. Column 12 contained positive controls (100 µl cells and 100 µl broth without extract in wells A&B), fluconazole control at a final concentration of 2 µg/ml for *C. albicans* and 32 µg/ml for *C. glabrata* (100 µl of fluconazole and 100 µl cell suspension in well C), 200 µl RPMI broth control (well D) and 200 µl saline control (well E). The broth and saline controls were included as sterility controls. 100 µl of cell suspension was then added to each well except the broth and saline control wells. Each well contained a final volume of 200 µl and the experiments were performed in separate plates for *C. glabrata* (ATCC 26512) and *C. albicans* (ATCC 90028). The plates were then sealed using sealing films and

incubated at 37°C for 24 and 48 h. After the incubation period, 40 µl of *p*-iodonitrotetrazolium chloride (INT) (a colometric agent), at a concentration of 0.2 mg/ml, was then added to each well and incubated for another 30 min at 37°C. This technique relied on the ability of viable cells to reduce the tetrazolium salt from yellow to pink/red. The MIC was determined as the lowest concentration of the extract showing no color change and exhibiting complete inhibition of fungal growth as seen by the lack of color change from yellow to pink.

5.3.5 MFC determination

The MFC was determined by sub-culturing a 10 µl aliquot from each negative well (well that did not produce any color change) and from a positive well (cell suspension control well) onto drug-free SGA. This was incubated for a further 18-24 h at 37ºC. The concentration that yielded no single bacterial colony on the solid medium was taken as the MFC (Costa, Endo *et al.* 2015).

5.3.6 Checkerboard assay

The checkerboard assay was used to assess the interaction between the ethanolic extract of *G. africana* and fluconazole as previously described (Hsieh, Yu *et al.* 1993; Petersen, Labthavikul *et al.* 2006), with some modifications as outlined in Chapter 2. This assay was employed to evaluate the effect of *G. africana* and fluconazole combinations against *Candid*a cell growth. In this method, like in Chapter 2, the ethanolic extract of *G. africana* was used to assess the extract-fluconazole combination effect. This method allowed for the evaluation of various extract-fluconazole combinations ranging from 4 x MIC to 1/16 x MIC. This broad range enabled the evaluation of antagonism and synergism, as well as any other effect, to be carried out. Both *G. africana* and fluconazole were tested on their own, on the same 96-well plate, for easy comparison. Two-fold serial dilutions of fluconazole were made across the X-axis Row A (2-11)

and two-fold serial dilutions of *G. africana* extract were made across the Y-axis column 1 (B-H). Fluconazole concentrations ranged from 0.12 to 64 µg/ml and *G. africana* concentrations ranged from 0.78 to 50 mg/ml. Both the extract and fluconazole were made up at concentrations that were double the desired concentration to be tested. This was done in order to ensure that the appropriate concentrations were obtained once the cell suspensions were added to the wells. For example, since the starting concentration of fluconazole was 64 µg/ml and that of *G. africana* was 50 mg/ml, the necessary starting concentrations were therefore 128 µg/ml and 100 mg/ml for fluconazole and *G. africana*, respectively. In order to carry out the checkerboard assay, two plate panels were used for both *Candida* strains. Panel 1 was used for the extract-fluconazole combination and panel 2 was used for the preparation of the fluconazole dilutions. In panel 1, 50 µl of RPMI 1640 media was added to all the wells except the wells in rows A (1-A11) and B (1-11) and column 1 (A-H). Once the broth was added, 50 µl of *G. africana* extract at a concentration of 200 mg/ml was then added to row C (2-11). Two fold serial dilutions were made, down the Y-axis, from each well in row C (2-11) down to row H (2-11) and 50 µl discarded from the last wells (to ensure that these wells only contained 50 µl of solution). Row B (2-11) contained 50 µl of the extract at a concentration of 200 mg/ml. This was done to ensure that the desired concentration was obtained once all the components were added. For panel 2, 100 µl of RPMI 1640 media was added to wells in columns 3-11. Fluconazole at a concentration of 256 µg/ml was then added to column 3 and two-fold serial dilutions made until column 11. Care was taken to ensure that 100 µl was discarded from column 11 and not added to column 12 as this column contained the control wells. Column 2 contained only 100 µl of fluconazole (256 µg/ml) with no RPMI 1640 media added. This was done to make sure that the final concentration of fluconazole and *G. africana*, in the combination assay, would be the appropriate range. In

addition, this plate layout also ensured that the fluconazole would be added to panel 1 (plate containing the extract) in the same way that it appeared in panel 2 (as the wells were corresponding to those in panel 1). Once the concentration panels were established, 50 µl of fluconazole in column 2 (in panel 2) was added to column 2 (in panel 1), and the same applied to all the other concentrations. Once this done, panel 1 now had both fluconazole and *G. africana* at double their starting concentration (128 µg/ml and 100 mg/ml for fluconazole and *G. africana*, respectively). 100 µl of yeast cell suspension was then added to the wells (except the sterility control wells) and this resulted in starting concentrations of 64 µg/ml for fluconazole and 50 mg/ml for *G. africana*. Control wells were also included in column 12 that contained the following controls: positive control (cell suspension and broth only, wells A&B); negative control (broth only) and de-ionized water control (water only). Well A1 was also used as a positive control well. All wells contained a final volume of 200 µl. The plates were then covered with a plastic film and incubated at 37ºC for 24 h. After the incubation period, 40 µl of 0.2 mg/ml of *p*-iodonitrotetrazolium chloride (INT) was then added and the plates were then incubated for another 30 min at 37ºC. After the incubation period, the plates were analyzed and the fractional inhibitory concentration indices (FICI) were determined.

5.3.6.1 *Calculating the Fractional Inhibitory Concentration Index (FICI)*

The FICI was used to estimate the interaction between fluconazole and *G. africana* in the checkerboard assay. It was determined using the fractional inhibitory concentrations (FICs) of fluconazole and *G. africana* calculated using their MICs alone as well as in combination. The FICs were calculated using the negative wells (wells that exhibited no growth) in the growth-no-growth interface. The FIC for a particular drug in a given well was established by dividing the concentration of that drug in that well by the control MIC of the test organism to that same drug.

The FIC index of a well was obtained by adding the FICs of each of the drugs present in the well. The FICI is calculated as follows:

$$\text{FIC index} = \text{FICx} + \text{FICy} = \frac{(X)}{(MICx)} + \frac{(Y)}{(MICy)}$$

The (X) is the MIC of drug X in combination; (MIC_x) is the MIC of drug X alone; FIC_x is the fractional inhibitory concentration of drug X; and (Y), (MIC_y) and FIC_y are established in the same way for Y as they are for X (Krogstad and Moellering 1986). The results were interpreted as follows: FICI $\leq$ 0.5 was defined as synergy, 0.5 < FICI < 1 as additive, 1 < FICI < 4 as indifference and antagonism as FICI > 4 (Olajuyigbe and Afolayan 2013).

5.3.7 Sensitire YeastOne colorimetric MIC procedure

This testing system was conducted in order to evaluate the susceptibility of *Candida* species to various antimicrobial agents. This method relies on the same principle as the broth microdilution assay and offers both qualitative and quantitative analysis of the MIC. Dried plates imbedded with antifungal agents were used. This method is a colorimetric microdilution assay that uses a colorimetric indicator to indicate yeast cell growth. The plates contain antifungal agents at various concentrations including the colorimetric indicator. The Sensititre susceptibility test was performed according to the manufacturer's instructions (TREK Diagnostics Systems, UK). Figure 5.1 shows the Sensititre YeastOne YO10 plate format for *Candida* species while Table 5.1 shows the dilution ranges of the antifungal agents present on the Sensititre plates. The Sensititre YeastOne YO10 plates (for evaluating the susceptibility of yeast isolates) were obtained from TREK Diagnostic Systems (UK).

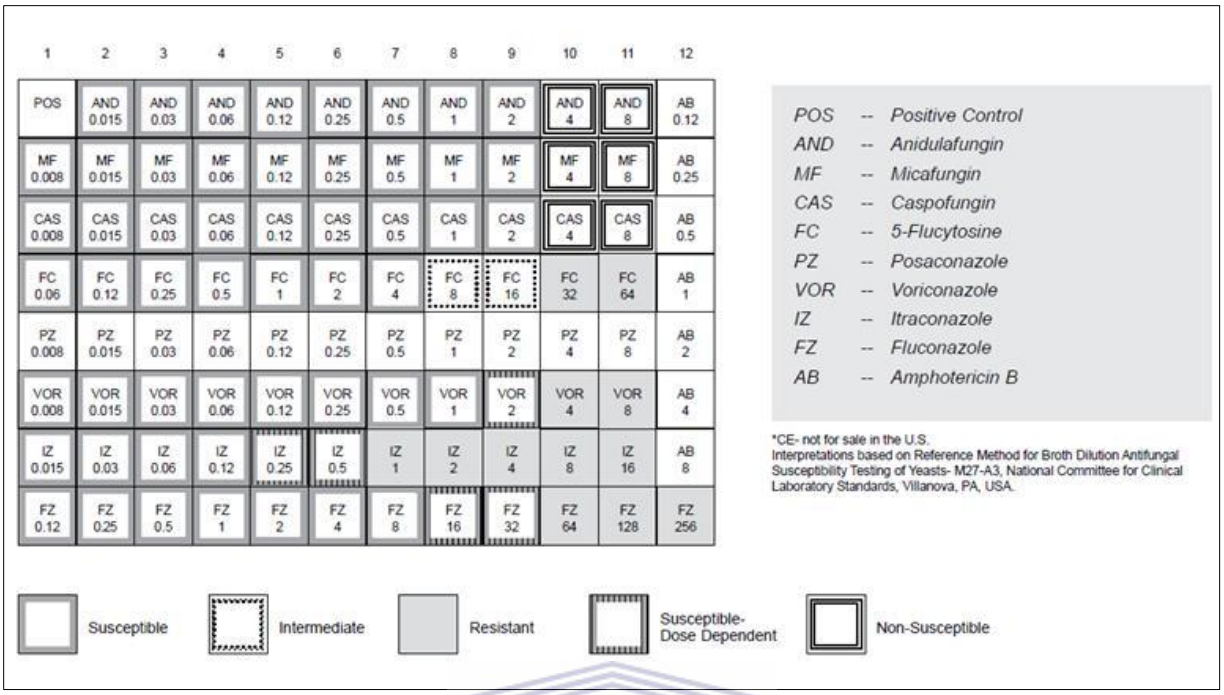

Figure 5.1: Sensititre YO10 plate format for *Candida* species.

Table 5.1: Dilution ranges of antifungal agents in the Sensititre YO10 plate.

Antifungal Agent	Dilution Range µg/ml
Amphotericin B	0.12 - 8
5-Flucytosine	0.06 - 64
Anidulafungin	0.015 - 8
Caspofungin	0.008 - 8
Micafungin	0.008 - 8
Fluconazole	0.12 - 256
Itraconazole	0.015 - 16
Posaconazole	0.008 - 8
Voriconazole	0.008 - 8

Before conducting the test, the broths were allowed to come to room temperature. The manufacturer recommended a final organism density of approximately 1.5-8 x 10^3 CFU/ml. Several well-isolated colonies were picked from a pure 24 h culture (colonies were > 1 mm in diameter) of the yeast isolates and emulsified in sterile PBS. The suspension was then vortex to mix the contents for approximately 15 s to ensure that a uniform suspension was obtained. Any

clumps present were allowed to settle prior to adjusting the density. The density of the suspension was adjusted to 0.5 McFarland standard using a nephelometer. Before adding the adjusted cell suspension to the broth, *G. africana* was added. Calculations were made to ensure that *G. africana* would be at the desired final concentration but without affecting the recommended final organism density. The concentrations of *G. africana* used in this susceptibility test were 6.25 (the MIC) and 12.5 (2 x MIC) mg/ml. The desired concentrations were obtained by removing a specific amount of broth from the tube and replacing it with the same volume of the extract. For example, in order to have a final extract concentration of 6.25 mg/ml, 344.4 µl of broth was removed from the tube and 344.4 µl of a *G. africana* ethanolic extract, at a concentration of 200 mg/ml, was added to that tube. This was done to make sure that the final volume in the tube was 11 ml and the final extract concentration was the desired one (either 6.25 or 12.5 mg/ml). Once this was done, 20 µl of the cell suspension was added to the 11 ml broth-extract solution to give an inoculum concentration of 1.5-8 x 10^3 CFU/ml. Control plates without the extract were also included. Care was taken to ensure that this was completed within 15 min. 100 µl was then transferred to the Sensititre plates (TREK Diagnostic Systems, UK) using a multichannel pipette. Following this step, the plates were sealed with the adhesive seal supplied. The plates were then incubated for 24 h at 37ºC. The plates were read manually by visually reading them under normal laboratory lighting. Yeast growth in the antifungal solutions was represented by a change in the colorimetric growth indicator from blue (negative) to red (positive). The manufacturer noted that some yeasts may show a purpling color as opposed to a completely red color. In addition, the manufacturer also explained that certain organisms may show purpling in wells containing posaconazole, voriconazole, fluconazole, itraconazole and ketoconazole. Furthermore, the manufacturer also recommended that the positive control wells

be examined after a 24 h incubation period especially when dealing with *Candida* species. They suggested that if the growth in the positive control wells was red, then antifungal endpoints could be interpreted. However, if the wells were still blue or purple, then the plates were to be re-incubated for an extra 24 h and re-examined. The MIC was recorded as the lowest concentration of antifungal agent inhibiting yeast growth as evident by the lack of development of a red or purple color in the growth well i.e. from a blue color. The intensity of color produced was compared to that of the positive control wells.

5.3.8 Microscopic analysis

5.3.8.1 *Light Microscopy*

Light microscopy was performed in order to assess the effect of the extract on the structure of the *Candida* strains after treatment. This was done after the yeast cells were exposed to the extract in the disk diffusion assay. Once the plates were incubated, macro-colonies (colonies outside the inhibition zone area) and micro-colonies (colonies from within the inhibition zone area) were picked and placed on slides. This was done for both *Candida* strains. The slides were then stained with 10% Lactophenol Cotton Blue (LCB) and viewed under 1000 X magnification. This was done in order to observe any morphological changes that may have been caused by the *G. africana* extract.

5.3.8.2 *Scanning Electron Microscopy (SEM)*

Scanning electron microscopy (SEM) was used to evaluate the effect of the extract on the *Candida* cells. SEM was performed on yeast cells treated with the extract in the disk diffusion assay. Specimens were fixed onto a glass cover slip using 2.5% gluteraldehyde in phosphate buffer saline (PBS) for 1 h. The cover slip was then washed twice with PBS and twice with

sterile distilled water in 5 min cycles. Samples were then dehydrated in graded concentrations of ethanol (50, 70, 90 and 100%) in 10 min cycles. After the dehydration process, samples were then removed from the 100% ethanol and placed into the critical point dryer (CPD) or air dried in order to remove any remnants of ethanol. Once all samples were dried, they were then sputter-coated with gold-palladium alloy and analyzed using the scanning electron microscope (X650 SEM).

5.4 RESULTS

5.4.1 Identification of *Candida* species

C. albicans (ATCC 90028) and *C. glabrata* (ATCC 26512) were identified using an oxoid chromogenic differential medium plate where *C. albicans* appeared as green colonies and *C. glabrata* appeared as beige/brown or purple/mauve colonies. Figure 5.2 represents the results obtained after the *Candida* species were streaked onto the oxoid chromogenic differential medium plate and incubated.

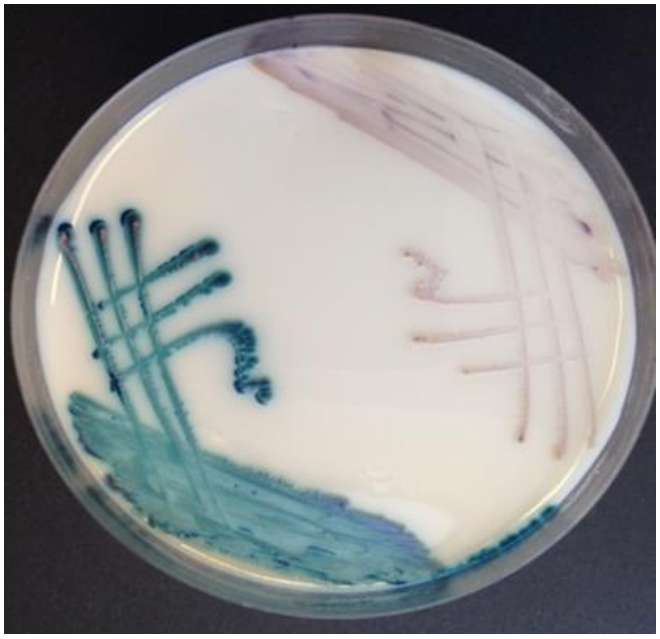

Figure 5.2: Identification of *Candida* species using Oxoid chromogenic differential medium plate. *Candida albicans* appear as green colonies and *Candida glabrata* appear as beige/brown or purple/mauve colonies.

5.4.2 Disk diffusion assay

The disk diffusion assay was utilized to investigate the antifungal activity of *G. africana* against *C. albicans* (ATCC 90028) and *C. glabrata* (ATCC 26512). The dried *G. africana* extract was dissolved in 50% ethanol to make up a 500 mg/ml stock solution. The results of the disk diffusion assay evaluating the effect of *G. africana* against the *Candida* strains are shown in Figures 5.3 and 5.4.

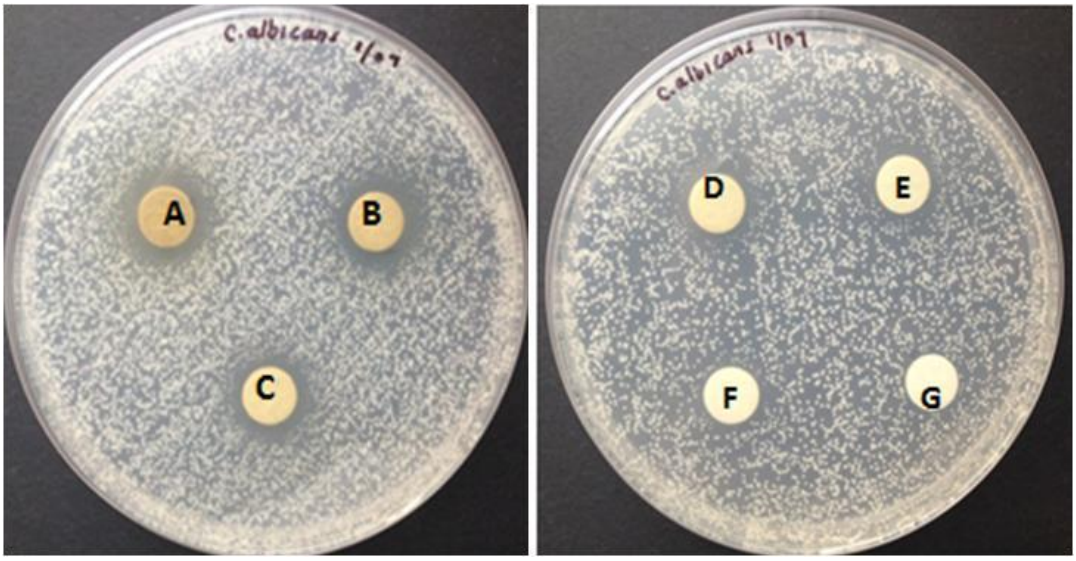

Figure 5.3: Inhibition zones of *C. albicans* treated with *G. africana* extract (3.91 to 250 mg/ml). The letters represent the following concentrations: A: 250 mg/ml; B: 125 mg/ml; C: 62.5 mg/ml; D: 31.25 mg/ml; E: 15.62 mg/ml; F: 7.81 mg/ml and G: 3.91 mg/ml.

Figure 5.3 shows small zones of inhibition, with the presence of micro-colonies, at concentrations of 15.6 to 250 mg/ml against *C. albicans* (ATCC 90028). *G. africana* concentrations of 7.8 and 3.9 mg/ml did not produce any visible zones of inhibition. The zones produced after treatment of *C. glabrata* (ATCC 26512) with *G. africana* were small and contained micro-colonies at concentrations of 31.25 to 250 mg/ml. The zone of inhibition produced after treatment with 15.62 mg/ml of *G. africana* was not well defined but micro-colonies were present. Concentrations of 7.8 and 3.91 mg/ml did not produce any visible inhibition zones (Figure 5.4).

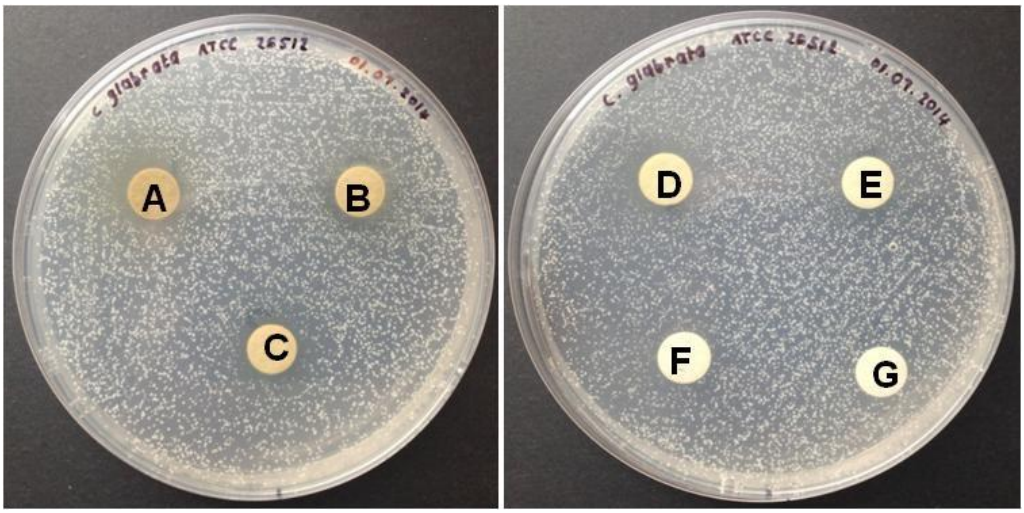

Figure 5.4: Inhibition zones of *C. glabrata* treated with *G. africana* extract (3.91 to 250 mg/ml). The letters represent the following concentrations: A: 250 mg/ml; B: 125 mg/ml; C: 62.5 mg/ml; D: 31.25 mg/ml; E: 15.62 mg/ml; F: 7.81 mg/ml and G: 3.91 mg/ml.

Control plates were also included that contained 50% ethanol and fluconazole (25 µg/ml). The control results are represented in Figures 5.5 and 5.6 for ethanol and fluconazole, respectively. There were no zones of inhibition present after exposure of both *Candida* strains to 50% ethanol. Treatment of the *Candida* species with fluconazole yielded an inhibition zone of 18 mm against *C. albicans* (ATCC 90028), with the presence of micro-colonies. However, there was only a small zone of inhibition produced when treated against *C. glabrata* (ATCC 26512).

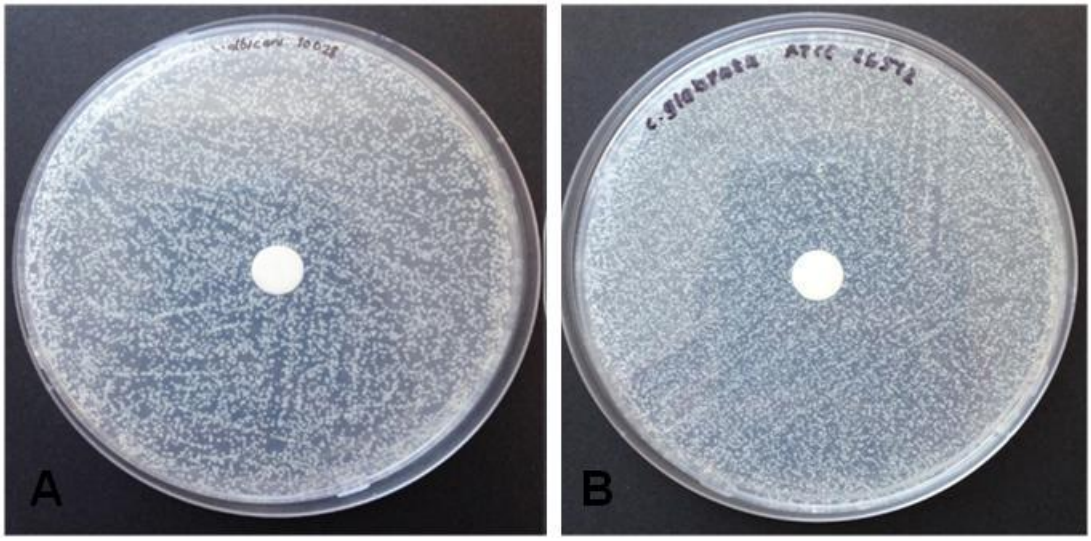

Figure 5.5: Treatment of *C. albicans* (A) and *C. glabrata* (B) with 50% ethanol. No zones of inhibition present for both *Candida* species.

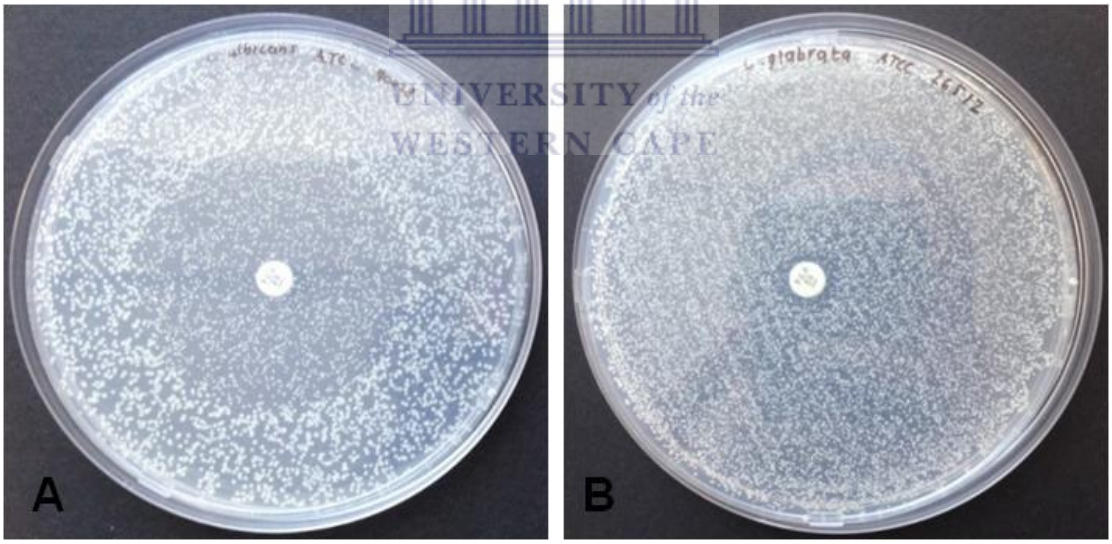

Figure 5.6: Treatment of *C. albicans* (A) and *C. glabrata* (B) with fluconazole (25 µg/ml). Fluconazole produced an inhibition zone of 18 mm with the presence of micro-colonies against *C. albicans* whereas there was only a small inhibition zone present when treated against *C. glabrata*.

5.4.3 Broth microdilution

The broth microdilution assay was used to evaluate the effect of both the dried and ethanolic *G. africana* extracts. The MIC of the dried *G. africana* extract against *C. albicans* (ATCC 90028) was 3.91 mg/ml (Figure 5.7) while the MIC of the dried extract against *C. glabrata* (ATCC 26512) was 1.95 mg/ml (Figure 5.8). The ethanolic extract produced an MIC of 6.25 mg/ml against both *C. albicans* (ATCC 90028) and *C. glabrata* (ATCC 26512) (Figures 5.9 and 5.10, respectively)

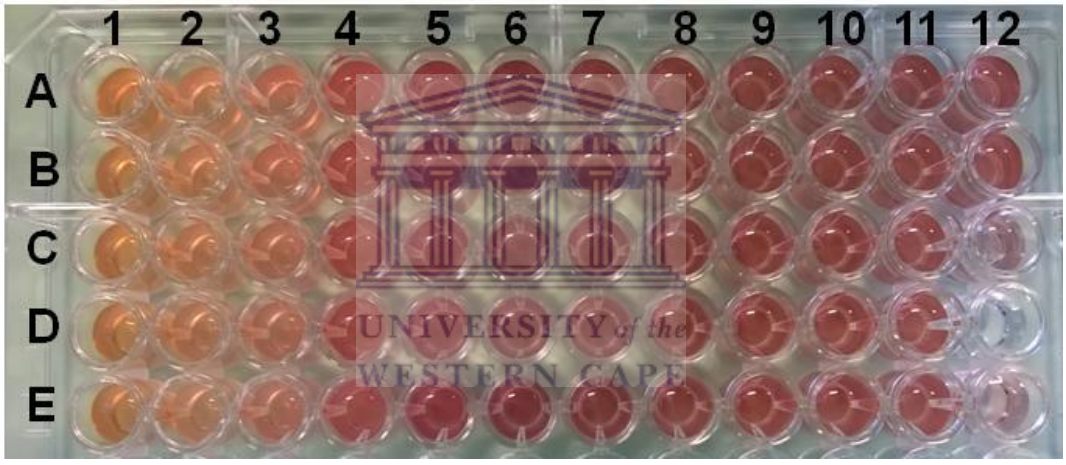

Figure 5.7: Broth microdilution results of *C. albicans* treated with the dried *G. africana* extract (0.015 to 15.63). Concentrations ranged from highest to lowest (columns 1-11, respectively). Column 12 contained control wells as follows: 12A&B: Positive control; 12C: Fluconazole control; 12D: Saline control and 12E: Broth control.

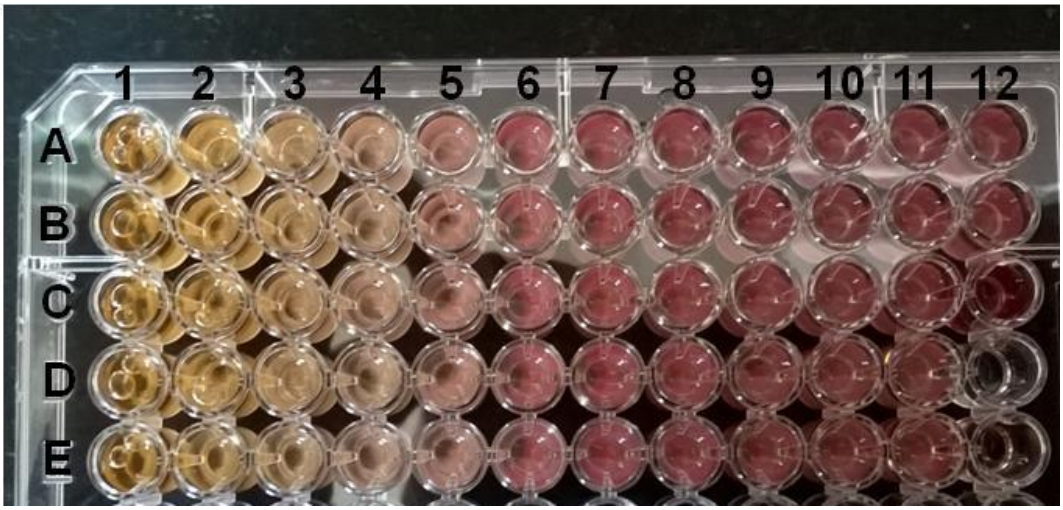

Figure 5.8: Broth microdilution results of *C. glabrata* treated with the dried *G. africana* extract (0.015 to 15.63). Concentrations ranged from highest to lowest (columns 1-11, respectively). Column 12 contained control wells as follows: 12A&B: Positive control; 12C: Fluconazole control; 12D: Saline control and 12E: Broth control.

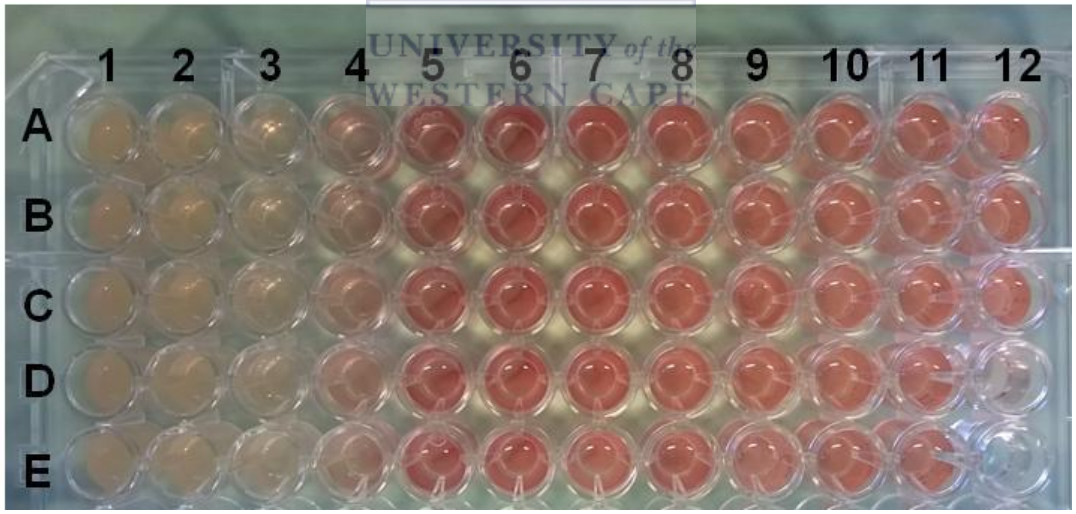

Figure 5.9: Broth microdilution results of *C. albicans* treated with *G. africana* ethanolic extract (0.024 to 25 mg/ml). Concentrations ranged from highest to lowest (columns 1-11, respectively). Column 12 contained the following control wells: 12A&B: Positive control; 12C: Fluconazole control; 12D: Broth control and 12E: Saline control.

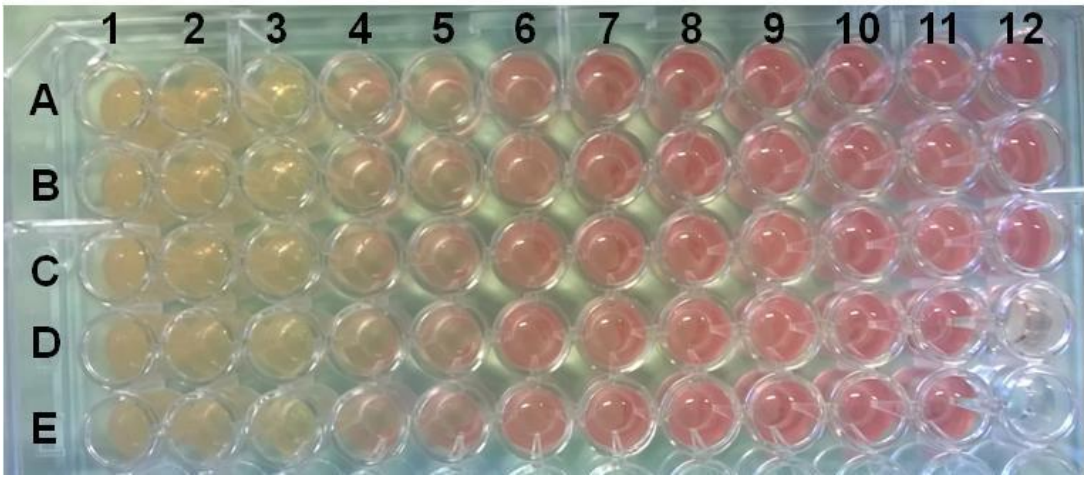

Figure 5.10: Broth microdilution results of *C. glabrata* treated with *G. africana* ethanolic extract (0.024 to 25 mg/ml). Concentrations ranged from highest to lowest (columns 1-11, respectively). Column 12 contained the following control wells: 12A&B: Positive control; 12C: Fluconazole control; 12D: Broth control and 12E: Saline control.

5.4.4 Minimum fungicidal concentration (MFC)

The MFCs of the dried and ethanolic extracts were evaluated in order to establish whether *G. africana* was fungistatic or fungicidal. Figure 5.11 shows the results obtained after sub-culturing aliquots from the negative wells (wells with no growth) in the microdilution assay onto SGA. The results revealed that the dried *G. africana* extract was fungistatic at all the concentrations (even the highest one used) as demonstrated by the presence of growth on the SGA plates.

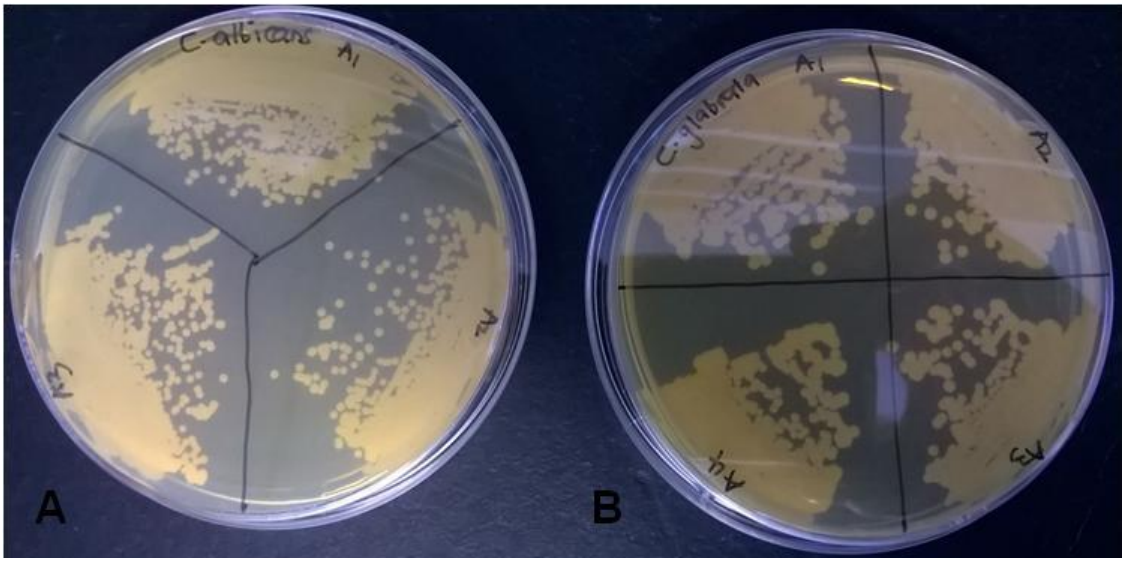

Figure 5.11: MFC results of *C. albicans* (A) and *C. glabrata* (B) treated with the dried *G. africana* extract (1.95 to 15.63 mg/ml). A1-A4 represents the different *G. africana* concentrations as follows: A1: 15.63 mg/ml; A2: 7.81 mg/ml; A3: 3.91 mg/ml and A4: 1.95 mg/ml.

The results obtained after sub-culturing the aliquot from the negative wells treated with the ethanolic extract are shown in Figure 5.12. The MFC of the ethanolic extract against *C. albicans* was 6.25 mg/ml and that of ethanolic extract against *C. glabrata* was 12.5 mg/ml. This showed that the MIC=MFC=6.25 mg/ml for *C. albiacans* whereas the MIC=6.25 mg/ml and MFC=12.5 mg/ml for *C. glabrata*.

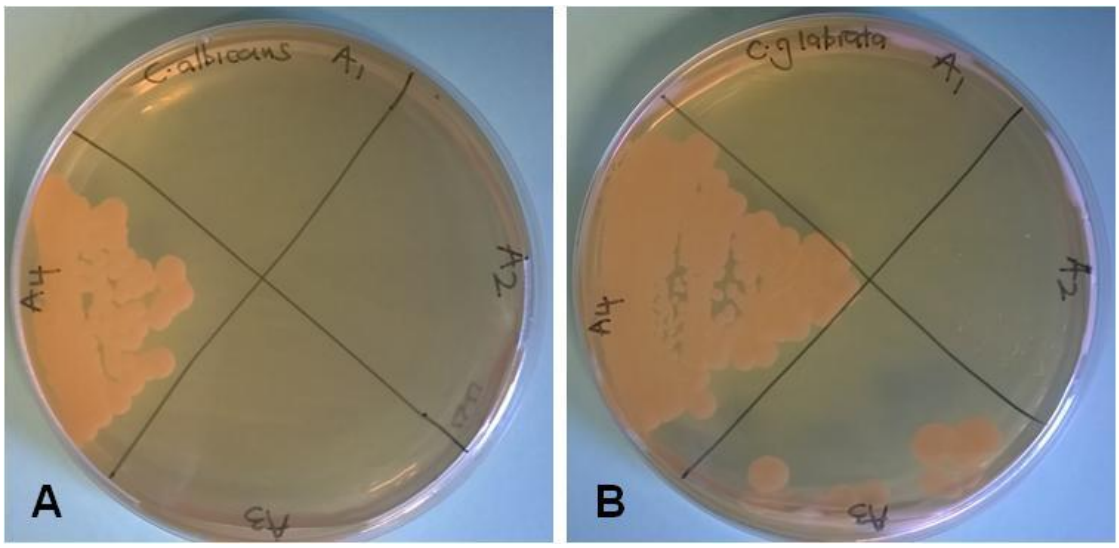

Figure 5.12: MFC results of *C. albicans* (A) and *C. glabrata* (B) treated with the *G. africana* ethanolic extract (3.12 to 25 mg/ml). A1-A4 represent the different *G. africana* concentrations as follows: A1: 25 mg/ml; A2: 12.5 mg/ml; A3: 6.25 mg/ml and A4: 3.12 mg/ml.

The MFC results demonstrated that the ethanolic extract exhibited greater antifungal activity compared to the dried extract. The ethanolic extract was fungicidal at concentrations of 6.25 and 12.5 mg/ml for *C. albicans* (ATCC 90028) and *C. glabrata* (ATCC 26512) respectively. The dried extract was fungistatic and not fungicidal even at a concentration of 15.63 mg/ml (the highest concentration used). It is for this reason that the ethanolic extract was used in the checkerboard assay and Sensititre susceptibility testing method in order to assess the combination effects of the extract and antifungal agents.

5.4.5 Checkerboard assay

The checkerboard assay was used to evaluate the effect of the extract-fluconazole combination on the *Candida* species. The effect of the combination of fluconazole and *G. africana* against *C. albicans* is shown in Figure 5.13. The plate layout was similar for both *C. albicans* (ATCC 90028) and *C. glabrata* (ATCC 26512). Row A (2-11) represented concentrations of fluconazole ranging from 64 to 0.125 µg/ml, respectively and Column 1 (B-H) represented *G. africana* concentrations ranging from 50 to 0.78 mg/ml, respectively. In order to calculate the FICI, the following parameters were needed: MIC of either fluconazole or *G. africana* alone as well as the MICs of both fluconazole and *G. africana* in combination for both *Candida* species. The MIC of fluconazole alone was 0.5 µg/ml and the MIC in combination was 0.125 µg/ml against *C. albicans* (ATCC 90028). The MIC of *G. africana* alone was 6.25 mg/ml and the MIC in combination was 0.78 mg/ml. With these parameters, the FICI was calculated as follows:

$$\text{FICI} = \frac{0.12\ \mu g/ml}{0.5\ \mu g/ml} + \frac{0.78\ mg/ml}{6.25\ mg/ml} = 0.24 + 0.12 = 0.36$$

The FICI value obtained was 0.36 which indicated that the interaction between fluconazole and *G. africana* against *C. albicans* (ATCC 90028) was synergistic since the value was < 0.5.

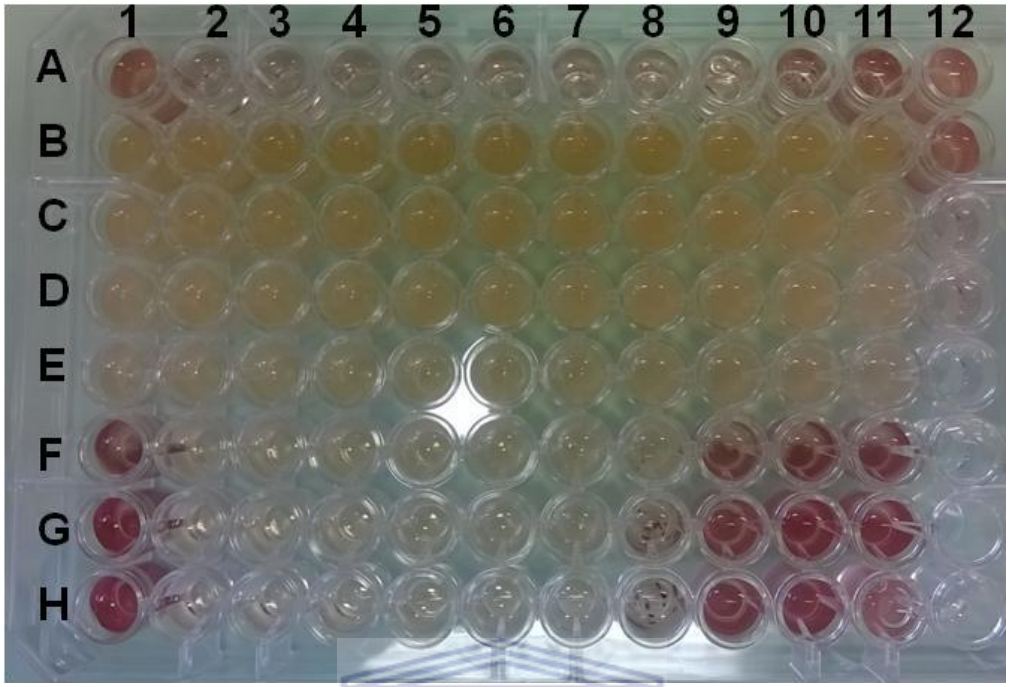

Figure 5.13: Checkerboard assay results of *C. albicans* treated with the *G. africana* ethanolic extract (0.78-50 mg/ml) and fluconazole (0.012-64 µg/ml). Row A (2-11) represents fluconazole and Column 1 (B-H) represents *G. africana*. The other wells represent various combinations of fluconazole and *G. africana*.

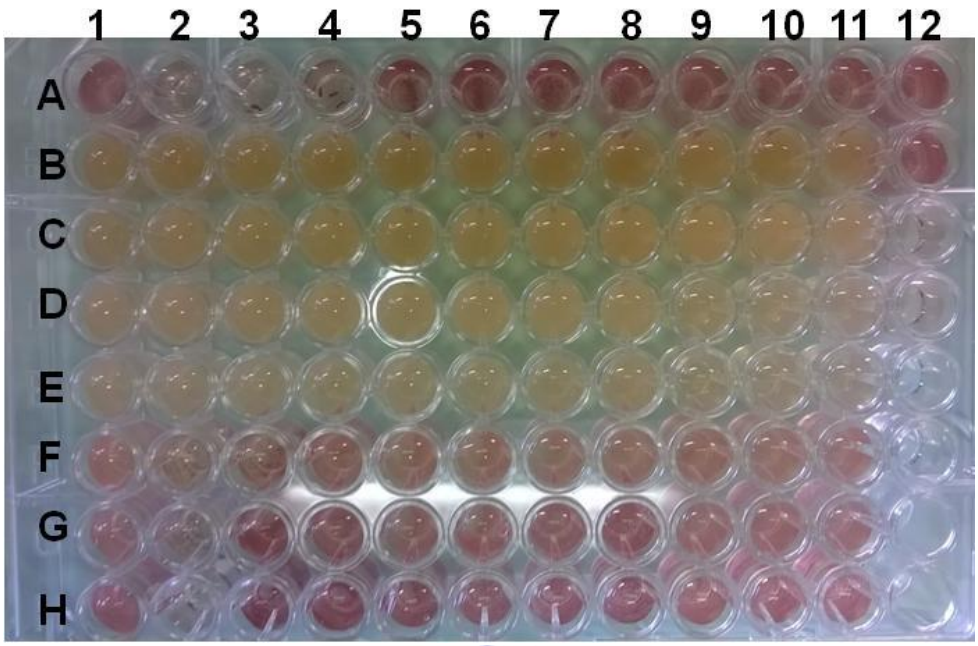

Figure 5.14: Checkerboard assay results of *C. glabrata* treated with the *G. africana* ethanolic extract (0.78-50 mg/ml) and fluconazole (0.012-64 µg/ml). Row A (2-11) represents fluconazole and Column 1 (B-H) represents *G. africana*. The other wells represent various combinations of fluconazole and *G. africana*.

Figure 5.14 shows the results obtained after treating *C. glabrata* (ATCC 26512) with the extract-fluconazole combinations. The results of the checkerboard assay revealed the following: the MIC of fluconazole alone was 64 µg/ml and the MIC in combination was 0.12 µg/ml. The MIC of *G. africana* alone and in combination was 6.25 mg/ml. Therefore, the FICI was calculated as follows:

$$\text{FICI} = \frac{0.12\ \mu\text{g/ml}}{64\ \mu\text{g/ml}} + \frac{6.25\ \text{mg/ml}}{6.25\ \text{mg/ml}} = 0.002 + 1 = 1.002$$

Since the FICI value obtained was 1.002, which was 1 < FICI < 4, the interaction was classified as being indifference. This meant that the interaction observed was as a result of the *G. africana* extract that had the greatest antifungal activity compared to fluconazole.

5.4.6 Sensititre YeastOne colorimetric MIC procedure

The Sensititre susceptibility test was used to evaluate the interaction between combinations of antifungal agents and *G. africana* against *C. albicans* (ATCC 90028) and *C. glabrata* (ATCC 26512). In this method, two *G. africana* concentrations (6.25 and 12.5 mg/ml) were combined with varying concentrations of different antifungal agents. Plates without the extract were also incorporated as controls in order to assess the effect of the individual drugs on the *Candida* species. Table 5.2 represents the MIC interpretive criteria as outlined by the CLSI.

Table 5.2: MIC Interpretive Criteria (µg/ml) for *Candida* species.

	MIC (µg/ml)						
	C. albicans			***C. glabrata***			**References**
Antifungal drug	**S**	**I**	**R**	**S**	**I**	**R**	
Anidulafungin	≤0.25	0.5	≥1	≤0.12	0.25	≥0.5	(Pfaller, Messer *et al.* 2013b)
Caspofungin	≤0.25	0.5	≥1	≤0.12	0.25	≥0.5	(Pfaller, Messer *et al.* 2013b)
Micafungin	≤0.25	0.5	≥1	≤0.06	0.12	≥0.25	(Pfaller, Messer *et al.* 2013b)
Itraconazole	≤0.12	0.25-0.5	≥1	≤2	–	>2	(Pfaller and Diekema 2012a)
Fluconazole	≤2	4	≥8	–	≤32	≥64	(Pfaller, Diekema *et al.* 2013a)
Posaconazole	≤0.06	–	>0.06	≤2	–	>2	(Pfaller and Diekema 2012a)
Voriconazole	≤0.12	0.25-0.5	≥1	≤0.5	–	>0.5	(Pfaller, Diekema *et al.* 2013a)
5-Flucytosine	≤4	8-16	≥32	≤4	8-16	≥32	(Pfaller, Espinel-Ingroff *et al.* 2012b)
Amphotericin	<1	–	≥1	<1	–	≥1	(Pfaller, Espinel-Ingroff

B	*et al.* 2012b)

S: susceptible; I: intermediate; R: resistant

The interpretation of possible test results that may be observed after the treatment of *Candida* species with various antifungal agents are summarized in Table 5.3 as outlined by the manufacturer.

Table 5.3: Illustration and interpretation of Sensititre susceptibility test results that may occur.

	Well Concentration (µg/ml)						
Result	**1**	**2**	**4**	**8**	**16**	**32**	**R= Red: Positive growth indicator** **B= Blue: Negative growth indicator**
A	**R**	**R**	**R**	**B**	**B**	**B**	Typical growth pattern; MIC endpoint is 8 µg/ml
B	**R**	**R**	**R**	**R**	**R**	**R**	Growth in all wells; MIC endpoint is > 32 µg/ml
C	**B**	**B**	**B**	**B**	**B**	**B**	No growth in any well; MIC endpoint is ≤ 1 µg/ml
D	**R**	**R**	**R**	**B**	**R**	**R**	"Skipped well". MIC endpoint is > 32 µg/ml. Disregard "skip" when wells on either have growth. If more than one "skip" should occur in a column, the test results are invalidated
E	**R**	**R**	**B**	**B**	**R**	**R**	Double "skipped well". The test should be repeated

Table 5.4: Sensititre susceptibility test results of *C. albicans* and *C. glabrata* treated with *G. africana* (6.25 and 12.5 mg/ml).

	MIC of antifungal drug (µg/ml)					
	C. albicans			***C. glabrata***		
Antifungal agent	**No Extract**	**Drugs + GA[a]**	**Drugs + GA[b]**	**No Extract**	**Drugs + GA[a]**	**Drugs + GA[b]**
5-Flucytosine	**= 0.5**	**≤ 0.06**	**≤ 0.06**	≤ 0.06	≤ 0.06	≤ 0.06
Amphotericin B	**= 0.5**	**≤ 0.12**	**≤ 0.12**	**= 2**	**≤ 0.12**	**≤ 0.12**
Anidulafungin	≤ 0.015	≤ 0.015	≤ 0.015	**= 0.06**	**≤ 0.015**	**≤ 0.015**
Caspofungin	**= 0.03**	**≤ 0.008**	**≤ 0.008**	**= 0.12**	**≤ 0.008**	**≤ 0.008**
Fluconazole	**= 0.25**	**≤ 0.12**	**≤ 0.12**	**= 16**	**≤ 0.12**	**≤ 0.12**
Itraconazole	≤ 0.015	≤ 0.015	≤ 0.015	**= 0.5**	**≤ 0.015**	**≤ 0.015**
Micafungin	≤ 0.008	≤ 0.008	≤ 0.008	**= 0.015**	**≤ 0.008**	**≤ 0.008**
Posaconazole	≤ 0.008	≤ 0.008	≤ 0.008	**= 1**	**≤ 0.008**	**≤ 0.008**
Voriconazole	≤ 0.008	≤ 0.008	≤ 0.008	**= 0.5**	**≤ 0.008**	**≤ 0.008**

[a] GA- *G. africana* at 6.25 mg/ml.
[b] GA- *G. africana* at 12.5 mg/ml
Values in bold represent changes in MIC observed after addition of the extract

Table 5.4 represents the results obtained after the treatment of *C. albicans* (ATCC 90028) and *C. glabrata* (ATCC 26512) with antifungal agents alone as well as treatment with extract-antifungal agent combinations. The results revealed that *G. africana* enhanced the antifungal activity of most of the antifungal agents as seen by a decrease in the antifungal MIC against the *Candida* species. *G. africana* enhanced the antifungal activity of 5-flucytosine, amphotericin B, caspofungin and fluconazole against *C. albicans* (ATCC 90028). In addition, *G. africana* enhanced the antifungal activities of amphotericin B, anidulafungin, caspofungin, fluconazole, itraconazolem micafungin, posaconazole and voriconazole against *C. glabrata*. This was significant as the MIC of fluconazole alone against *C. glabrata* (ATCC 26512) in the microdilution assay, checkerboard assay and Sensititre susceptibility test was ≥16 µg/ml, but reduced significantly in the combination assays.

5.4.7 Microscopic analysis

5.4.7.1 *Light microscopy*

Light microscopy was carried out in order to examine the effect of the extract on the structure of the yeast cells. Figure 5.15 shows the appearance of the untreated cells obtained from colonies outside the zones of inhibition (macro-colonies) and stained with 10% LCB stain. Cells appeared large, had an intact morphology without any disruption to the structure.

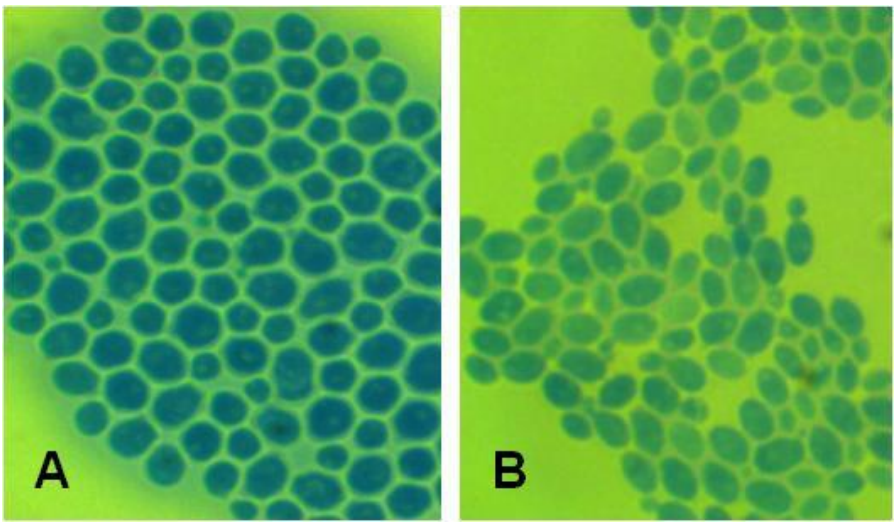

Figure 5.15: Light microscopic images of untreated *C. albicans* (A) and *C. glabrata* (B) cells. Cells were obtained from macro-colonies stained 10% LCB stain. Magnification was 1000 X and slides were viewed under oil immersion.

Figure 5.16 shows the result obtained after the cells were treated with *G. africana* at a concentration of 250 mg/ml. The cells were collected from micro-colonies and stained with 10% LCB. The cells appeared smaller and fewer in number compared to the untreated cells.

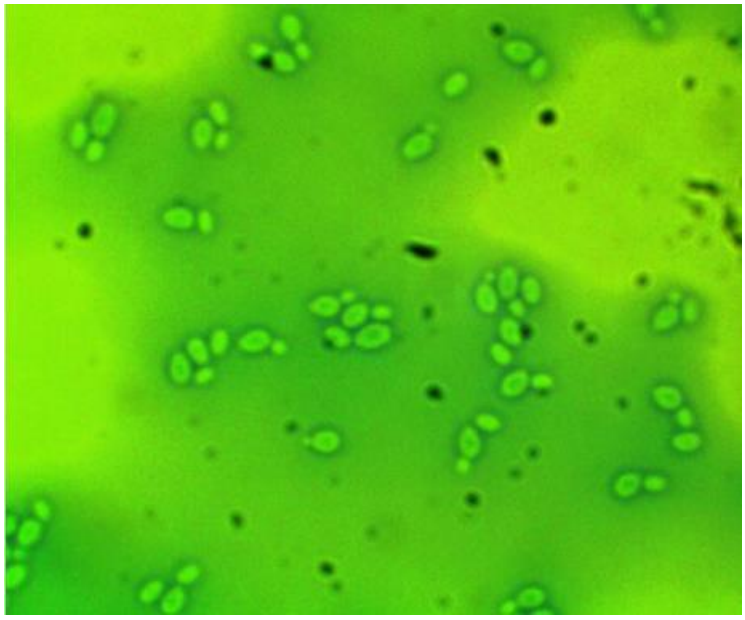

Figure 5.16: Light microscopic image of *Candida* cells. Cells were obtained from micro-colonies after treatment with *G. africana* concentration (250 mg/ml) and stained with 10% LCB and viewed at 1000 X magnification. Cells appear smaller and less in number compared to the untreated cells.

5.4.7.2 ***Scanning Electron Microscopy (SEM)***

Scanning electron microscopy was also employed to examine the structural changes of *Candida* cells after treatment with the extract. Figure 5.17 shows the appearance of the *Candida* cells prior to treatment. The cells appeared rounded with smooth surfaces. Figures 5.18 and 5.19 represent scanning electron micrographs of *C. albicans* (ATCC 90028) and *C. glabrata* (ATCC 26512) cells after treatment with *G. africana* at a concentration of 250 mg/ml, respectively. The structure of both *Candida* cells appeared distorted and only remnants of other cells could be seen.

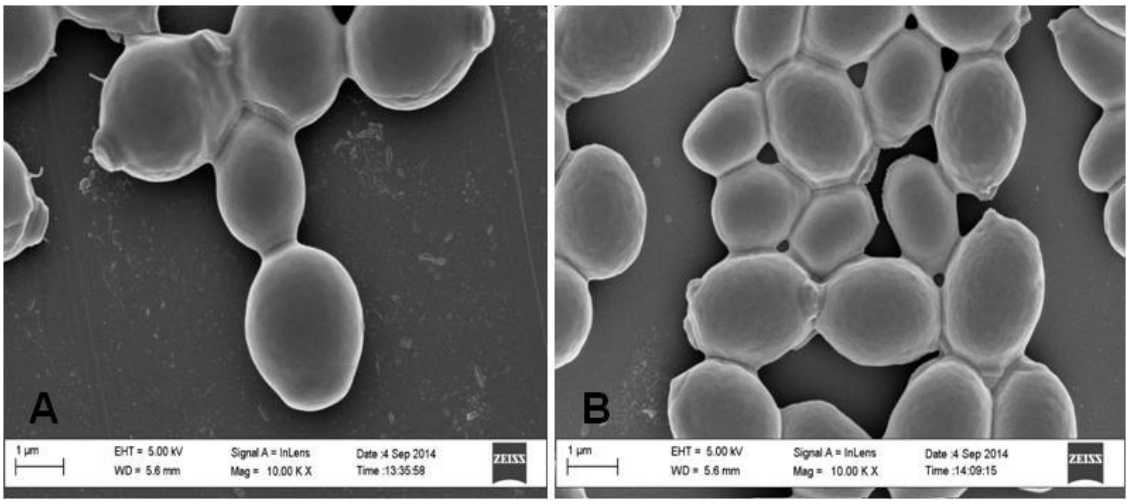

Figure 5.17: Scanning electron micrograph of untreated *C. albicans* (A) and *C. glabrata* (B) at a magnification of 10 000 X. Cells are rounded and smooth with signs of budding.

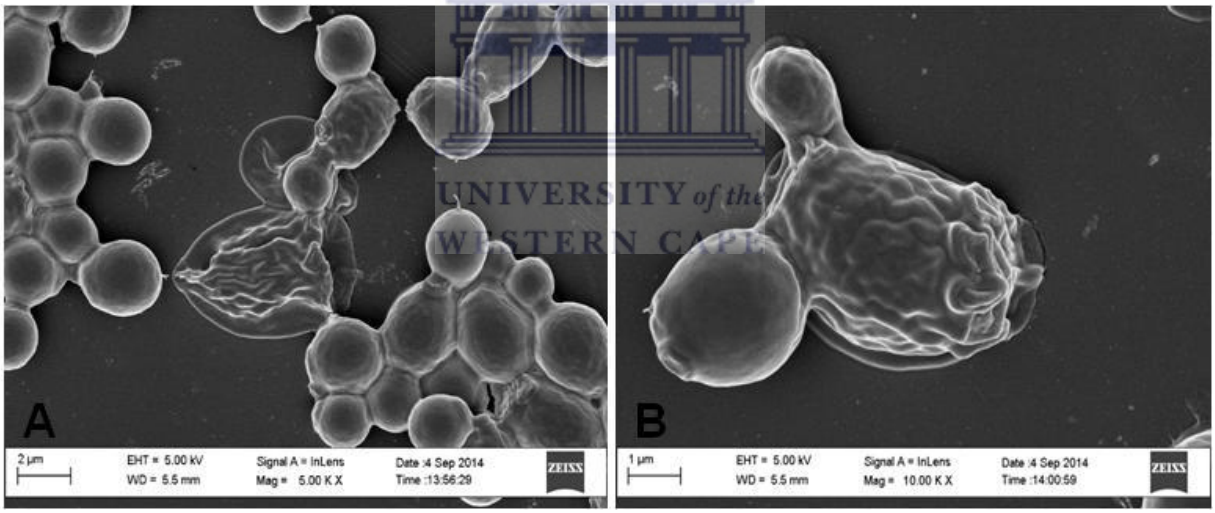

Figure 5.18: Scanning electron micrograph of *C. albicans* treated with *G. africana* extract (250 mg/ml). Cells were viewed at a magnification of 5 000 X (A) and 10 000 X (B). Cells appeared desiccated and craggy after treatment. The structural appearance was rough compared to the untreated cells.

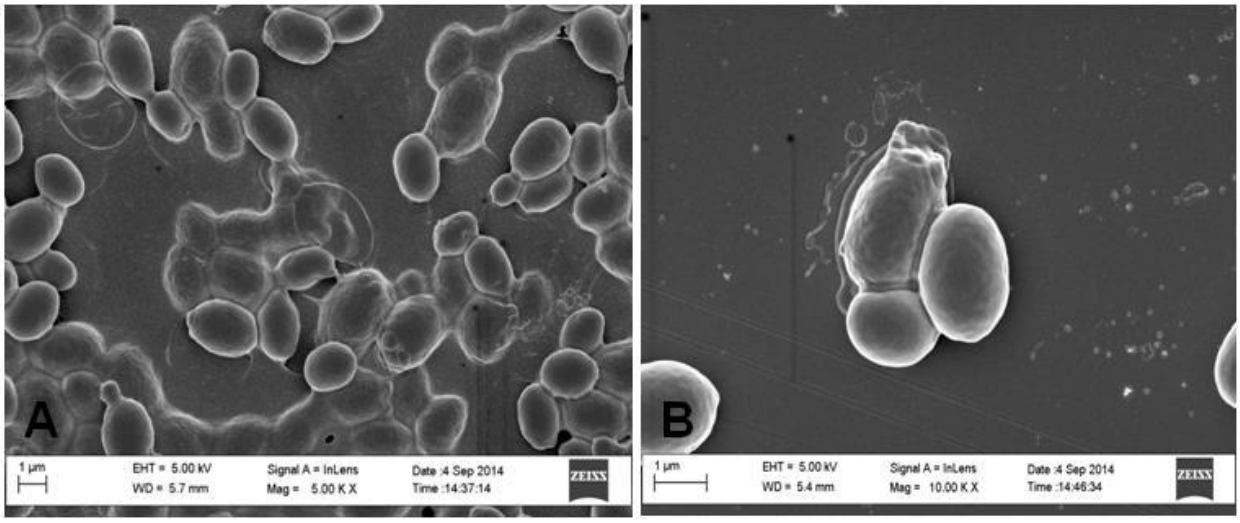

Figure 5.19: Scanning electron micrograph of *C. glabrata* treated with *G. africana* extract (250 mg/ml). Cells were viewed at a magnification of 5 000 X (A) and 10 000 X (B). Some cells appeared distorted while only remnants of other cells could be seen. The structural appearance of the cells was rough compared to the untreated cells.

5.5 Discussion

The appearance of *Candida* species that are resistant to a number of antifungal agents is as a result of the rise in the use these antifungal agents (Pemmaraju, Pruthi *et al.* 2013). An example of an infection caused by *Candida* species, particularly *C. albicans*, is oral candidiasis. This infection is normally associated with HIV-positive patients as a result of their weakened immune system. In addition, oral candidiasis can result in high mortality rates (Pomarico, de Souza *et al.* 2010; Abrantes, McArthur *et al.* 2014). Treatment of candidiasis is usually achieved by the administration of amphotericin B and fluconazole with fluconazole frequently being given as the first line of treatment when dealing with systemic infections. Unfortunately, *Candida* strains are developing resistance to fluconazole (Abrantes, McArthur *et al.* 2014). The rise in multi-drug resistant organisms prompted health institutions to propose the use of combination therapy in an

effort to avoid these organisms from developing resistance and increase the effectiveness of the drugs against infectious agents (Sukandar, Kurniati *et al.* 2016). Medicinal plants possess numerous health beneficial compounds that can be used in the development of new antimicrobial agents (Sukandar, Kurniati *et al.* 2016). In this, study, the antifungal activities and synergistic effects of *G. africana* and fluconazole were evaluated using the disk diffusion, broth microdilution and checkerboard assay. Fluconazole was selected because it is extensively used in the treatment of fungal infections as a result of its high bioavailability and low toxicity (Kohli, Smriti Mukhopadhyay *et al.* 2002). The Sensititre susceptibility testing method was used to evaluate the effect of combinations of the ethanolic extract with a selection of antifungal drugs. The effect of the *G. africana* extract on the structure of the *Candida* cells was assessed using light microscopy and scanning electron microscopy.

The results of the disk diffusion assay showed that the *G. africana* extract was not as effective in inhibiting the growth of the *Candida* strains, even at a concentration of 250 mg/ml, as seen by the lack of large inhibition zones. This lack of inhibitory activity could be attributed to the fact that natural plant products tend to diffuse out the disks much slower than conventional antimicrobial agents (Klancnik, Piskernik *et al.* 2010). In addition, polarity also influences the rate of diffusion of a compound with more polar compounds diffusing out of the disk faster than less polar compounds (Moreno, Scheyer *et al.* 2006). The results of the broth dilution assay revealed that the dried *G. africana* was more effective than the ethanolic as seen by the lower MIC against the *Candida* strains. The MIC of the dried extract against *C. albicans* (ATCC 90028) was 3.91 mg/ml while that of the ethanolic extract was 6.25 mg/ml. The MIC of the dried extract against *C. glabrata* (ATCC 26512) was 1.95 mg/ml while that of the ethanolic extract was 6.25 mg/ml. A number of studies have been carried to evaluate the antifungal potential of medicinal

plants (Isa, Awouafack *et al.* 2014; Goncalves, Piras *et al.* 2015; Sharifzadeh, Khosravi *et al.* 2015). For instance, a study conducted to assess the anticandidal activity of *Ecballium elaterium* fruit extracts and bifonazole against *C. albicans* isolates revealed that the fruit extracts exhibited good anticandidal activity with MIC values that ranged from 0.048 to 6.25 mg/ml (Adwan, Salameh *et al.* 2011). Another study carried out by Martins *et al.* (Martins, Barros *et al.* 2015) evaluated the antifungal activity of ten plant extracts, frequently used in traditional medicine, against nineteen *Candida* strains. Results showed that two hydro methanolic extracts of *Juglans regia* and *Eucalyptus globulus* leaves demonstrated good antifungal activity against all the *Candida* strains tested.

The MFCs were determined for both the dried and ethanolic extracts. The dried extract was fungistatic, and this was shown by the presence of colonies when sub-cultured on SGA at even the highest concentration (15.63 mg/ml). On the other hand, the ethanolic extract was fungicidal at concentrations of 6.25 and 12.5 mg/ml for *C. albicans* (ATCC 90028) and *C. glabrata* (ATCC 26512), respectively. This result showed that the ethanolic extract had a greater ability to kill the yeast cells compared to the dried extract. The drying process of the extract could have had an effect on the activity of some of the compounds found in the extract resulting in reduced activity. It was for this reason that the ethanolic extract was used in the checkerboard assay and Sensititre susceptibility test. A study carried out by Avijgan *et al.* (Avijgan, Mahboubi *et al.* 2014) evaluated the synergistic anticandidal activity of *Echinophora platyloba* DC ethanolic extract against 27 *C. albicans* clinical isolated. The results indicated MIC values of 3.1 to 6.25 mg/ml and MFC values of 6.2 to 12.5 mg/ml. The significant antifungal effect of the ethanolic extract compared to extracts prepared from other solvents can also be seen with other medicinal plants. A study carried out on ethanolic and methanolic leaf extracts of *Pogostemon parviflorus* Benth.

revealed that the ethanolic extract had greater antifungal activity compared to the methanolic extract. This could be seen from the mean MICs of the ethanolic extract against *Candida* species, such as *C. albicans* and *C. glabrata*, of 5.7 mg/ml compared to that of 6.6 mg/ml of the methanolic extract (Najafi and Sadeghi-Nejad 2011).

The checkerboard assay results showed that the interaction between *G. africana* and fluconazole against *C. albicans* (ATCC 90028) was synergistic as evident by the FICI of 0.36 which was < 0.5. A number of studies evaluating the synergistic effects of medicinal plants and antifungal drugs, such a fluconazole, have been conducted. For example, a study carried out on glabridin (a compound found in *Glycyrrhiza glabra*) against *Canadida* species, such as drug-resistant *C. albicans*, revealed that the combination of fluconazole and glabridin demonstrated synergistic effects (Liu, Li *et al.* 2014). Another study evaluating the antimicrobial interaction of ethanolic extracts of *Zingiber officinale* var rubrum rhizome, *Boesenbergia pandurata* rhizome and *Stevia rebaudiana* leaves with certain antibiotics against infectious mouth microbes revealed that the combination of all the plant extracts with ketoconazole showed a synergistic effect against *C. albicans* (Sukandar, Kurniati *et al.* 2016). A study conducted by Costa and colleagues also demonstrated that the combination of plant extracts with antifungal agents such as fluconazole and nystatin exhibited synergistic effects against *C. albicans* with FICI of less than 0.5 (Costa, Endo *et al.* 2015). A study focusing on the evaluation of the combination of berberine (an alkaloid used in traditional Chinese medicine) and fluconazole against fluconazole-resistant *C. albicans* also showed a synergistic effect (Li, Xu *et al.* 2013). In the present study, the interaction of *G. africana* and fluconazole against *C. glabrata* (ATCC 26512) was indifference as seen by the FICI value of 1.002. This result was similar to a study conducted to investigate the interaction between ferulic acid and caspofungin against *Candida albicans* and *Candida glabrata*

that showed that there was a synergistic effect against *C. albicans* (FICI of < 0.5). However, the interaction was not synergistic against *C. glabrata* (FICI of > 0.5) (Canturk 2017). The Sensititre susceptibility test showed that the combination of *G. africana* and the antifungal agents was more effective against the *Candida* strains than the antifungal agents on their own. This could be seen in the reduction of the MIC of most of the antifungal agents for both *C. albicans* (ATCC 90028) and *C. glabrata* (ATCC 26512). This was an important result because *C. glabrata* (ATCC 26512) was showing less susceptibility to the antifungal agents compared to *C. albicans* (ATCC 90028).

The microscopic analysis revealed that *G. africana* had an effect on the yeast cells. This could be seen by the distorted structure of the cells and the cell remnants that were present after treatment. These results were seen despite *G. africana* not showing much antifungal activity against the *Candida* strains in the disk diffusion assay. The results observed after microscopic analysis of yeast cells following treatment with *G. africana* are similar to those obtained after treatment of *C. albicans* with *Euphorbia hirta* L. leaf extract. Transmission electron micrographs taken after treatment of *C. albicans* with the *E. hirta* leaf extract revealed significant alterations to the microstructure of the *C. albicans* cells. These alterations included changes in the morphology of the cells, lysis and total disintegration of the cells after 36 h of exposure to the extract. The control cells, on the other hand, demonstrated the usual *Candida* morphology with a regular undamaged cell wall (Basma, Zuraini *et al.* 2011).

5.6 Conclusion

The results obtained in this study indicate that *G. africana* is a potential source of new antifungal agents. The results revealed that *G. africana* enhanced the antifungal activities of fluconazole

and other antifungal agents in both the checkerboard assay and the Sensitire susceptibility test as seen by the reduction of the MICs. The ethanolic *G. africana* extract also demonstrated fungicidal effects against the *Candida* strains. The fungicidal effects as well as the synergistic effects exhibited by the *G. africana* extract support the growing need for the use of medicinal plant extracts as therapeutic agents in the fight against multi-drug resistant pathogens. However, additional research such as exploring the effect of *G. africana* on clinical *Candida* isolates as well other fungal species of medical importance would be essential in future studies.

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6 CHAPTER 6: Effect of *Galenia africana* alone and in combination with doxorubicin on human breast cancer (MCF-7) and normal human fibroblast (KMST-6) cells lines

6.1 Abstract

Background: Breast cancer is considered to be the second leading cause of cancer-related deaths among women worldwide. Despite the availability of several chemotherapeutic drugs, they are normally associated with severe adverse effects and toxicity due to them not being able to differentiate cancer cells from normal cells. In addition, the increased resistance to anticancer drugs is a growing challenge. Medicinal plant extracts have shown potential as alternative sources for the treatment of breast cancer. *Galenia africana* is a woody shrub reported to have various therapeutic effects including antibacterial and antifungal properties. However, current research about the mechanisms of its anticancer potential is limited.

Objective/Purpose: This study was aimed at investigating the cytotoxicity of *G. africana* alone and in combination with doxorubicin in human breast cancer cell lines (MCF-7) and normal fibroblast (KMST-6) cells. This study also investigated the ability of *G. afrciana* to alter gene expression, induce apoptosis and impact the cell cycle process of MCF-7 cells.

Methods: The cytotoxic effects of the *G. africana* extract alone and in combination with doxorubicin were evaluated using the MTT **(**3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. The ability of *G. africana* to have an effect on gene expression, induce apoptosis and affect the cell cycle was assessed using the RT^2 Profiler PCR Array.

Results: Results from the MTT assay revealed that *G. africana* extract inhibited the proliferation of MCF-7 and KMST-6 in a dose-dependent manner. The extract demonstrated a greater anticancer effect against the MCF-7 than the KMST-6 cells as determined by the IC_{50} values. The underlying mechanism of growth inhibition in MCF-7 cells involved the up-regulation of genes involved in apoptosis such as *BCL2L11*, *CASP2*, *CASP7/9* and cellular senescence such as *IGFBP* 3, 5 and 7. Furthermore, *G. africana* caused the inhibition of angiogenesis as indicated by a down-regulation of *ANGPT1* and *ANGPT2*. In addition, *G. africana* affected the cell cycle as indicated by the down-regulation of *STMN1* and *WEE1* genes as well as metabolism as indicated by the down-regulation of *ACLY, ACSL4* and *PFKL* genes.

Conclusion: Overall, this study demonstrates the potential applications of *G. africana* as an anticancer drug for breast cancer treatment. In addition, the synergistic effect exhibited when combined with doxorubicin suggests that it can complement current chemotherapy.

6.2 Introduction

Cancer has been a global healthcare problem for years and a large amount of money has been invested in investigating it (Prakash, Kumar *et al.* 2013). Breast cancer is the major type of cancer diagnosed in women and accounts for the highest number of deaths in women worldwide (Abdulrahman and Rahman 2012; Seymour, Wainstein *et al.* 2016). Statistical data obtained from the South African National Cancer Registry 2010 indicates that 1 in 35 women is at risk of having breast cancer at some point in their lives (Parag and Buccimazza 2016). Reports have shown that as much as 10% of breast cancer cases are due to germline mutations in genes that are prone to cancer such as *BRCA1* and *BRCA2* (Diamond, Sutphen *et al.* 1998; Seymour, Wainstein *et al.* 2016). In addition, older women are at a higher risk of developing breast cancer as the risk increases with age (Bernardi, Errante *et al.* 2008). Despite the availability of screening plans and

the introduction of more chemotherapeutic agents, particularly in developed countries, cancer resistance has still been an important healthcare crisis (Ahmad 2013). It has been observed that in 30% of women presenting with early-stage breast cancer, the disease is persistent. Furthermore, drug resistance develops in no less than one quarter of all cases (Gonzalez-Angulo, Morales-Vasquez *et al.* 2007; Musgrove and Sutherland 2009).

Plants and plant derived compounds have been used, for centuries, for their healing properties and incorporated as therapeutic agents. Approximately 80-85% of the population worldwide depends on herbal medicines, in the form of plant extracts or their active compounds, to treat numerous ailments (Tomlinson and Akerele 1998; Elujoba, Odeleye *et al.* 2005; Ignacimuthu, Ayyanar *et al.* 2006). Medicinal plants have been used in the treatment of various infections including cancers (Kuete, Wabo *et al.* 2011). Some of the chemotherapeutic drugs that are derived from medicinal plants include vincristine, irinotecan, etoposide and paclitaxel (Da Rocha, Lopes *et al.* 2001). The majority of chemotherapeutic agents are associated with numerous extremely uncomfortable side effects. This is the reason why medicinal plants and plant derived products are being used in an effort to fight cancer (Prakash, Kumar *et al.* 2013).

In this study, the anticancer activity of *G. africana* was assessed using the MTT assay. The gene expression was assessed in MCF cells after treatment using the RT PCR Array profiler.

6.3 Materials and Methods

6.3.1 Plant material extraction and preparation

A 20% (w/v) *G. africana* extract was commercially prepared with 60% ethanol using the dried leaves and shoots. The drying process was conducted by Brenn-O-Kem (Pty) Ltd (Wolseley, South Africa) and involved the use of a commercial vacuum drying chamber to dry the ethanolic

extract under reduced pressure at a temperature of 50 ºC. This was done in order to dehydrate the extract and get rid of any excess absorbed solvent and water and to attain a crystal-form of the extract that would be used in subsequent experiments. In order to be used in the MTT assay, the crystals were reconstituted in Dulbecco's Modified Eagle Medium (DMEM) (Life technologies, UK) to make a stock solution of 60 mg/ml. This was done by weighing out 800 mg of the *G. africana* crystals and reconstituting them in 13.3 ml of DMEM to have a concentration of 60mg/ml. Once the crystals were weighed out, they were placed in a 15 ml greiner tube containing the media (13.3 ml) and sterile forceps were used to crush them. This process was performed in a laminar flow. The tube with the reconstituted extract was then centrifuged at maximum speed for 7 min so as to obtain a clear liquid. This clear extract liquid was then sterile filtered using 0.45 µm sterile filters. This liquid extract was used as a stock solution and was stored at 4ºC. A sample of the reconstituted dried extract was sent to Cape Peninsula University of Technology (CPUT) (Bellville, South Africa) for analysis in order to determine the chemical composition. Once analysis was done, a certificate of analysis was supplied (see Appendix V).

6.3.2 Cell culture conditions

The human breast cancer cell line (MCF-7) (ATCC HTB-22) and the immortalized human normal fibroblast cells (KMST-6) were obtained from Prof Meyer (Biotechnology Department, University of the Western Cape, Bellville, South Africa). The MCF-7 and KMST-6 cells were grown in DMEM (Life technologies, UK) supplemented with 10% v/v fetal bovine serum (FBS) (Gibco, UK), 100 U/ml of penicillin (Gibco, Life Technologies), and 100 μg/ml of streptomycin (penstrep) (Gibco, UK) as a complete growth medium. Cells were maintained in complete growth media in 75 cm^2 flasks and incubated in a humidified incubator at 37°C with 5% CO_2. Doxorubicin was supplied by Sigma-Aldrich, USA.

6.3.3 Cytotoxic assay (MTT assay)

In vitro cytotoxicity of *G. africana* alone, doxorubicin alone as well as a combination of the two was carried out using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT assay) (Sigma-Aldrich) based on methods described in previous studies (Ayob, Mohd Bohari *et al.* 2014). This assay was performed after 12, 24, 36 and 48 h time periods. Prior to conducting the MTT assay, the laminar flow was cleaned with 70% ethanol and all materials to be used in this assay were sterilized, by using UV lighting, within the laminar flow. In addition, the microscope and all other working surfaces were sterilized prior to conducting the assay. In this assay, cells were harvested once they reached a confluency of 70-80% and this was confirmed by observing them under the microscope. Upon being confluent, excess media was removed from the flask and the cells were washed 3 times with 5 ml of phosphate buffered saline (PBS) (Life Technologies, USA). After washing the cells, any excess PBS was removed and 1-3 ml of trypsin (Lonza, Belgium) was added to the flask. Care was taken to ensure that the trypsin covered the entire surface of the flask. The flask was then incubated for 2-3 min at 37ºC in a humidified incubator with 5% CO_2. The flask was removed from the incubator and viewed under the microscope to check if the cells had detached from the surface of the flask. Once cells were detached, 5 ml of complete media was added in order to neutralize the trypsin. The cell suspension was transferred to a 15 ml greiner tube and centrifuged at 500 rpm for 5 min. The media was removed and the cells were re-suspended in 1 ml of complete media. Cells were then counted by removing 20 µl of cells and adding this to 20 µl of trypan blue in a 1.5 ml micro centrifuge tube and gently pipetted to mix. Cells were aseptically counted using a counting chamber, referred to as a hemocytometer, and the number of cells per ml was recorded. The cells were diluted using

complete media. 100 µl of cells, at a density of 2.5 x 10^3 cells, were added into each well of the 96-well flat bottom plate and incubated for 24 h at 37ºC in a humidified incubator with 5% CO_2.

The following day, the cells were treated with the *G. africana* extract at concentrations ranging from 1.25 to 20 mg/ml (made up in DMEN) and doxorubicin at concentrations of 0.1, 0.5, 2.5 and 5 µM (reconstituted in DMSO (Sigma-Aldrich, USA) but working solutions made up in DMEM). This was done by adding 100 µl of either the extract or doxorubicin to the cells and incubating for 12, 24, 36 and 48 h (for treatment with the extract) and 12 and 24 h (for treatment with doxorubicin). On the third day, a stock solution of 5 mg/ml MTT was prepared in PBS and filter sterilized using 0.22 µm sterile filters. The tube containing the MTT was covered in foil as it is light sensitive. 20 µl of 5 mg/ml MTT (final concentration of 0.5 mg/ml in incubation media) was then added to each well and the plates were incubated, covered in foil, for 4 h at 37ºC in a humidified incubator with 5% CO_2. The above mentioned processes were performed aseptically in the laminar flow. Control wells were also included that contained staurosporine (Sigama-Aldrich, USA) (positive apoptosis control), cells only (negative control), as well as pinocembrin (Sigma-Aldrich, USA) and naringenin (Sigma-Aldrich, USA) (components found in the extract). After the incubation period, the media was carefully removed without disturbing the cells and the MTT crystals in the wells. 100 µl of isopropanol was added to each well to dissolve the MTT formazan crystals and incubated for 20-30 min in a shaking incubator. The plates were read at 570 nm using a microplate reader (Glomax Multi Detection System, Promega, Madison, USA) and the absorbance readings recorded.

6.3.3.1 *Statistical analysis*

The cell viabilities of the treated MCF-7 and KMST-6 cell lines were calculated as follows:

$$\text{Cell viability (\%)} = \frac{\text{Mean OD}}{\text{Control OD}} \text{ x } 100\%$$

The cell viability results were then used to determine the IC_{50} values of the extract and doxorubicin as well as their combination against MCF-7 and the KMST-6 cells. The IC_{50} values were determined using nonlinear regression (log (inhibitor) vs. response-variance slope) in GraphPad Prism 5. The IC_{50} values represented the concentrations of the extract required to decrease the absorbance readings of treated cells by 50% compared to the untreated cells (cells only control) (Ahmad, Ali *et al.* 2005). Grouped data was analyzed using two-way analysis of variance (ANOVA). The analysis included row means with SD, multiple *t*-tests (one-per row) and multiple comparisons. A p value ≤ 0.05 was considered to be statistically significant. The IC_{50} values obtained after the treatment of cells with *G. africana* alone, doxorubicin alone as well as the combination of the two were used for subsequent experiments.

6.3.4 Purification of Total RNA from animal cells using the Spin Technology

Purification of total RNA was done using the RNeasy Mini kit (Qiagen, Germany). Use of the correct quantity of starting material was essential in obtaining the optimal yield and purity of RNA. According to the protocol, the minimum amount of starting material is usually 100 cells while the maximum starting material will depend on a number of factors. These factors include RNA content of the cell type, the RNA binding capacity of the RNeasy spin column (100 µg RNA) and the volume of Buffer RLT required for efficient lysis (the maximum volume of Buffer RLT that can be used limits the maximum amount of starting material to 1 x 10^7 cells). In addition, RNA content can vary significantly between cell types. Table 2 (page 17 of the RNeasy Mini Handbook) outlined the typical yields of total RNA that would be obtained with the RNeasy Mini spin column kit. Since this study focused on MCF-7 cell lines, which were not

included in Table 2, the protocol recommended starting with no more than 3-4 x 10^6 cells. Prior to extraction and purification of RNA, cells were grown in 6 well plates, with a diameter of 9-9.5 cm^2, at a density of 0.3 x 10^6 per well for the four groups. The number of cells at confluency was expected to be 1-1.2 x 10^6 cells per well.

The cells were harvested and the required number of cells determined. The medium was aspirated and the cells were washed with PBS. The PBS was then aspirated and 2 ml of 0.25% trypsin was added. Once the cells detached from the wells, medium containing serum was added to inactivate the trypsin. This was then transferred to a polypropylene centrifuge tube and centrifuged at 300 x g for 5 min. After centrifuging, the supernatant was completely aspirated. The appropriate volume of Buffer RLT to be added to the pelleted cells was outlined in Table 5 of the RNeasy Mini Handbook. The pellet was loosened thoroughly by flicking the tube. Since the staring cell density was < 5 x 10^6, 350 µl of Buffer RLT was added. This was vortexed in order to facilitate efficient lysis and increase RNA yields. The lysate was homogenized by passing it at least 5 times through a blunt 20-gauge needle (0.9 mm diameter) fitted to an RNase-free syringe. 350 µl of 70% ethanol (Merck, Germany) was added to the homogenized lysate and mixed well by pipetting (some precipitation was visible after addition of ethanol but this did not affect the procedure). 700 µl of the sample, including the precipitate was transferred to an RNeasy spin column placed in a 2 ml collection tube. The lid was closed gently and this was then centrifuged for 15 s at ≥ 8000 x g (≥ 10 000 rpm). The flow-through was discarded. Since the volume exceeded 700 µl, successive aliquots were centrifuged in the same RNeasy spin column and the flow-through discarded after each centrifugation step.

An optional on-column DNase digestion genomic DNA elimination step was performed using the High Pure RNA isolation kit (Roche, USA). 90 µl of DNase Incubation Buffer was pipetted

into a sterile reaction tube and 10 µl of DNase I added. This was mixed thoroughly and the solution (entire volume) pipetted in the upper reservoir of the RNeasy spin column and incubated for 60 min at 15-25°C. 500 µl Buffer RPE was then added to the RNeasy spin column. The lid was closed gently and the spin column centrifuged for 15 s at ≥ 8000 x g (≥ 10 000 rpm) to wash the spin column membrane. The flow-through was discarded and the collection tube reused. Another 500 µl of Buffer RPE was added to the spin column. The lid was closed gently and centrifuged for 2 min at ≥ 8000 x g (≥ 10 000 rpm) to wash the membrane. This longer centrifugation step dried the spin column membrane ensuring that no ethanol was carried over during RNA elution which would interfere with downstream reactions. After centrifugation, the spin column was carefully removed from the collection tube to avoid contact with the flow-through otherwise carryover of ethanol would occur. The spin column was then placed in a new collection tube, the lid closed gently and centrifuged at full speed for 1 min. This step eliminated any possible carryover of Buffer RPE or residual flow-through remaining on the outside of the spin column. The spin column was then placed in a new 1.5 ml collection tube. 30 µl of RNase-free water was added directly to the spin column membrane. The lid was carefully closed and centrifuged for 1 min at ≥ 8000 x g (≥ 10 000 rpm) to elute the RNA. This was then used to synthesize cDNA.

6.3.5 Determination of RNA quantity and quality

RNA samples were sent to Stellenbosch University (Stellenbosch, South Africa) to be analyzed using the Aligent 2100 Bioanalyzer system per the manufacturer's instructions (Aligent Technologies, USA). This instrument utilized the RNA integrity number (RIN) to measure the integrity of the RNA while also giving the RNA concentration. The RIN value was calculated based on the association between the areas of 18S rRNA and 28S rRNA (Schroeder, Mueller *et*

al. 2006). Furthermore, if a ratio of 28S:18S bands is 2 or higher, then the RNA is considered to be of high quality (Schroeder, Mueller *et al.* 2006). RIN values are classified using a numbering system from 1 to 10. 1 denotes the most degraded RNA while 10 denotes the most intact RNA (Ondracek, Cheng *et al.* 2015). In addition, RIN values above 8 demonstrate that the RNA obtained after the extraction method employed was not degraded (Ruocco, Costantini *et al.* 2017).

6.3.6 cDNA synthesis using the RT2 First Strand Kit and quantification

The RT^2 First Strand kit (Qiagen, USA) was essential for acquiring optimal results and for detecting the reverse transcription controls contained in the RT^2 Profiler PCR Array. The protocol recommended using a starting total RNA concentration of 0.5 µg for the 96-well plate formats for first-time users. Since this was the first time this kit was used, 0.5 µg total RNA was the starting concentration for cDNA synthesis.

The reagents of the RT^2 First Strand kit were thawed and briefly centrifuged to bring the contents to the bottom of the tubes. The genomic DNA elimination mix was prepared (Table 6.1) as outlined in the RT^2 Profiler PCR Array Handbook. The elimination mix was mixed gently by pipetting up and down and subsequently centrifuged briefly.

Table 6.1: Genomic DNA elimination mix.

Component	Amount
RNA	0.5 µg
Buffer GE	2 µl
RNase-free water	variable
Total volume	10 µl

The genomic DNA elimination mix was incubated at 42°C for 5 min and then immediately placed on ice for at least 1 min. This was followed by the preparation of the reverse-transcription mix (Table 6.2).

Table 6.2: Reverse-transcription mix.

Component	Volume for 1 reaction (µl)	Volume for 2 reactions (µl)	Volume for 4 reactions (µl)
5x Buffer BC3	4	8	16
Control P2	1	2	4
RE3 Reverse Transcriptase Mix	2	4	8
RNase-free water	3	6	12
Total volume	10	20	40

Since cDNA from the four groups was required, the volume for 4 reactions was used (total volume of 40 µl). 10 µl of reverse-transcription mix was added to each tube containing 10 µl genomic DNA elimination mix and mixed gently by pipetting up and down several times. This was incubated for exactly 15 min at 42°C. After incubation, the reaction was immediately stopped by incubating it at 95°C for 5 min. 91 µl of RNase-free water was then added to each reaction and mixed by pipetting up and down several times. The reactions were placed on ice for use in the real-time PCR step. cDNA was quantified using the NanoDrop 2000 spectrophotometer.

6.3.7 Real-Time PCR for RT2 Profiler PCR Array Formats A, C, D, E, F, G

This array relied on the use of RT^2 SYBR Green Mastermixes (Qiagen, Germany) to obtain accurate results from the RT^2 Profiler PCR Array (Qiagen, Germany). It was crucial to ensure that the RT^2 SYBR Green Mastermix and the RT^2 Profiler PCR Array were suitable for the real-time cycler being used. The format of the RT^2 Profiler PCR Array was indicated by the last letter

of the catalog number. A summary of genes present in the RT² Profiler PCR Array are summarized in Appendix VI. To prepare the PCR components mix, the RT^2 SYBR Green Mastermix was briefly centrifuged for 10-15 s to bring the contents to the bottom of the tube. A total volume of 2700 µl of the PCR components mix was prepared (Table 6.3) for each experimental group in a 5 ml tube as described in the RT^2 Profiler PCR Array Handbook.

Table 6.3: PCR components mix.

Component	**Array format : 96-well A, C, D, F**
2x RT^2 SYBR Green Mastermix	1350 µl
cDNA synthesis reaction	102 µl
RNase-free water	1248 µl
Total volume	2700 µl

The RT^2 Profiler PCR Array was carefully removed from the sealed bag. This was followed by the addition of 25 µl of PCR components mix to each well of the PCR Array plate. Tips were changed following each pipetting step to avoid cross-contamination between wells. PCR Array plates were then carefully and tightly sealed with the optical adhesive film supplied. The PCR Array plates were centrifuged for 1 min at 1000 x g at room temperature (15-25°C) to remove bubbles as they would interfere with results. The PCR Array plates were then placed on ice while the PCR cycling program was being set up. Figure 6.1 shows the PCR Array plate layout. The real-time cycler was programmed according to the conditions outlined in the RT^2 Profiler PCR Array Handbook. The recommended cycling conditions for the Roche LightCycler 480 are outlined in Table 6.4. The ramp rate was adjusted to 1.5°C/s. A dissociation (melting) curve analysis was performed to verify the PCR specificity using the real-time cycler software.

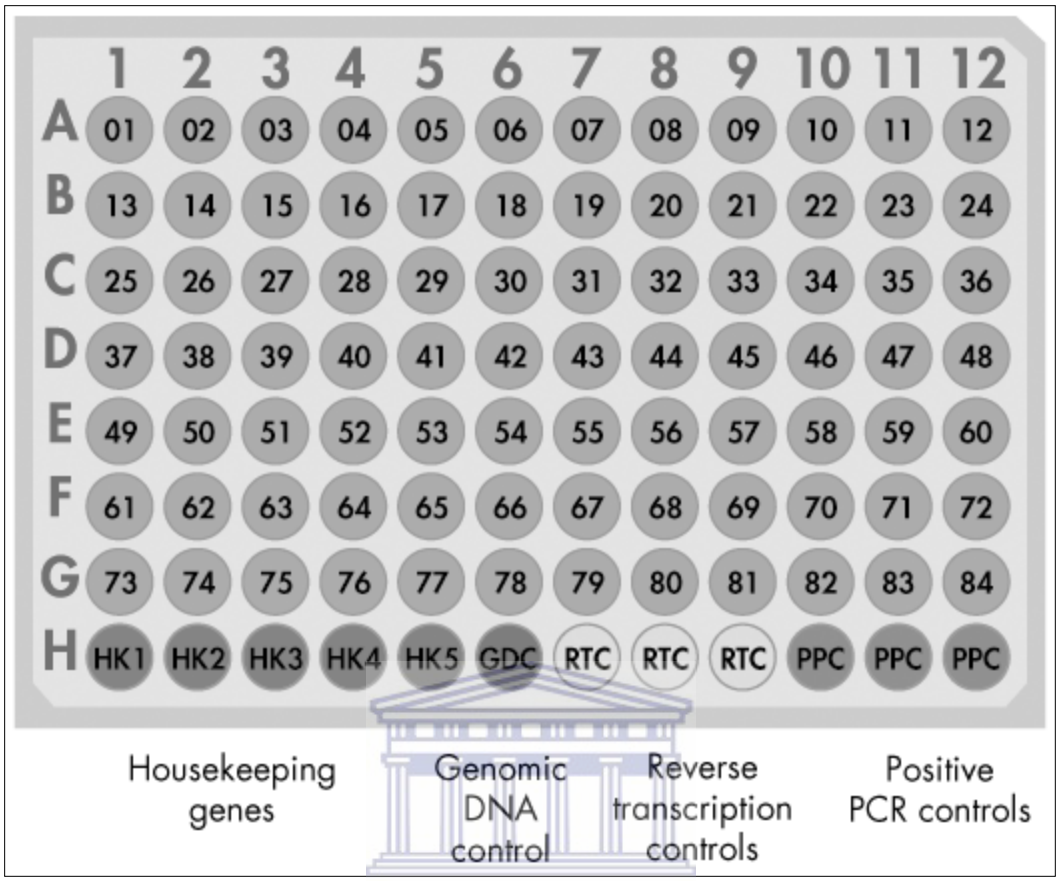

Figure 6.1: RT^2 Profiler PCR Array layout. Wells A1 to G12 each contain a real-time PCR assay for a pathway/disease/functionally related gene. Wells H1 to H5 contain a housekeeping gene panel to normalize array data (HK1–5). Well H6 contains a genomic DNA control (GDC). Wells H7 to H9 contain replicate reverse-transcription controls (RTC). Wells H10 to H12 contain replicate positive PCR controls (PPC).

Table 6.4: Cycling conditions for Roche LightCycler 480.

Cycles	Duration	Temperature	Comments
1	10 min	95°C	HotStart DNA Taq Polymerase is activated by this heating step.
45	15s	95°C	
	1 min	60°C	Perform fluorescence data collection.

After programming the cycling conditions, the PCR Array plate was placed into the real-time cycler. The threshold cycle (C_T) for each well was calculated using the real-time cycler software. The second derivate max setting was used to calculate the C_T. Once the C_T values for all wells were obtained, they were exported to a blank Excel spreadsheet for use in the SABiosciences PCR Array Data Analysis Web-based software.

6.3.7.1 *RT^2 Profiler PCR Array Data Analysis*

Data analysis was performed according to the RT^2 Profiler PCR Array Data Analysis v3.5 Handbook. The C_T values were calculated for each well using the second derivate maximum setting (recommended analysis method for the LightCycler 480 software). The second derivate maximum setting is recommended because the algorithm is based on the kinetics of the PCR. The C_T value in this method is identified as the point where the reaction's fluorescence reaches the maximum of the second derivative of the amplification curve, which corresponds to the point where the acceleration of the fluorescence signal is at its maximum. As a result, this crossing point should always be located in the middle of the log-linear portion of the PCR amplification plot. The advantage of this analysis method is that it requires little user input. The C_T values were then exported from the experimental run into an excel spreadsheet. These C_T values were analyzed to calculate changes in gene expression. After being exported from the qPCR instrument, the C_T values were formatted into a new Excel spreadsheet. This was done to enable the PCR Array Data Analysis Webportal to correctly read and import data as the Webportal only accepts Excel spreadsheets. All plates and wells were copied into a single worksheet. The raw C_T values for each sample were in a single column that lined up correctly with the correct well location on the PCR Array. Once the spreadsheet was correctly filled with the required information, the data was uploaded onto the Data analysis Webportal. An automated data

analysis system was employed using the Qiagen online software. A data QC step was performed to ensure that each sample passed the PCR Array reproducibility, RT (reverse transcription) efficiency, and Genomic DNA contamination quality controls. Data normalization was performed using the housekeeping genes before analysis was carried out. The data was reviewed to see each group's distribution of threshold cycle values and the average raw data in each group. Analysis was performed and the fold regulation, fold change, average ΔC_T and the $2^{\wedge}- \Delta C_T$ reviewed from the calculations processed by the software from the imputed data. Plots and charts were formulated using the defined groups and the specified fold-change boundary (threshold) for the genes of interest. The figures/images were then saved and gene expression was analyzed by screening genes to verify the target differentially expressed genes compared to the control.

6.4 Results

6.4.1 Cytotoxic assay (MTT assay)

The results of the MTT assay revealed that *G. africana* had a more cytotoxic effect on the MCF-7 cells compared to the KMST-6 cells. This was evident from the high IC_{50} values obtained after treatment of the KMST-6 cells with the extract at the four different time lines. The IC_{50} values obtained after a 12 h treatment of KMST-6 and MCF-7 cells with different *G. africana* concentrations were 4.161 and 1.635 mg/ml, respectively whereas those obtained after a 24 h treatment period were 3.625 and 1.692 for KMST-6 and MCF-7 cells, respectively. Treatment of both KMST-6 and MCF-cells with the extract for 36 h produced IC_{50} values of 2.367 and 1.484 mg/ml, respectively. The IC_{50} values obtained after treating the KMST-6 and MCF-7 cells for 48 h with the extract were 1.983 and 1.25 mg/ml, respectively (Figure 6.2).

Figure 6.3 represents the results obtained after the treatment of the two cell lines with four doxorubicin concentrations for 12 and 24 h. The IC_{50} values could not be determined after 12 h of treatment with doxorubicin as the cell viabilities were above 50% at all the concentrations for both cell lines. However, the IC_{50} value obtained after 24 h of treatment of MCF-7cells was 0.3 µM whereas that obtained after treating the KMST-6 cells was 18.93 µM.

Figure 6.4 represents the effect of the combination of *G. africana* with doxorubicin after a 12 h treatment period. Treatment of KMST- 6 and MCF-7 cells with a combination of *G. africana* and 0.1 µM doxorubicin produced IC_{50} values of 6.989 and 1.842 mg/ml, respectively. Treatment with *G. africana* and 0.5 µM doxorubicin produced IC_{50} values of 6.257 and 2.311 mg/ml for KMST-6 and MCF-7 cells, respectively. Treatment with *G. africana* and 2.5 µM doxorubicin produced IC_{50} values of 5.025 and 1.969 mg/ml for KMST-6 and MCF-7 cells, respectively.

Figure 6.5 represents the effect of the combination of *G. africana* with doxorubicin after a 24 h treatment period. Results obtained after a combination of *G. africana* and 0.1 µM doxorubicin produced IC_{50} values of 8.814 and 1.144 mg/ml for KMST-6 and MCF-7 cells, respectively. Treatment with *G. africana* and 0.5 µM doxorubicin produced values of 4.134 and 1.228 mg/ml for KMST-6 and MCF-7 cells, respectively. Treatment with *G. africana* and 2.5 µM doxorubicin produced IC_{50} values of 3.885 mg/ml and 0.116 mg/ml for KMST-6 and MCF-7 cells, respectively.

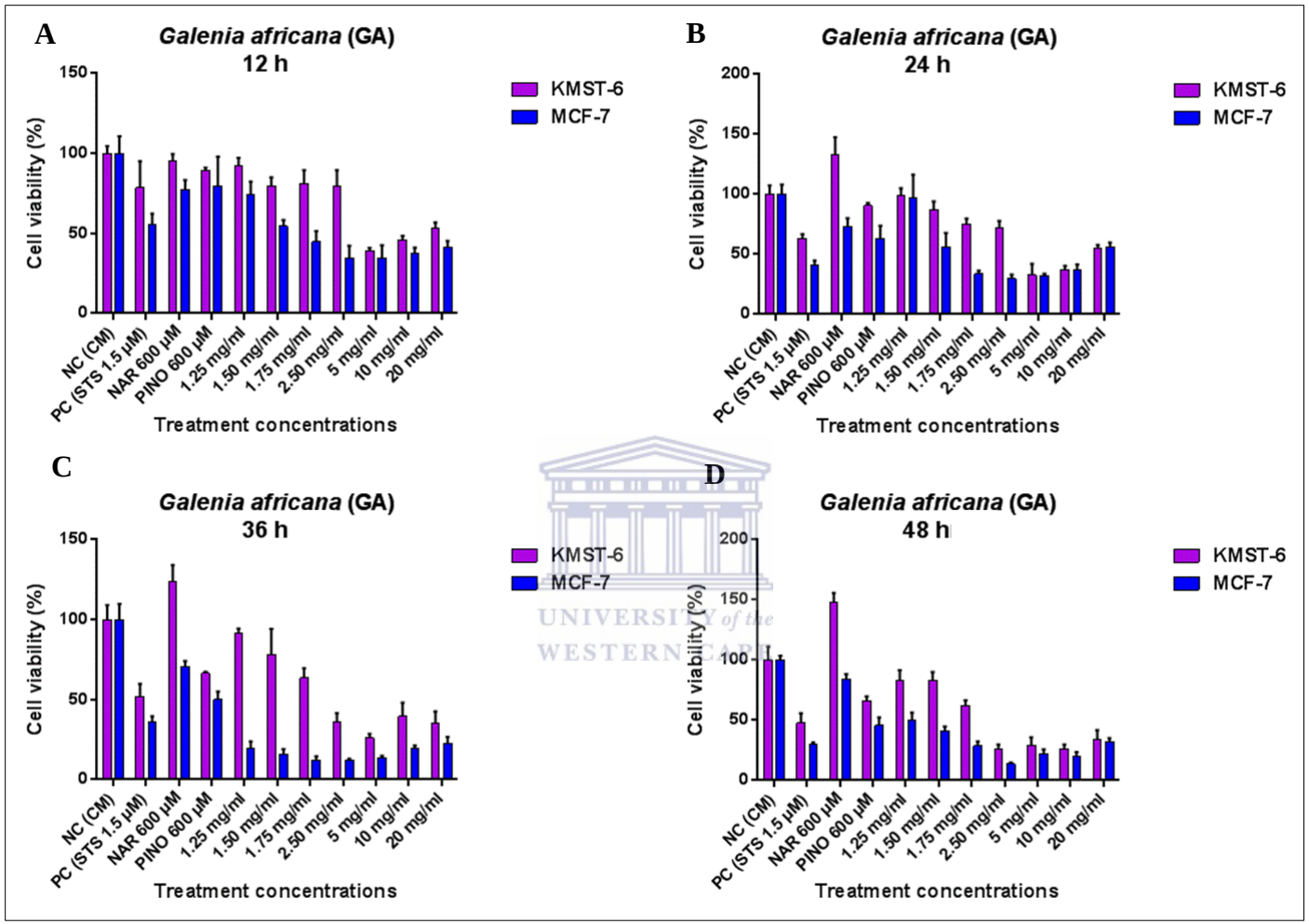

Figure 6.2: Cell viabilities of KMST-6 and MCF-7 cells treated with *G. africana* (1.25 to 20 mg/ml). (A) 12 h of treatment; (B) 24 h of treatment; (C) 36 h of treatment and (D) 48 h of treatment. Values are expressed as mean ± SD (n=5). $p < 0.001$ ($p < 0.05$).

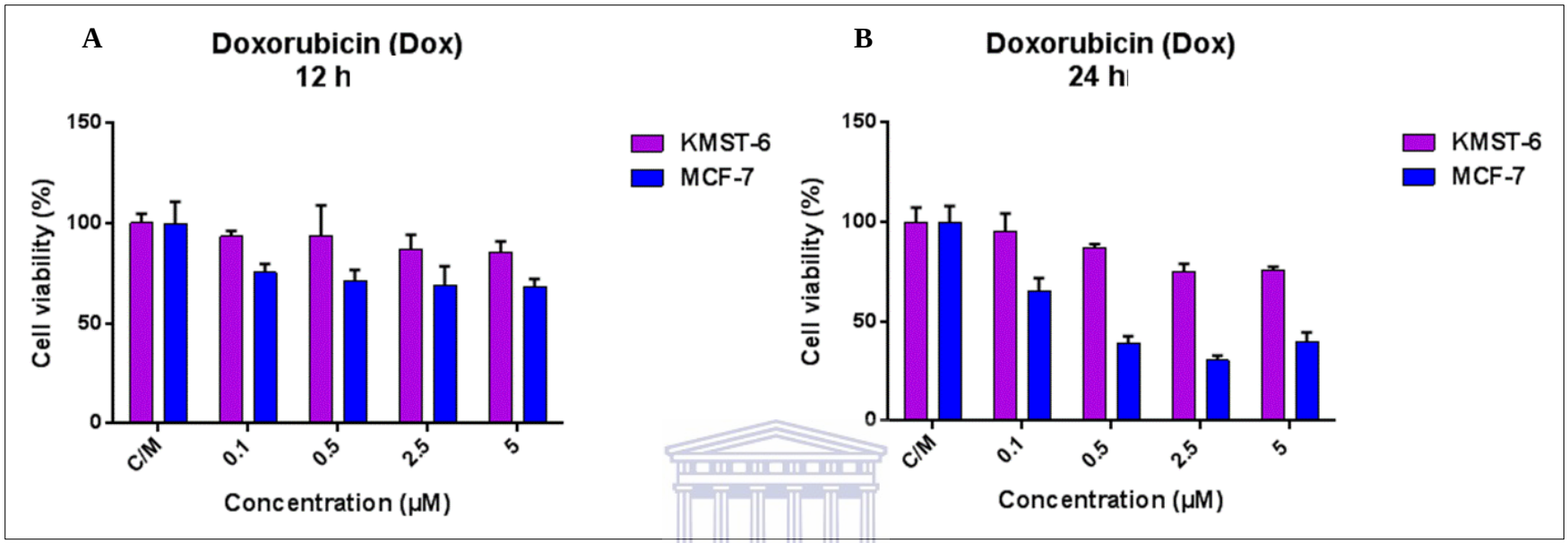

Figure 6.3: Cell viabilities of KMST-6 and MCF-7 cells treated with doxorubicin (0.1 to 5 µM). (A) 12 h of treatment and (B) 24 h of treatment. Values are expressed as mean ± SD (n=5). $p < 0.001$ ($p < 0.05$).

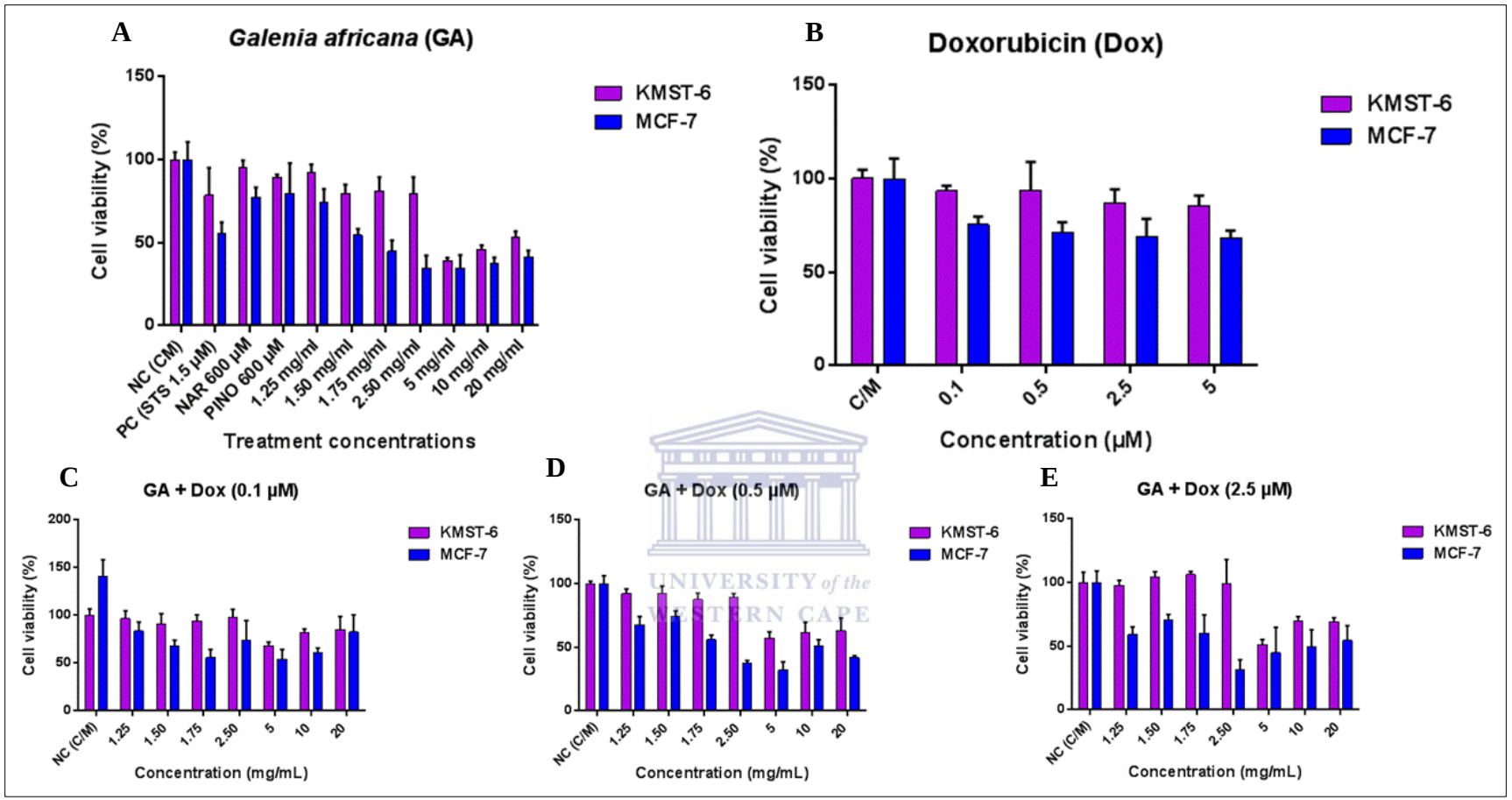

Figure 6.4: 12 h treatment of KMST- and MCF-7 cells with combinations of *G. africana* (1.25 to 20 mg/ml) and doxorubicin (0.1, 0.5 and 2.5 µM). (A) *G. africana* alone, (B) doxorubicin alone, (C) *G. africana* and doxorubicin (0.1 µM), (D) *G. africana* and doxorubicin (0.5 µM) and (E) *G. africana* and doxorubicin (2.5 µM). Values are expressed as mean ± SD (n=5). $p < 0.001$ ($p < 0.05$).

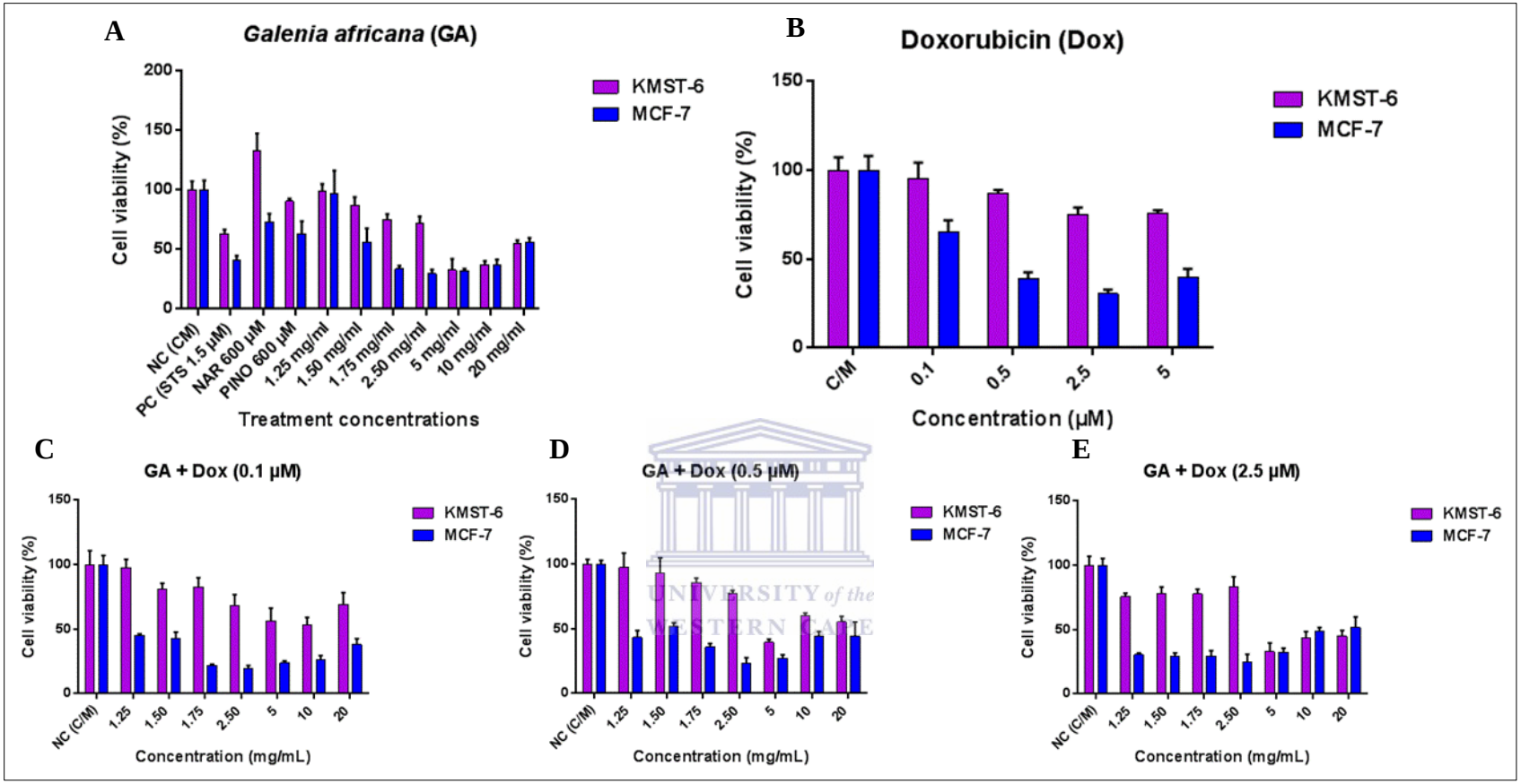

Figure 6.5: 24 h treatment of KMST- and MCF-7 cells with combinations of *G. africana* (1.25 to 20 mg/ml) and doxorubicin (0.1, 0.5 and 2.5 µM). (A) *G. africana* alone, (B) doxorubicin alone, (C) *G. africana* and doxorubicin (0.1 µM), (D) *G. africana* and doxorubicin (0.5 µM) and (E) *G. africana* and doxorubicin (2.5 µM). Values are expressed as mean ± SD (n=5). $p < 0.001$ ($p < 0.05$)**.**

Table 6.5: Comparison of IC_{50} values of MCF-7 and KMST-6 cells lines treated with *G. africana*, doxorubicin and a combination of the two after 12, 24, 36 and 48 h.

	IC_{50} values							
	KMST-6				**MCF-7**			
Treatment	**12 h**	**24 h**	**36 h**	**48 h**	**12 h**	**24 h**	**36 h**	**48 h**
G. africana alone	4.161	3.625	2.367	1.983	1.635	1.692	1.484	1.25
Doxorubicin alone	–	18.93	ND	ND	–	0.30	ND	ND
0.1 µM Dox + *G. africana*	6.989	8.814	ND	ND	1.842	1.144	ND	ND
0.5 µM Dox + *G. africana*	6.257	4.134	ND	ND	2.311	1.228	ND	ND
2.5 µM Dox + *G. africana*	5.025	3.885	ND	ND	1.969	0.116	ND	ND

ND- Not determined, as the effect of doxorubicin alone, as well as the combination of *G. africana* and doxorubicin against the cells was only evaluated after 12 and 24 h. The effect observed was significant enough for experiments not to go beyond these two time lines.

Table 6.5 shows the IC_{50} values obtained after treating the KMST-6 and MCF-7 cells with *G. africana* alone (12, 24, 36 and 48 h), doxorubicin alone (12 and 24 h) as well as a combination of the two (12 and 24 h). The IC_{50} values obtained in the combination experiments demonstrated the changes in the concentration of *G. africana* required to have an effect on cell viability. The IC_{50} values of *G. africana* alone and doxorubicin alone after a 24 h treatment period were 1.692 mg/ml and 0.3 µM, respectively. For the combination study, it was observed that the IC_{50} value of the extract reduced from 1.692 (when used alone) to 1.144 mg/ml when combined with 0.1 µM doxorubicin after 24 h of treatment. This result also revealed that there was an increase in the activity of both the extract and doxorubicin as the IC_{50} value of the extract reduced with a doxorubicin concentration of 0.1 µM, which was

lower than its IC_{50} of 0.3 µM. These IC_{50} concentrations were used for the RNA extraction process.

6.4.2 Determination of RNA quantity and quality

The RNA quality and concentration was determined using the Aligent 2100 Bioanalyzer. Results revealed that the RNA obtained from the untreated cells had two distinct bands whereas the RNA obtained from the three treatment groups showed smearing (Figure 6.6). Figure 6.7 shows results of the RNA obtained from MCF-7 cells treated with a combination of *G. africana* and doxorubicin (MCF CB) and *G. africana* alone (MCF GA) that had RIN values of 2.6 and 2.4, respectively. This showed that the RNA was degraded as a result of the treatment. Figure 6.8 shows results of the RNA obtained from MCF-7 cells treated with doxorubicin (MCF_Dox) and the untreated cells (MCF_UT) that produced RIN vales of 2.7 and 8.9, respectively. These results showed that the untreated cells had RNA that was not degraded as demonstrated by the RIN values. The RNA concentrations were 255 ng/µl for doxorubicin, 237 ng/µl for *G. africana*, 259 ng/µl for the combination of the two and 1110 ng/µl for the untreated cells.

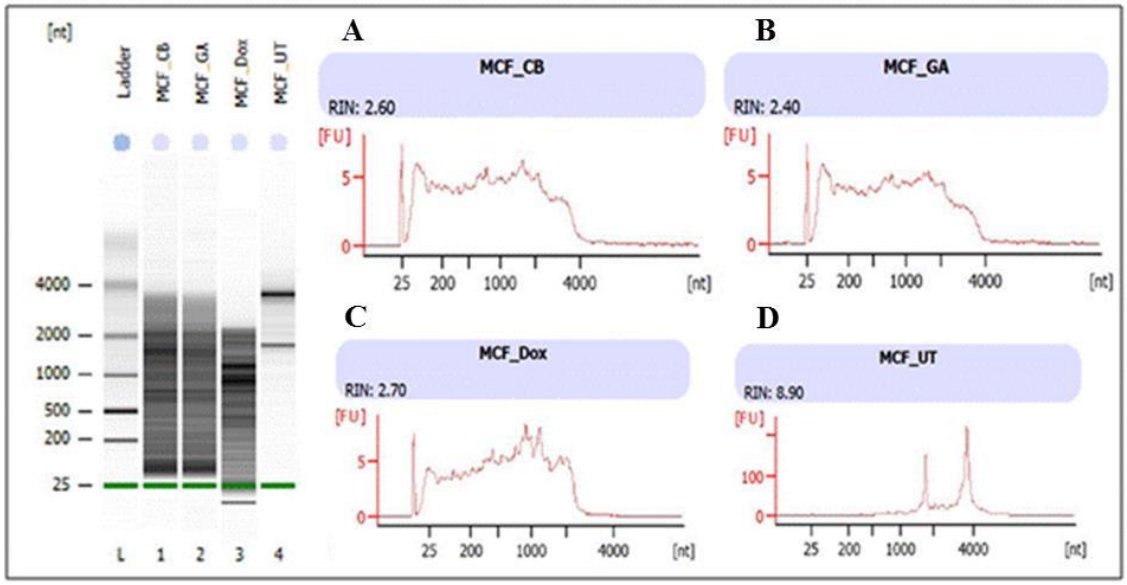

Figure 6.6: Agilent Bioanalyzer electropherograms and electrophoresis file run summaries of MCF-7 cells. (A) treatment with a combination of *G. africana* (1.144 mg/ml) and doxorubicin (0.1 µM) (MCF_CB), (B) treatment with *G. africana* only (1.692 mg/ml) (MCF_GA), (C) treatment with doxorubicin only (0.3 µM) (MCF Dox) and (D) untreated cells (MCF_UT). RIN values are also reported.

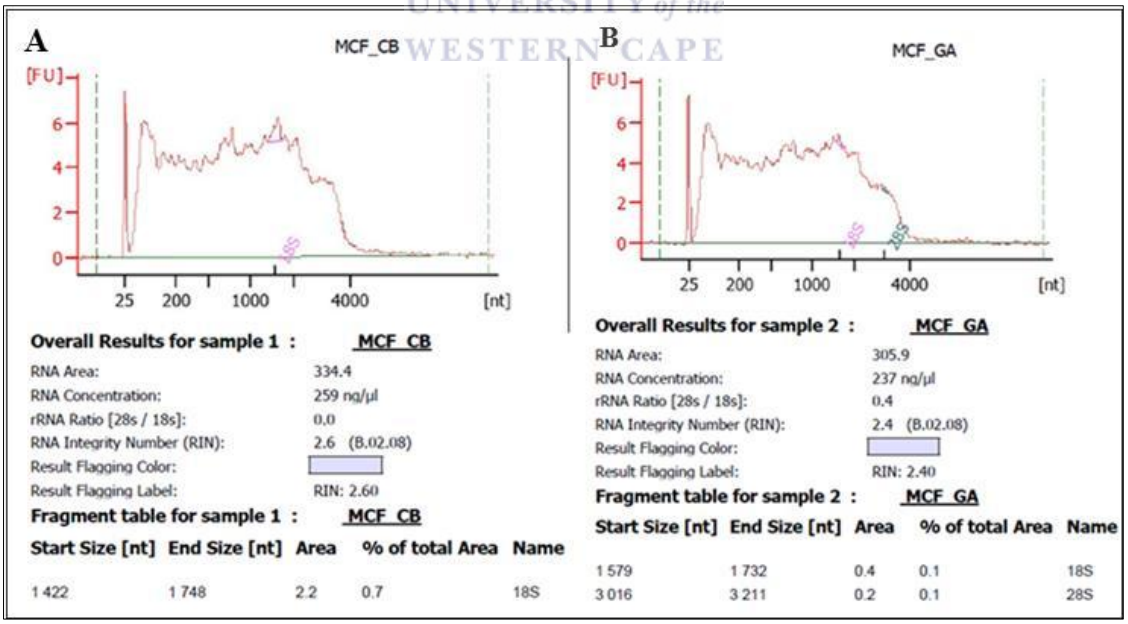

Figure 6.7: Agilent Bioanlyzer electropherograms of MCF-7 cells. **(A)** treatment with a combination of *G. africana* (1.144 mg/ml) and doxorubicin (0.1 µM) (MCF_CB) and **(B)** treatment with *G. africana* only (1.692 mg/ml). RIN values, RNA concentrations and rRNA (28s/18s) ratios are also reported.

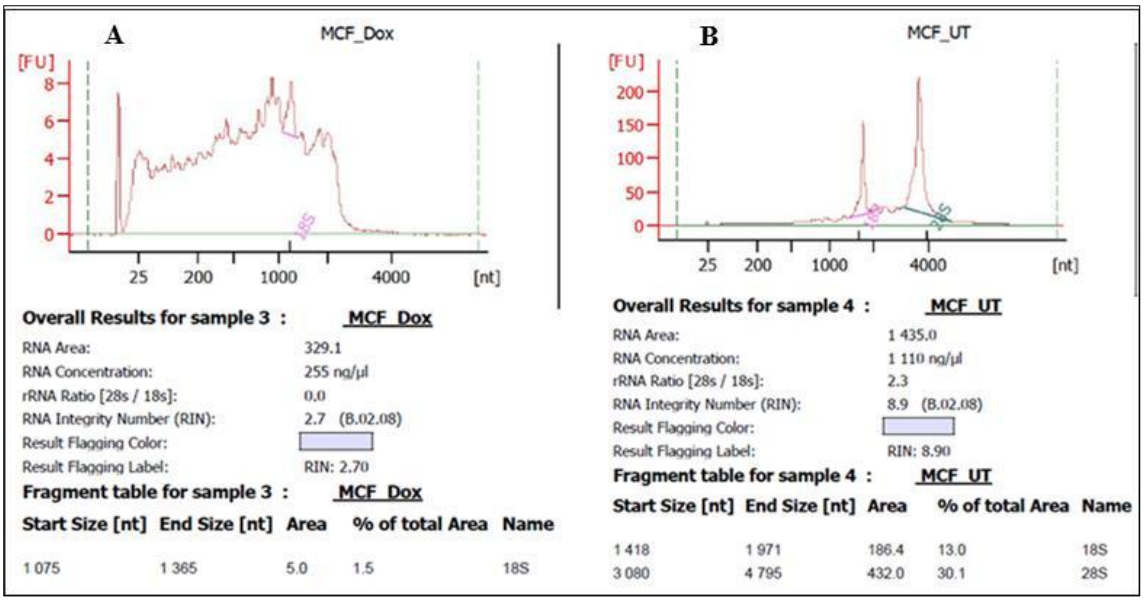

Figure 6.8: Agilent Bioanlyzer electropherograms of MCF-7 cells. (A) treatment with doxorubicin only (0.3 µM) (MCF Dox) and (B) untreated cells (MCF_UT). RIN values, RNA concentrations, 28s:18s ratios are also reported.

6.4.3 cDNA quatification (NanoDrop)

After obtaining the RNA, it was then used to synthesize cDNA. The concentrations of the cDNA are outlined in Table 6.6. Despite the RNA concentration for the untreated cells being higher than the treatment groups, the cDNA concentration was within the same range of 200 to 225 ng/µl. This was important so as to ensure that the same amount of cDNA was used for subsequent experiments.

Table 6.6: cDNA results obtained after quantification using the NanoDrop 2000 Spectrophotometer.

Sample	Concentration (ng/µl)
MCF CB[a]	212
MCF GA[b]	224.5
MCF Dox[c]	213.7
MCF UT[d]	212.8

[a] MCF-7 cells treated with a combination of *G. africana* and doxorubicin

[b] MCF-7 cell treated with *G. africana* only

[c] MCF-7 cell treated with doxorubicin only
[d] Untreated MCF-7 cells

6.4.4 RT PCR Array

Figure 6.9 and Table 6.7 outline the distribution of genes after treating the MCF-7 cells with *G. africana* alone (Group 1), doxorubicin alone (Group 2) and a combination of the two (Group 3) for 24 h. The Control Group included untreated MCF-7 cells.

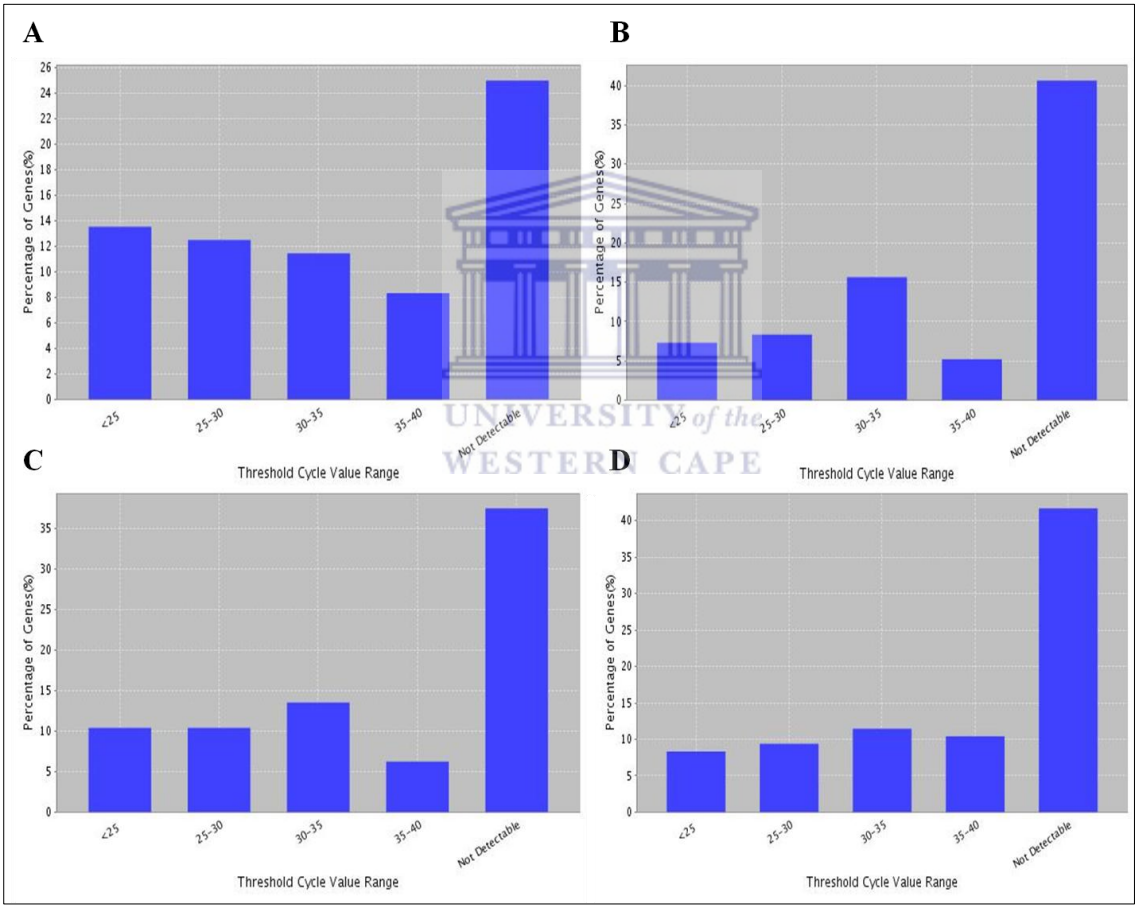

Figure 6.9: Percent distribution of genes based on threshold cycle value ranges in MCF-7 cells. Cells were treated with the IC_{50} values of *G. africana*, doxorubicin and a combination of the two after 24 h. (A) Control Group (untreated), (B) Group 1 (cells treated with *G. africana* only); (C) Group 2 (cells treated with doxorubicin only) and (D) Group 3 (cells treated with a combination of the two).

Table 6.7: Distribution of threshold cycle (C_t) value ranges in treatment Groups 1, 2 and 3 and the Control Group.

Distribution of C_t Values				
C_t Range	**Control Group**	**Group 1**	**Group 2**	**Group 3**
<25	13	7	10	8
25-30	12	8	10	9
30-35	11	15	13	11
Absent Calls	32	44	42	50
Percent Distribution of C_t Values				
<25	13.54%	7.29%	10.42%	8.33%
25-30	12.50%	8.33%	10.42%	9.38%
30-35	11.46%	15.62%	13.54%	11.46%
Absent Calls	33.33%	45.83%	43.75%	52.08%

Tables 6.8 and 6.9 represent genes that were down- and up-regulated as result of the different treatments, respectively. Classification of genes as being down- or up-regulated was achieved by comparing their expression levels to the control group. There were a total of 19 genes that were down-regulated and these genes were similar across the treatment groups. There were a total of 15 genes that were up-regulated as a result of treatment. It was also observed that these genes were similar across the treatment groups. These results demonstrated that *G. africana* may have a similar mode of action as doxorubicin.

Table 6.8: Comparison of down-regulated genes in Groups 1 (*G. africana* only), Group 2 (doxorubicin only) and Group 3 (combination of the two).

Gene	Group 1	Group 2	Group 3
ACLY	+	+	+
ACSL4	+	+	+
ANGPT1	+	+	+
ANGPT2	+	+	+
BIRC3	+	+	+
BMI1	+	+	+
CCND3	+	+	+
CDH2	+	—	+
FGF2	+	+	+
FLT1	+	+	+
G6PD	+	+	+

LDHA	+	+	+
MAPK14	+	+	+
PFKL	+	+	+
SNAI1	+	+	+
SOD1	+	+	+
STMN1	–	–	+
UQCRFS1	+	+	+
WEE1	+	+	+

+ gene present; – gene absent

Table 6.9: Comparison of up-regulated genes in Groups 1 (*G. africana* only), Group 2 (doxorubicin only) and Group 3 (combination of the two).

Gene symbol	Group 1	Group 2	Group 3
BCL2L11	+	+	+
CASP2	+	+	+
CASP7	+	+	+
CASP9	+	+	+
DDB2	+	+	+
DDIT3	–	+	+
DSP	+	+	+
FASLG	+	+	+
GADD45G	+	+	+
IGFBP3	–	–	+
IGFBP5	+	+	+
IGFBP7	+	+	+
OCLN	+	+	+
PINX1	+	+	+
PPP1R15A	+	+	+

+ gene present; – gene absent

6.5 Discussion

In this study, the cytotoxic effects of *G. africana*, doxorubicin and a combination of the two were evaluated. *G. africana* was chosen for this study due to its antimycobacterial activities (Mativandlela, Meyer *et al.* 2008), cytotoxicity capabilities (Mativandlela, Muthivhi *et al.* 2009), antifungal properties (Fielding, Knowles *et al.* 2015) and wound healing properties (Watt and Breyer-Brandwijk 1962). The stock solutions of the extract and doxorubicin were

prepared in DMEM. Despite doxorubicin initially being reconstituted in DMSO at concentrations as high as 100 mg/ml, the final concentration of DMSO was not more than 0.2% and not toxic to the cells as described in previous studies (Graidist, Martla *et al.* 2015). Therefore, it was concluded that the cytotoxic effects observed were due to doxorubicin and not the solvent. This study utilized the MTT cell viability assay to assess the cytotoxicity of *G. africana* alone, doxorubicin alone and a combination of the two against MCF-7 (human breast cancer) and KMST-6 (normal fibroblast) cells. This was done in order to establish the individual cytotoxic effects of *G. africana* and doxorubicin as well as the effect of their combination. MCF-7 cells were used in this study because they are the most frequently used *in vitro* models for breast cancer studies (Lacroix and Leclercq 2004). The non-cancerous normal fibroblasts KMST-6 cells were used as controls. The survival of the MCF-7 and KMST-6 cells after treatment with the *G. africana* extract (after 12, 24, 36 and 48 h) and doxorubicin (after 12 and 24 h) was determined. In addition, the survival of the two cell lines after treatment with a combination of the *G. africana* and doxorubicin after 12 and 24 h was also determined. The responses of MCF-7 and KMST-6 cells to increasing concentrations of the extract and doxorubicin are shown in Figures 6.1 and 6.2, respectively. The results showed that the extract had a biphasic effect on both cell lines at all time points (with the greatest effect seen on MCF-7 cells), whereas the growth of both cell lines decreased considerable in a dose-dependent manner (with the greatest effect seen on MCF-7) after treatment with doxorubicin. The IC_{50} values obtained after treatment of MCF-7 and KMST-6 cells with various concentrations of the *G. africana* extract, doxorubicin and a combination of the two are shown in Table 6.5. The results showed that the IC_{50} values obtained after treatment of the MCF-7 cells with the extract were much lower than those of the KMST-6 cells. This indicated that the extract had a greater cytotoxic effect on the MCF-7cells than the KMST-6 cells. These results were similar to those obtained by Abdullah and colleagues, who

demonstrated that the *Mangifera indica* extract was significantly cytotoxic to MDA-MB-231 and MCF-7 breast cancer cells, while being less toxic to normal breast cells (Abdullah, Mohammed *et al.* 2014). IC_{50} values of doxorubicin could not be obtained after 12 h of treatment because none of the concentrations reduced the growth of the cells by 50%. However, after 24 h of treatment, the IC_{50} value obtained for the MCF-7 cells was also lower than that of the KMST-6 cells. The combination interaction that produced the greatest activity at the lowest doxorubicin dose (0.1 µM) was seen after 24 h of treatment. The IC_{50} value of *G. africana* reduced from 1.692 to 1.144 mg/ml when used alone and in combination with doxorubicin (0.1 µM), respectively. The IC_{50} value of the extract increased from 3.625 to 8.814 mg/ml, for KMST-6 cells, when used alone and in combination with doxorubicin (0.1 µM) respectively, after 24 h of treatment. This synergistic effect could be attributed to the interaction between the extract and doxorubicin which contributed to the increased cytotoxic effect seen in the combination studies. Despite the extract having an IC_{50} value of 0.116 mg/ml, after 24 h of treatment, when combined with 2.5 µM of doxorubicin, the doxorubicin concentration was too high and this study aimed at using the lowest concentration of doxorubicin. Even though doxorubicin is widely used to treat numerous types of cancer, it is associated with adverse effects such as cardiotoxicity (Antonowa, Asbahr *et al.* 2017).

Plant extracts are considered to be effective anticancer agents only if they destroy cancer cells without causing too much damage to normal cells (Lacroix, Toillon *et al.* 2006). Phytochemical analysis of *G. africana* showed that it was mainly composed of flavonoids (Ticha, Klaasen *et al.* 2015). Flavonoids have been shown to possess numerous biologically active compounds. These compounds contribute to flavonoids having the ability to promote health and prevent diseases (Mikell and Khana 2012). Some of the flavonoids found in *G. africana* were similar in structure to chrysin, sakuranetin and pinocembrin (Ticha, Klaasen *et al.* 2015). Results from a study conducted to assess whether chrysin increased TRAIL-

induced cell death revealed that it enhanced TRAIL-induced apoptosis in human cancer cell lines such as Hela (cervical cancer) cells. Furthermore, it was shown that the apoptosis was caspase-dependent. Chrysin has also been shown to stimulate cell cycle arrest (Xin Li, Wang *et al.* 2011). It has also been shown that chrysin is more toxic to cancer cells than to normal cells. Studies have demonstrated that normal cells, such as epithelial and fibroblasts, exhibited increased resistance to the cytotoxic effect of chrysin compared to cancer cells (Kasala, Bodduluru *et al.* 2015). Pinocembrin has been shown to be cytotoxic to cancer cells, such as HCT-116 and SW480 (colorectal adenocarcinoma), MCF-7 (human breast cancer) and Hela (cervical cancer), but less toxic to normal cells, such as human umbilical cord endothelial cells (Kumar, Biswas *et al.* 2017). The results obtained from these studies explain why the *G. africana* extract exhibited strong anticancer properties as chrysin and pinocembrin constitute part of the chemical compounds found in *G. africana*.

The IC_{50} values obtained after 24 h of treatment were selected for the RT PCR profiler array to analyze gene expression. This array allowed for the determination of genes that would be up or down regulated in comparison to the untreated group (control group). The results of the RT PCR array showed that there was no significant difference in the number of genes that were up and down regulated between the *G. africana* extract and doxorubicin treated groups. The genes that were down- and up-regulated after exposure of the MCF-7 cells to the different treatment groups are shown in Tables 6.8 and 6.9, respectively. Tables 6.10 and 6.11 show the fold changes and functions of the genes that were down- and up-regulated, respectively, after 24 h of treatment. The results revealed that the genes that were down regulated (Table 6.10) included those that played a role in angiogenesis (*ANGPT1*, *ANGPT2* and *FLT1*), apoptosis (*BIRC3*), cell cycle (*CCND3*, *STMN1* and *WEE1*), cellular senescence (*MAPK14* and *SOD1*), epithelial-to-mesenchymal transition (EMT) (CDH2 and SNAI1), hypoxia signaling (*LDHA*) and metabolism (*ACLY*, *ACSL4*, *G6PD*, *PFKL* and *UQCRFS1*).

The results further revealed that the genes that were up-regulated (Table 6.11) included those that played a role in apoptosis (BCL2L11, CASP2, CASP7, CASP9 and FASLG), cellular senescence (IGFBP3, IGFBP5 and IGFBP7), DNA damage and repair (DDB2, DDIT3, GADD45G and PPP1R15A) and epithelial-to-mesenchymal transition (OCLN).

Table 6.10: Comparison of fold regulation changes of down-regulated genes in Groups 1, 2 and 3 (fold change < 1 or negative fold change).

	Fold change			
Gene symbol	**Group 1[a]**	**Group 2[b]**	**Group 3[c]**	**Function**
ACLY	-4.01	-1.06	-1.88	ATP-citrate lyase (ACLY) is a cytosolic enzyme responsible for the production of acetyl CoA (essential for the generation of fatty acids and cholesterol) from citrate. ACLY is over-expressed in numerous types of cancers. When it is under-expressed or inhibited, it causes proliferation in cancer cells to cease both *in vitro* and *in vivo* (Zaidi, Swinnen *et al.* 2012).
ACSL4	4.04	-1.50	-2.64	Acyl-CoA synthetase long-chain family member 4 Catalyzes the biosynthesis of lipids by generating fatty acyl-CoA esters from fatty acids. They also function in the breakdown of fatty acids. This enzyme is up-regulated in breast, colon and liver cancers (Wu, Li *et al.* 2013; Wu, Deng *et al.* 2015)
ANGPT1	-1.99	-6.04	-1.92	Angiopoietin 1 stimulates the assembly and maturation of blood vessels by enabling endothelial cells to survive stressful conditions (Davis, Aldrich *et al.* 1996).
ANGPT2	-2.56	-1.17	-1.77	Angiopoietin 2 stimulates endothelial cells to respond to angiogenic factors like VEGF (vascular endothelial growth factor). It also enables the vascular system to form branches and grow (Kim, Kim *et al.* 2000; Daly, Pasnikowski *et al.* 2006)
BIRC3	-3.59	-3.62	-4.82	BIRC3 is a member of a group of proteins referred to as inhibitors of apoptosis proteins (IAP) (Smolewski and Robak 2011; Saleem, Qadir *et al.* 2013). These proteins inhibit apoptosis and exert their functions by preventing the activity of caspase 3/7. BIRC3 has been shown to be highly expressed in numerous types of cancers (Gan, Liu *et al.* 2016) such as breast (Lu, Ning *et al.* 2016; Wang, Xu *et al.* 2016), pancreatic (Gan, Liu *et al.* 2016) and bladder (Kim, Ho *et al.* 2016).
BMI1	-4.15	-1.71	-1.69	BMI1 is a member of a group of proteins known as the Polycomb Group (PcG) gene family that act by altering the structure of chromatin thus regulating the transcription process of several genes (Cao, Bombard *et al.* 2011). This gene also functions in maintaining the integrity of stem cells in addition to cancer progression (Valk-Lingbeek, Bruggeman *et al.*

				2004). Studies have shown that BMI1 is up-regulated in cancers such as breast cancer (Cao, Bombard *et al.* 2011). In addition to the above mentioned functions, studies have indicated that BMI1 enhances angiogenesis, enables tumors to become more aggressive, and causes glioma cells to become resistant to apoptosis through the activation of resistance pathways (Paranjape, Balaji *et al.* 2014).
CCND3	-2.49	-1.21	-2.73	Cyclin D3 plays a role in the cell cycle and controls/regulates the action of Cyclin-dependent kinases 4 and 6 (Cdk4 and Cdk6). This gene is Up-regulated cancers such as bladder carcinoma in situ, a number of human B-lymphoid malignancies and breast cancer (Chi, Huang *et al.* 2015).
CDH2	-2.84	—	-2.46	CDH2 is a transmembrane protein responsible for cell-cell adhesion. It is up-regulated in cancers such as gastric, pancreatic and hepatocellular cancer (Yan, Yan *et al.* 2015).
FGF2	-9.60	-2.35	-1.27	FGF2 plays a role in angiogenesis, stimulates blood vessel development, endothelial cell growth and wound healing. It is also an effective angiogenic factor (Massabeau, Rouquette *et al.* 2009)
FLT1	-9.88	-2.90	-9.38	This gene plays a role in regulating angiogenesis, cell survival, and cell migration. It facilitates the ability of cancers to invade different tissue. FLT1 also functions in enhancing the growth of endothelial cells (Hoffmann, Goekkurt *et al.* 2013).
G6PD	-1.92	-1.23	-1.11	This gene codes for the glucose-6-phosphate dehydrogenase (G6PD) enzyme that plays a role in catalyzing the initial step of the pentose phosphate pathway. This pathway is essential for the production of nicotinamide adenine dinucleotide phosphate (NADPH), a coenzyme involved in the synthesis of lipids and nucleic acids, as well as ribose which is also required for the synthesis of DNA. It has been reported that there is a link between G6PD deficiency and reduced susceptibility to developing colorectal cancer (Luzzatto 2001; Dore, Davoli *et al.* 2016). Furthermore, results from a study conducted on breast cancer patients showed that increased expression of G6PD was linked to an increased risk of recurrent metastasis and poor progression-free survival in primary breast carcinoma (Pu, Zhang *et al.* 2015).
LDHA	-3.72	-1.36	-2.13	LDHA codes for an enzyme that plays a role in aerobic glycolysis as well as tumor development. It is responsible for catalyzing the final step in the aerobic glycolytic pathway (Xian, Liu *et al.* 2015). LDHA is highly expressed in pancreatic cancer (Shi, Cui *et al.*

				2014) and breast cancer (Zhao, Zhou *et al.* 2009). Studies indicated that down-regulation of LDHA caused a reduction in the ability of cancer cells to become malignant and significantly slowed down the tumor development process (Fantin, St-Pierre *et al.* 2006).
MAPK14	-12.41	1.52	-3.34	Studies carried out on various cancer cell lines have demonstrated that MAPK14 functions in enhancing cancer cell migration, enabling the spread of tumors and metastasis (Emerling, Platanias *et al.* 2005; Junttila, Ala-Aho *et al.* 2007).
PFKL	-4.10	-3.64	-5.03	PFKL is involved in catalyzing the phosphorylation of fructose-6-phosphate to fructose-1,6-bisphosphate, which is essential for glycolysis to occur (Wegener and Krause 2002). It has been shown that inhibiting the expression of PFKL prevented cell growth as well as their ability to become tumorgenic (Yi, Clark *et al.* 2012).
SNAI1	-1.47	-2.21	-2.53	SNAI1 belongs to a family of transcription factors. It acts by repressing the function of E-cadherin and is believed to play a role in enabling tumor cells become metastatic (Schwock and Geddie 2011). SNAI1 has been implicated in a number of conditions which include tissue fibrosis and cancers such as breast and ovarian cancer (Schwock and Geddie 2011). It also regulates and stimulates epithelial-mesenchymal transition (EMT) and facilitates the spread of cancer cells such as head and neck squamous cell carcinoma (HNSCC) (Ota, Masui *et al.* 2016).
SOD1	-7.75	-1.87	-3.71	SOD1 is essential for converting superoxide to hydrogen peroxide. It is predominantly found in the cytoplasm though it is also present in the inter-membrane space (IMS) (Papa, Manfredi *et al.* 2014). A study conducted on lung cancer cells revealed that up-regulation of SOD1 enhanced proliferation of the cancer cells and caused decreased apoptosis (Somwar, Erdjument-Bromage *et al.* 2011).
STMN1	—	—	-2.91	This gene codes for a protein that functions in regulating the assembly and disassembly of the mitotic spindles. It also enables the process of mitosis to take place and stimulates the proliferation of malignant tumor cells. This proliferation of tumor cells is uncontrolled and l leads to an increase in the number of tumor cells (Karst, Levanon *et al.* 2011). STMN1 is also regarded as an oncoprotein (Steinmetz 2007). A study conducted by Kang and colleagues revealed that there was an increase in the expression of STMN1 in 80% of primary gastric adenocarcinomas at protein level and in 56% at mRNA level (Kang, Tong

				et al. 2012). Furthermore, it has been shown that silencing STMN1 or reducing its expression increased the susceptibility of gastric cancer cells to docetaxel treatment (Meng and Tao 2015). Studies have shown that the expression of STMN1 increased in cancers such as breast and lucng cancer. Studies have also shown that this gene ehnaced the abiliy of the the cancer cells to grow and spread causing advanced disease and increased chances of death (Guo, Luo *et al.* 2016).
UQCRFS1	-1.86	-2.09	-1.77	UQCRFS1 is the major subunit of the the cytochrome bc1 complex (complex III) of the mitochondrial respiratory chain responsible for generating electrochemical potential linked to adenosine triphosphate (ATP) production (Jun, Kim *et al.* 2012). Experiments conducted on breast cancer cell lines that involved knocking out the UQCRFS1 gene showed that there was reduction in the mitochondrial membrane potential and reduced ability of cancer cells to invade tissues (Owens, Kulawiec *et al.* 2011). This demonstrated that up-regulation of UQCRFS1 can enhance production of oxygen radicals, speed up cancer cell growth and increase the spread of cancer cells through the rapid production of ATP (Jun, Kim *et al.* 2012).
WEE1	-1.08	-1.37	-2.89	Regulation of the cell cycle is necessary to prevent cells from becoming genomically unstable. In cancer cells, this regulatory mechanism is lost and hence, leads to genomic instability (Williams and Stoeber 2012). DNA damage is assessed and verified in the G_1 and G_2 phases of the cell cycle. However, the G_1 phase is absent in cancer cells and thus depend on G_2 arrest for growth (Vriend, De Witt Hamer *et al.* 2013). WEE1 functions in maintaining the integrity of the genetic material and regulates the G_2 phase. This gene is up-regulated in numerous cancer including osteosarcoma (PosthumaDeBoer, Wurdinger *et al.* 2011) and breast cancer (Wang, Huang *et al.* 2011).

[a] Cells treated with *G. africana* only

[b] Cells treated with doxorubicin only

[c] Cells treated with a combination of *G. africana* and doxorubicin

─ gene absent

Table 6.11: Comparison of fold regulation changes of up-regulated genes in Groups 1, 2 and 3 (fold change >1 or positive fold change).

	Fold change			
Gene symbol	**Group 1[a]**	**Group 2[b]**	**Group 3[c]**	**Function**
BCL2L11	8.03	2.65	8.46	BCL2L11 belongs to the pro-apoptotic family and is responsible for initiating the mitochondrial apoptotic pathway (Bouillet, Metcalf *et al.* 1999). It also plays a role in tumor suppression and acts as a tumor suppressor (Tagawa, Karnan *et al.* 2005). Inhibition or deletion of the BCL2L11 gene results in resistance mechanisms in cells thus enabling them not to respond to various pro-apoptotic agents such as calcium ionophores (Bouillet, Metcalf *et al.* 1999; Herold, Stuchbery *et al.* 2014).
CASP2	8.03	2.65	8.46	CASP2 is involved in apoptosis resulting from various intrinsic and extrinsic factors such as DNA damage, reactive oxygen species (ROS) and cytoskeletal disturbances (Kumar 2009). It also functions as a tumor suppressor (Puccini, Dorstyn *et al.* 2013). Resistance to chemotherapeutic agents in conditions such as childhood acute lymphoblastic leukemia (ALL) had been linked to the down-regulation or under-expression of CASP2 (Holleman, den Boer *et al.* 2005). CASP2 has also been shown to play a role in cell growth and transformation regulation (Gitenay, Lallet-Daher *et al.* 2014).
CASP7	8.03	2.65	8.46	CASP7 belongs to a family of genes needed for the maintenance of homeostasis by means of cell death regulation and inflammation. Caspases act on peptide bonds, via hydrolysis, which results in the activation of chemicals that play a role in apoptosis and inflammation (McIlwain, Berger *et al.* 2013; Chaudhary, Madhukrishna *et al.* 2016). Caspases that play a role in apoptosis can either be classified as being initiator caspases or executioner caspases. Executioner caspases are activated by initiator caspases (McIlwain, Berger *et al.* 2013). CASP7 codes for a protease enzyme that is an example of an executioner caspase. Activation of executioner caspases, like caspase-7, leads to the activation of additional executioner caspases that also play a role in apoptosis (McIlwain, Berger *et al.* 2013).

CASP9	8.03	2.65	8.46	Apoptosis is a process that involves both the intrinsic and the extrinsic pathways and is necessary for controlling the amount and quality of cells (Druškovič, Šuput *et al.* 2006). CASP9 encodes caspase-9, which is the main enzyme involved in the intrinsic apoptotic pathway. Activation of caspase-9 leads to the activation of caspase-3 resulting in apoptosis (Kim, Srivastava *et al.* 2015). Caspase-9 inhibition causes testicular tumors to become resistant to apoptosis (Mueller, Voigt *et al.* 2003). It has been shown that the mode of action of several cytotoxic agents is the initiation of cell death through the activation of the cytochrome c/Apaf-1/Caspase- 9-dependent intrinsic pathway (Debatin 2004). Studies have shown that natural compounds are beneficial in the fight against cancer as they have the potential to prevent cancer growth, cancer spread as well as drug resistance. They inhibit cancer development in many types of cancers by triggering apoptosis through the activation of caspase-9 (Kim, Srivastava *et al.* 2015).
DDB2	8.03	2.65	8.46	The prevalence and death rates of metastatic breast cancer are on the rise and knowing the exact mechanism by which metastasis takes place is very important. The ability of breast cancer to become metastatic and invade neighboring and distant tissues is coupled with changes in cellular structure and cellular communication. Data has shown that DDB2 is able to decrease the invasive capabilities of mammary tumors (Ennen, Klotz *et al.* 2013; Zhao, Han *et al.* 2014). A study conducted to evaluate the tumor suppressive role of the DDB2 gene revealed that mice that lacked the gene were prone to UV-induced carcinogenesis and they easily had malignant tumors (Yoon, Chakrabortty *et al.* 2005; Itoh, Iwashita *et al.* 2007).
DDIT3	–	1.37	1.35	DDIT3 codes for a protein that is involved in stress related responses. The expression of DDIT3 is stimulated by DNA damage, hypoxia, ER stress as well as starvation (Jauhiainen, Thomsen *et al.* 2012). Stimulated expression of DDIT3 results in the inhibition of the cell cycle process in some cells with apoptosis occurring in other types of cells. It is for this reason that DDIT3 is regarded as an apoptotic transcription factor (Zinszner, Kuroda *et al.* 1998; Wu, Sun *et al.* 2014).
DSP	8.03	2.65	8.46	Cancers become metastatic when the cell-cell adhesion properties at the original tumor location are lost (Behrens 1999). Desmosomes are specific cellular components

				that are responsible for cell-cell adhesion and studies have indicated that they play a role in regulating cell motility, growth, apoptosis and differentiation (Yang, Chen *et al.* 2012). DSP belongs to the plankin family and the proteins are extensively expressed in several tissues. Reports from numerous studies have shown that a decrease in the number of desmosomes resulted in tumor cells being invasive (Yang, Chen *et al.* 2012). Down-regulation of desmosomal proteins was also observed in breast cancer (Oshiro, Kim *et al.* 2005), cervical carcinoma (Alazawi, Morris *et al.* 2003) and pancreatic cancer (Hamidov, Altendorf-Hofmann *et al.* 2011).
FASLG	8.03	2.65	8.46	The FASLG gene codes for a protein that is a member of the tumor necrosis factor (TNF) family. The primary function of the encoded transmembrane protein is the initiation of apoptosis triggered by binding to FAS (Xu, Zhou *et al.* 2013).
GADD45G	14.68	1.13	–	GADD45G gene plays a role in a number of processes such as cellular stress responses and tumor suppression (Liebermann and Hoffman 2007; Tamura, de Vasconcellos *et al.* 2012). It has been shown that there is an interaction between the GADD45G gene and numerous other proteins that are responsible for DNA repair, apoptosis, cell cycle control and senescence (Johnen, González-Silva *et al.* 2013). A reduction in the expression levels of GADD45G has been linked to several types of cancers (Zhang, Yang *et al.* 2014)
IGFBP3	–	–	1.83	IGFBP3 codes for the Insulin-like growth factor binding protein 3 (IGFBP3) which belongs to the IGF family. IGFBP3 is involved in cellular proliferation, differentiation, apoptosis, and mammary carcinogenesis (Schedlich and Graham 2003). Studies have indicated that IGFBP3 helps the transforming growth factor-1 to function in inhibiting cell growth and it also stimulates apoptosis by means of the tumor suppressor gene (p53) in breast cancer patients (Marshman and Streuli 2002). Reduced IGFBP3 levels have been linked to an increase in the risk of cancer development. This has been seen in cases where genetic polymorphisms in IGFBP3 have resulted in changes in the circulating levels of IGFBP3 thus increases the chances of developing different types of carcinomas (Yang, Liu *et al.* 2014) including breast cancer (Safarinejad, Shafiei *et al.* 2011; Ma, Kang *et al.* 2015)
IGFBP5	8.03	2.65	8.46	IGFBP5 codes for a protein that belongs to the Insulin-like growth factor binding

				protein (IGFBP) family. Studies have shown that the up-regulation of IGFBP5 in breast cancer cell models induced apoptosis and inhibited cell proliferation. Furthermore, it inhibited tumor cell growth in *in vivo* studies (Butt, Dickson *et al.* 2003).
IGFBP7	8.03	2.65	8.46	IGFBP7 also encodes a protein that is a member of the IGFBP family. IGFBP7 is involved in the regulation of cell proliferation, cell differentiation, cell adhesion, cell senescence and angiogenesis in a wide range of cancer cells. It has also been shown to possess tumor suppressing capabilities in several types of cancers (Chen, Cui *et al.* 2011).
OCLN	8.03	2.65	8.46	OCLN is a gene that encodes the transmembrane protein called occludin. Occludin is involved in the formation and maintenance of tight junctions. It is for this reason that any alteration to the structure or loss of occluding could result in enhanced epithelial leakage thereby promoting metastasis in cancers. Changes in occludin expression have been shown to be associated with enhanced invasion, reduction in cell adhesion and decrease in tight junction composition in breast cancer tissue. In addition, evidence showed that patients with invasive tumors had considerably low occluding levels suggesting that proper tight junction function is essential in preventing metastasis (Martin, Mansel *et al.* 2010).
PINX1	8.03	2.65	8.46	PINX1 has been shown to inhibit the function of telomerase and also plays a role in tumor suppression (Zhou and Lu 2001). Studies have also indicated that PINX1 is capable of inhibiting metastasis in breast cancer which subsequently has an effect on the development and advancement of the cancer (Shi, Cao *et al.* 2015).
PPP1R15A	8.03	2.65	8.46	PPP1R15A codes for a protein that plays a role in inhibiting cell growth (Zhan, Lord *et al.* 1994) and is also involved in E.R (endoplasmic reticulum) mediated cell death (Sano and Reed 2013). Studies have demonstrated that PPP1R15A induces apoptosis in specific cell lines after the administration of alkylating agents and ionizing ration (Tanaka, Ito *et al.* 2015).

[a] Cells treated with *G. africana* only

[b] Cells treated with doxorubicin only

[c] Cells treated with a combination of *G. africana* and doxorubicin
– gene absent

Numerous studies have been conducted to investigate the effect of medicinal plants on the gene expression of human breast cancer cells. A study carried out to investigate the effect of *Euphorbia tirucalli* extracts in breast cancer cell lines revealed that the extracts inhibited cell proliferation in dose-dependent manner (Choene and Motadi 2016). It was established that the over-expression of *p21* contributed to the proliferation inhibition. Furthermore, pro-apoptotic genes such as *Bax* and caspase-8 were significantly up-regulated in cells treated with plant extracts (Choene and Motadi 2016). Another study that investigated the effect of soy isoflavones on the gene expression of MCF-7, MDA-MB-231 and MCF-10a cell lines found that there was a significant change in the expression of about 246 genes. These genes played a role in cell communication, biodegradation, lipid metabolism, signal transduction and cell growth/death (Satih, Chalabi *et al.* 2010). Orangi *et al.* (Orangi, Pasdaran *et al.* 2016) investigated the cytotoxic effects of *Scrophularia oxysepala* methanolic subfractions as well as the mechanisms responsible for cell death in MCF-7 cells. They observed that caspase-8 was up-regulated while the expression of BCL-2 was down-regulated. A study evaluating the anticancer effect of *Vernonia amygdalina* in MCF-7 and MDA-231 cells showed that *p53* and *p21* were up-regulated and this resulted in cell growth arrest. It was also shown that cyclin D1 and cyclin E were down-regulated. Apoptosis was considered to be caspase-dependent (Wong, Woo *et al.* 2013).

6.6 Conclusion

This study demonstrated that *G. africana* had significant anticancer potential on the MCF-7 cells with minimal effects on the KMST-6 cells. The IC_{50} values for the KMST- cells were higher than those of the MCF-7 cells at the different time-points suggesting that the extract could have minimal effects on other normal cells. *G. africana* also had an effect on gene expression causing the up-regulation of genes involved in apoptosis and cellular senescence while causing down-

regulation of genes involved in angiogenesis and metabolism. Evaluation of gene expression using the RT PCR array was a preliminary study and was used as a screening process. Further studies will be conducted based on these results. These results can subsequently be used to investigate the mode of action of *G. africana*. Future studies will include the use of at least three normal cells as controls as well as the detection of apoptotic markers to confirm whether the plant extract induces cell apoptosis.

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7 CHAPTER 7: Conclusion and recommendations

7.1 Overview

Drug resistance is a global problem that arises when diseases no longer respond to therapeutic agents normally used in their treatment. Initially, drug resistance was observed in bacteria that exhibited reduced susceptibility to particular antibiotics. However, other microorganisms have also developed resistance to therapeutic agents. This trend can also be seen in diseases such as cancer (Housman, Byler *et al.* 2014). Treatment failure and deferred treatment often lead to high death rates (Kumar, Haery *et al.* 2006; Ferrer, Martin-Loeches *et al.* 2014). Unfortunately, new antimicrobial agents are not being produced fast enough to match the rate at which these microorganisms develop resistance to these drugs (Karam, Chastre *et al.* 2016).

Medicinal plants have been used for centuries for the treatment of various ailments (Lixandru, Dracea *et al.* 2010). Reports have indicated that therapeutic drugs derived from medicinal plants have proved to be safer than synthetic drugs and produce no side effects (Bansod and Rai 2008). A number of medicinal plants have been investigated to assess their antimicrobial and anticancer activities. A study carried out by Rous and colleagues on the antibacterial and antifungal activities of essential oils showed that these oils had considerable antibacterial and antifungal activities against the tested pathogens including *S. aureus*, *C. albicans* and *C. glabrata* (Rouis, Abid *et al.* 2013). The assessment of medicinal plants for their cytotoxic effects against breast cancer cells lines revealed that they were cytotoxic against these cells and also had an effect on the structure of the cells (Vijayarathna and Sasidharan 2012).

Galenia africana has been shown to possess various health benefits. It has been utilized in the treatment of wounds, relief of eye inflammation, venereal diseases, ringworms as well as in the

treatment of toothaches (Watt and Breyer-Brandwijk 1962). However, despite medicinal plants being used in the treatment of a vast number of diseases, investigation on the potential toxicity of these plants is limited even though numerous studies have been conducted to assess their health beneficial properties. Reports have indicated that a large number of medicinal plants result in severe toxicity when used (Fennell, Light *et al.* 2004; Wojcikowski, Johnson *et al.* 2004). As a result, more studies are being conducted to investigate the possible toxic effects of medicinal plants (Nath and Yadav 2015).

The present study evaluated the potential antimicrobial, antifungal and anticancer properties of *G. africana* using various techniques. Prior to conducting the study, toxicity studies were conducted in order to ascertain the level of toxicity that *G. africana* may have. This was done using acute toxicity and skin irritation and sensitization studies.

The evaluation of the toxicity of the ethanolic extract of *G. africana* was conducted according to the OECD guidelines and determined using the acute oral (OECD guideline No. 423 (OECD 2001), acute dermal (OECD guideline No. 402 (OECD 1987; OECD 2015a) and skin sensitization toxicity studies (OECD test guideline No. 429 (OECD 2010) in animal models and skin irritation test in reconstructed human *epidermis* models (OECD 439 (OECD 2015b). The results of the acute oral and dermal toxicity studies showed that the median lethal dose (LD_{50}) of the extract in the rats was higher than 2000 mg/kg. Furthermore, macroscopic analysis, at necropsy, revealed that there were no abnormalities observed. The skin sensitization study was done using the local lymph node assay (LLNA). Treatment with *G. africana* concentrations of 50, 100 and 200 mg/ml resulted in stimulation index (SI) values of 1.3, 0.9 and 1.3, respectively. It was concluded that since the *G. africana* extract did not result in a SI value of ≥ 3 in any of the groups (50, 100 and 200 mg/ml, respectively), it was regarded as not having the potential to

cause skin sensitization. The results of the skin irritation assay revealed that both the concentrated and the diluted *G. africana* extracts were non-irritant to the EpiSkin® models. The outcomes of this study demonstrated that the ethanolic extract of *G. africana* did not result in any *in vivo* danger.

The antibacterial activity of *G. africana* against methicillin-susceptible (ATCC 25923) and methicillin-resistant (ATCC 33591) *S. aureus* (MSSA and MRSA, respectively) was evaluated using the broth microdilution assay, checkerboard assay and Sensititre susceptibility test. The MBC of *G. africana* was also determined. The MIC obtained after treatment of the bacterial cultures with the ethanolic extract was 3.12 mg/ml for both strains The MBC of the ethanolic extract against MSSA (ATCC 25923) was 6.25 mg/ml while that of the MRSA (ATCC 33591) was 12.5 mg/ml. Results of the checkerboard assay revealed that there was an additive effect (FICI 0.64) between the extract and ampicillin against MSSA (ATCC 25923). However, the effect seen with the extract-ampicillin combination was indifference (FICI 1.002) against MRSA (ATCC 33591). The results from the Sensititre susceptibility test showed that the extract enhanced the antimicrobial activity of several drugs against both strains.

The antifungal activity of *G. afrciana* against *C. albicans* (ATCC 90028) and *C. glabrata* (ATCC 26512) was assessed using the disk diffusion, broth microdilution assay, checkerboard assay and Sensititre susceptibility test. The effect of *G. africana* on fungal growth was evaluated by determining the MFC. The effect of the extract on the structure of the *Candida* cells was also investigated. After the incubation period, small zones of inhibition and micro-colonies were seen at *G. africana* concentrations of 15.6 to 250 mg/ml. No zones of inhibition were seen at concentrations of 7.8 and 3.9 mg/ml. The ethanolic extract produced an MIC of 6.25 mg/ml against both strains. The MFC of the ethanolic extract against *C. albians* (ATCC 90028) was

6.25 mg/ml and that of ethanolic extract against *C. glabrata* (ATCC 26512) was 12.5 mg/ml. Results of the checkerboard assay showed that the interaction between fluconazole and *G. africana* against *C. albicans* (ATCC 90028) was synergistic (FICI value of 0.36 which was < 0.5.) The effect of the combination of fluconazole and *G. africana* against *C. glabrata* (ATCC 26512) produced an FICI value of 1.002, which was considered to be indifference as this value was in the range of 1 < FICI < 4. The Sensititre susceptibility testing results revealed that *G. africana* enhanced the antifungal activity of the antifungal agents as seen by a decrease in the MICs of the antifungal agents against the *Candida* species. Microscopic analysis revealed that cell structures appeared distorted, with leakages of cell constituents as well as loss of cell contents. Remnants of cells could also be seen under the microscope. The untreated *Candida* cells appeared round with smooth surfaces without any distortion to their morphology. The results obtained indicate that *G. africana* is a potential source of new antibacterial and antifungal agents.

G. africana alone, doxorubicin alone as well as their combinations were tested for *in vitro* cytotoxicity, using MCF-7 and KMST-6 cells by the MTT assay. The effect of *G. africana* on gene expression was also assessed. The results demonstrated that *G. africana* had significant anticancer potential on the MCF-7 cells with minimal effects on the KMST-6 cells. The IC_{50} values for the KMST- cells were higher than those of the MCF-7 cells at the different time-points suggesting that the extract could have minimal effects on other normal cells. *G. africana* also had an effect on gene expression causing the up-regulation of genes involved in apoptosis and cellular senescence while causing down-regulation of genes involved in angiogenesis and metabolism.

Collectively, these results show that *G. africana* has tremendous potential in complementary and alternative medicine for the treatment of bacterial and fungal infections as well as for the treatment of cancer. The toxicity studies also revealed that *G. africana* did not result in any adverse effects and there were no signs of toxicity in both the animal and human skin models.

7.2 Recommendations

The outcomes of this study demonstrated that the ethanolic extract of *G. africana* did not result in any *in vivo* danger. Pharmacological and biochemical investigations will be essential in elucidating the mechanism of action and will be beneficial in utilizing this plant as a therapeutic agent. Furthermore, a detailed experimental analysis of the chronic toxicities is important to support these findings. Toxicity assessment of medicinal plants already in use, including the ones not yet commercialized, is important in evaluating their safety and sensitizing potential at dosages for which these formulations are being used. Clinical trials have not yet been performed on this medicinal plant and will be essential. This study supplies additional information on the activity of *G. africana* and contributes to the knowledge of antimicrobial properties of plants commonly found in South Africa. However, additional research such as exploring the mechanism of action and performing *in vivo* studies to corroborate the antimicrobial potential of the extract alone and in combination with antibacterial and antifungal agents will have to be conducted. Evaluation of gene expression using the RT PCR array was a preliminary study and was used as a screening process. Further studies will be conducted based on these results. These results can subsequently be used to investigate the mode of action of *G. africana*.

7.3 References

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8 APPENDICES

APPENDIX I

Certificate of Analysis for *G. africana* Extract

extracting the essence of africa

CERTIFICATE OF ANALYSIS

Product code	:	PJ0070/08
Batch No.	:	06/08/08
Product	:	Galenia africana herba sicc Ø 0.2g/ml
Production date	:	February 2008
Expiry date	:	February 2013

Characteristic		Specification	Result
Plant material used for extraction	:	Galenia africana herba	Pass
Content	:	Galenia africana herba sicc Ø 0.2g/ml	Pass
Appearance	:	A yellow green to brown green liquid	Pass
***Odour* & Taste**	:	Aromatic, alcohol specific odour	Pass
Specific gravity	:	Record only	0.8611
Total solids	:	>1.0%m/m	1.74%m/m
TLC	:	Compare to reference run at the same time under the same conditions	Not Tested

Storage
Storage Conditions : Closed container, cool (10 - 25°C) and dry conditions

PAARL, May 2009

R Abrahams
Afriplex
Analytical Department

Afriplex
PO Box 3386, Paarl 7620, South Africa Tel: +27 21 872 4978 Fax: +27 21 872 1930

APPENDIX II

GLP Certificate

THE DEPARTMENT OF HEALTH OF THE GOVERNMENT OF THE UNITED KINGDOM

GOOD LABORATORY PRACTICE

STATEMENT OF COMPLIANCE
IN ACCORDANCE WITH DIRECTIVE 2004/9/EC

TEST FACILITY	TEST TYPE
Charles River Laboratories **Tranent** **Edinburgh** **EH33 2NE**	**Analytical Chemistry** **Clinical Pathology** **Biological Safety Tests** **Ecosystems** **Environmental Fate** **Environmental Toxicity** **Mutagenicity** **Phys/Chem. Testing** **Toxicology** **Virology**

DATE OF INSPECTION

5th August 2008

A general inspection for compliance with the Principles of Good Laboratory Practice was carried out at the above test facility as part of the UK GLP Compliance Programme.

At the time of inspection no deviations were found of sufficient magnitude to affect the validity of non-clinical studies performed at these facilities.

20/10/08

Dr. Andrew J. Gray
Head, UK GLP Monitoring Authority

MHRA

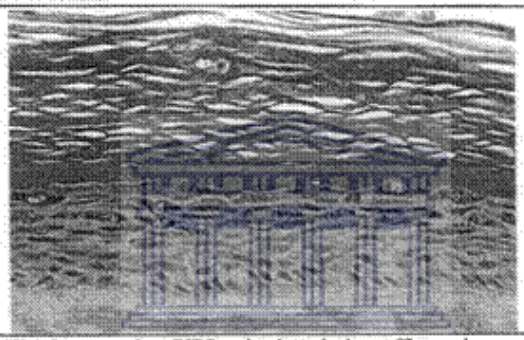
APPENDIX III

EpiSkin® Technical Data, Safety Sheet and Certificate of Analysis

SkinEthic laboratories

TECHNICAL DATA, SAFETY SHEET AND CERTIFICATE OF ANALYSIS
RECONSTRUCTED HUMAN EPIDERMIS

CCE-037/10

Description:	**Episkin Small Model** 0.38 cm² reconstructed epidermis of normal human keratinocytes. Cells are grown on a collagen matrix, airlifted for 13 days.
Usage:	**FOR SCIENTIFIC USE ONLY - PRODUCT OF HUMAN ORIGIN**
Storage:	This product was prepared and packaged using aseptic techniques. Store in an incubator at 37° C, 5% CO_2 with saturated humidity.
Passage:	Second (Strains n° : 99-KERA-016, 99-KERA-049, 00-KERA-017, 08-KERA-004)
Batch N°:	**09-EKIN-024**
Origin:	Adult donors.
Histology:	 **Control n° E09305**
Quality Controls:	**. Histology scoring** (HES stained vertical paraffin sections, n = 6) : specification ≥ 19.5, **result = 22.6 ± 0.5 , CV = 2.2 %** Well-differentiated epidermis consisting of a basal layer, several spinous and granular layers and a thick stratum corneum **. IC 50 determination** (SDS concentration, MTT test, n = 14) : specification ≥ 1.5 mg/ml, **result = 2.1 mg/ml** **. Statistical Analysis :** → Histology : probability 0.95 that 100 % of the batch > 20 → IC 50 : probability 0.95 that IC 50 ≥ 2.0 mg/ml (threshold value)
Biological safety:	On blood of the same donors, we have verified: . the absence of HIV1 and 2 antibodies (Abbott + Centaur Bayer) . the absence of hepatitis C antibodies (Centaur Bayer) . the absence of hepatitis B antigen HBs (Centaur Bayer) On epidermal cells of the same donors, we have verified: . the absence of bacteria, fungus and mycoplasma
Expiration date	Day of shipping + 1 week

Lyon, June 16, 2009.
Certified and released by
Carole Amsellem, Quality Control Manager

Manufactured in accordance to the ISO9001 quality system of Episkin.

45, rue St. Philippe - 06000 NICE – France
Téléphone : +33 (0)4 93 97 77 27 - Fax : +33 (0)4 93 97 77 28
S.A. au capital de 470.010 € - Code NAF 7219 Z - RCS Nice B 388 097 537
E-mail : infos@skinethic.com Site Web : www.skinethic.com

APPENDIX IV

Certificate of analysis for *G. africana*

Oxidative Stress Research Centre

Centre Manager
Prof. Jeanine Marnewick
Tel: (021) 953 8416
e-mail: marnewickJ@cput.ac.za

Lab Manager
Fanie Rautenbach
Tel: (021) 953 8418
e-mail: rautenbachF@cput.ac.za

Fax: (021) 953 8490

Cape Peninsula University of Technology

Certificate of Analysis

Assay for: UWC – Kraalbos
Jeremy Klaasen

Sample Name	7-OH-Flav (mg/L)	Pinocembrin (mg/L)	2,4-Dihidrochalcone (mg/L)	Chrysin (mg/L)	Polyphenols (mg/L)
20% ethanolic extract	N.D.	741.60	227.69	530.10	1740
GA-MHB (*G. africana* in MHB)	N.D.	36.46	13.99	8.96	1119
GA-RPMI (*G. africana* in RPMI)	N.D.	50.22	14.32	6.031	941

N.D. = none detected

Assay conditions

HPLC column : YMC - Pack Pro C18
Mobile phase A: Water + 0.05% TFA
Mobile phase B: Methanol + 0.05% TFA
Mobile phase C: Acetonitrile + 0.05%
TFA Runtime: 30 minutes
Column Temperature : 30°C Flow rate : 1 mL/min
UV detection : 280 and 320nm
Injection : 20µL
Sample preparation : None

Analysis date : 05/10/2015
Analysed by : G.S. Rautenbach
Authorised by : J.L. Marnewick

G.S. Rautenbach
Laboratory Manage

APPENDIX V

Certificate of analysis for *G. africana*

Oxidative Stress Research Centre

Centre Manager
Prof. Jeanine Marnewick
Tel: (021) 953 8416
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Lab Manager
Fanie Rautenbach
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Cape Peninsula University of Technology

Certificate of Analysis

Assay for: UWC – Kraalbos
Jeremy Klaasen

Sample Name	7-OH-Flav (mg/l)	Pinocembrin (mg/l)	2,4-Dihidrochalcone (mg/l)	Chrysin (mg/l)	Polyphenols (mg/l)
20% Crystals *G. africana*	N.D.	8144	6292	1726	21976
Sample Name	**7-OH-Flav (mg/g)**	**Pinocembrin (mg/g)**	**2,4-Dihidrochalcone (mg/g)**	**Chrysin (mg/g)**	**Polyphenols (mg/g)**
20% Aqueous extract	N.D.	8.84	4.67	1.34	20.21

N.D. = none detected

Assay conditions

HPLC column : YMC - Pack Pro C18
Mobile phase A: Water + 0.05% TFA
Mobile phase B: Methanol + 0.05% TFA
Mobile phase C: Acetonitrile + 0.05%
TFA Runtime: 30 minutes
Column Temperature : 30°C Flow
rate : 1 mL/min
UV detection : 280 and 320nm
Injection : 20µL
Sample preparation : None

Analysis date : 05/10/2015
Analysed by : G.S. Rautenbach
Authorised by : J.L. Marnewick

G.S. Rautenbach
Laboratory Manage

APPENDIX VI

Summary of genes in RT² Profiler PCR Array

Position	UniGene	GenBank	Symbol	Description
A01	Hs.387567	NM_001096	ACLY	ATP citrate lyase
A02	Hs.268785	NM_004458	ACSL4	Acyl-CoA synthetase long-chain family member 4
A03	Hs.441047	NM_001124	ADM	Adrenomedullin
A04	Hs.369675	NM_001146	ANGPT1	Angiopoietin 1
A05	Hs.583870	NM_001147	ANGPT2	Angiopoietin 2
A06	Hs.728891	NM_001160	APAF1	Apoptotic peptidase activating factor 1
A07	Hs.632446	NM_001668	ARNT	Aryl hydrocarbon receptor nuclear translocator
A08	Hs.298280	NM_004046	ATP5A1	ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit 1, cardiac muscle
A09	Hs.250822	NM_003600	AURKA	Aurora kinase A
A10	Hs.469658	NM_006538	BCL2L11	BCL2-like 11 (apoptosis facilitator)
A11	Hs.127799	NM_001165	BIRC3	Baculoviral IAP repeat containing 3
A12	Hs.380403	NM_005180	BMI1	BMI1 polycomb ring finger oncogene
B01	Hs.63287	NM_001216	CA9	Carbonic anhydrase IX
B02	Hs.368982	NM_032982	CASP2	Caspase 2, apoptosis-related cysteine peptidase
B03	Hs.9216	NM_001227	CASP7	Caspase 7, apoptosis-related cysteine peptidase
B04	Hs.329502	NM_001229	CASP9	Caspase 9, apoptosis-related cysteine peptidase
B05	Hs.303649	NM_002982	CCL2	Chemokine (C-C motif) ligand 2
B06	Hs.376071	NM_001759	CCND2	Cyclin D2
B07	Hs.534307	NM_001760	CCND3	Cyclin D3
B08	Hs.524947	NM_001255	CDC20	Cell division cycle 20 homolog (S. cerevisiae)
B09	Hs.464829	NM_001792	CDH2	Cadherin 2, type 1, N-cadherin (neuronal)
B10	Hs.390736	NM_003879	CFLAR	CASP8 and FADD-like apoptosis regulator
B11	Hs.401903	NM_004255	COX5A	Cytochrome c oxidase subunit Va
B12	Hs.705379	NM_000098	CPT2	Carnitine palmitoyltransferase 2
C01	Hs.700338	NM_000107	DDB2	Damage-specific DNA binding protein 2, 48kDa
C02	Hs.728989	NM_004083	DDIT3	DNA-damage-inducible transcript 3
C03	Hs.4747	NM_001363	DKC1	Dyskeratosis congenita 1, dyskerin
C04	Hs.519873	NM_004415	DSP	Desmoplakin
C05	Hs.108371	NM_001950	E2F4	E2F transcription factor 4, p107/p130-binding
C06	Hs.2303	NM_000799	EPO	Erythropoietin
C07	Hs.469872	NM_000122	ERCC3	Excision repair cross-complementing rodent repair deficiency, complementation group 3 (xeroderma pigmentosum group B complementing)
C08	Hs.258429	NM_000123	ERCC5	Excision repair cross-complementing rodent repair deficiency, complementation group 5
C09	Hs.644231	NM_005239	ETS2	V-Ets erythroblastosis virus E26 oncogene homolog 2 (avian)
C10	Hs.2007	NM_000639	FASLG	Fas ligand (TNF superfamily, member 6)
C11	Hs.284244	NM_002006	FGF2	Fibroblast growth factor 2 (basic)
C12	Hs.654360	NM_002019	FLT1	Fms-related tyrosine kinase 1 (vascular endothelial growth factor/vascular permeability factor receptor)
D01	Hs.436448	NM_005251	FOXC2	Forkhead box C2 (MFH-1, mesenchyme forkhead 1)
D02	Hs.461047	NM_000402	G6PD	Glucose-6-phosphate dehydrogenase
D03	Hs.9701	NM_006705	GADD45G	Growth arrest and DNA-damage-inducible, gamma
D04	Hs.512382	NM_000408	GPD2	Glycerol-3-phosphate dehydrogenase 2 (mitochondrial)
D05	Hs.440438	NM_173849	GSC	Goosecoid homeobox

APPENDIX VI (Continued)

Summary of genes in RT² Profiler PCR Array

Position	UniGene	GenBank	Symbol	Description
D06	Hs.517581	NM_002133	HMOX1	Heme oxygenase (decycling) 1
D07	Hs.450230	NM_000598	IGFBP3	Insulin-like growth factor binding protein 3
D08	Hs.607212	NM_000599	IGFBP5	Insulin-like growth factor binding protein 5
D09	Hs.479808	NM_001553	IGFBP7	Insulin-like growth factor binding protein 7
D10	Hs.479756	NM_002253	KDR	Kinase insert domain receptor (a type III receptor tyrosine kinase)
D11	Hs.654380	NM_000526	KRT14	Keratin 14
D12	Hs.2795	NM_005566	LDHA	Lactate dehydrogenase A
E01	Hs.166091	NM_002312	LIG4	Ligase IV, DNA, ATP-dependent
E02	Hs.180878	NM_000237	LPL	Lipoprotein lipase
E03	Hs.145442	NM_002755	MAP2K1	Mitogen-activated protein kinase kinase 1
E04	Hs.514012	NM_002756	MAP2K3	Mitogen-activated protein kinase kinase 3
E05	Hs.485233	NM_001315	MAPK14	Mitogen-activated protein kinase 14
E06	Hs.477481	NM_004526	MCM2	Minichromosome maintenance complex component 2
E07	Hs.689823	NM_002417	MKI67	Antigen identified by monoclonal antibody Ki-67
E08	Hs.513667	NM_003946	NOL3	Nucleolar protein 3 (apoptosis repressor with CARD domain)
E09	Hs.592605	NM_002538	OCLN	Occludin
E10	Hs.255093	NM_002626	PFKL	Phosphofructokinase, liver
E11	Hs.252820	NM_002632	PGF	Placental growth factor
E12	Hs.490991	NM_017884	PINX1	PIN2/TERF1 interacting, telomerase inhibitor 1
F01	Hs.654484	NM_002690	POLB	Polymerase (DNA directed), beta
F02	Hs.631593	NM_014330	PPP1R15A	Protein phosphatase 1, regulatory (inhibitor) subunit 15A
F03	Hs.594481	NM_002575	SERPINB2	Serpin peptidase inhibitor, clade B (ovalbumin), member 2
F04	Hs.532768	NM_002615	SERPINF1	Serpin peptidase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium derived factor), member 1
F05	Hs.23348	NM_005983	SKP2	S-phase kinase-associated protein 2 (p45)
F06	Hs.473721	NM_006516	SLC2A1	Solute carrier family 2 (facilitated glucose transporter), member 1
F07	Hs.48029	NM_005985	SNAI1	Snail homolog 1 (Drosophila)
F08	Hs.360174	NM_003068	SNAI2	Snail homolog 2 (Drosophila)
F09	Hs.253790	NM_178310	SNAI3	Snail homolog 3 (Drosophila)
F10	Hs.443914	NM_000454	SOD1	Superoxide dismutase 1, soluble
F11	Hs.376984	NM_006941	SOX10	SRY (sex determining region Y)-box 10
F12	Hs.209983	NM_005563	STMN1	Stathmin 1
G01	Hs.531085	NM_005994	TBX2	T-box 2
G02	Hs.89640	NM_000459	TEK	TEK tyrosine kinase, endothelial
G03	Hs.508835	NM_007110	TEP1	Telomerase-associated protein 1
G04	Hs.442707	NM_017489	TERF1	Telomeric repeat binding factor (NIMA-interacting) 1
G05	Hs.301419	NM_018975	TERF2IP	Telomeric repeat binding factor 2, interacting protein
G06	Hs.496191	NM_012461	TINF2	TERF1 (TRF1)-interacting nuclear factor 2
G07	Hs.370267	NM_003747	TNKS	Tankyrase, TRF1-interacting ankyrin-related ADP-ribose polymerase
G08	Hs.329327	NM_025235	TNKS2	Tankyrase, TRF1-interacting ankyrin-related ADP-ribose polymerase 2
G09	Hs.170107	NM_006003	UQCRFS1	Ubiquinol-cytochrome c reductase, Rieske iron-sulfur polypeptide 1
G10	Hs.435215	NM_005429	VEGFC	Vascular endothelial growth factor C
G11	Hs.249441	NM_003390	WEE1	WEE1 homolog (S. pombe)
G12	Hs.356076	NM_001167	XIAP	X-linked inhibitor of apoptosis
H01	Hs.520640	NM_001101	ACTB	Actin, beta
H02	Hs.534255	NM_004048	B2M	Beta-2-microglobulin
H03	Hs.592355	NM_002046	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
H04	Hs.412707	NM_000194	HPRT1	Hypoxanthine phosphoribosyltransferase 1
H05	Hs.546285	NM_001002	RPLP0	Ribosomal protein, large, P0
H06	N/A	SA_00105	HGDC	Human Genomic DNA Contamination
H07	N/A	SA_00104	RTC	Reverse Transcription Control
H08	N/A	SA_00104	RTC	Reverse Transcription Control
H09	N/A	SA_00104	RTC	Reverse Transcription Control
H10	N/A	SA_00103	PPC	Positive PCR Control
H11	N/A	SA_00103	PPC	Positive PCR Control
H12	N/A	SA_00103	PPC	Positive PCR Control