PHYTOCHEMICAL ANALYSIS AND BIOLOGICAL ACTIVITIES OF CRUDE EXTRACTS FROM SELECTED *TULBAGHIA SPECIES*.

BY

SAMKELISO TAKAIDZA **(**209101296)

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Department of Biotechnology

Faculty of Applied and Computer Sciences

Vaal University of Technology

Vanderbijlpark

Gauteng, South Africa

PROMOTER: PROFESSOR M. PILLAY

CO-PROMOTER: Dr F. MTUNZI

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DECLARATION

This work has not previously been accepted in substance for any degree and is not being concurrently submitted in any candidature for any degree.

Signed…….. Date…….....

STATEMENT 1

This thesis is being submitted in partial fulfilment of the requirements for the Doctor of Philosophy (PhD): Biotechnology.

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STATEMENT 2

This thesis is the result of my own independent investigation, except where otherwise stated. Other sources are acknowledged by giving explicit references. A bibliography is appended.

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DEDICATION

This Thesis is dedicated to my entire Moyo and Takaidza family who have been a great pillar of strength throughout my study.

It's not about how far you go in life, but with whom you walk with.

ABSTRACT

The genus *Tulbaghia* has been used in traditional medicine to treat various ailments such as fever, earache, tuberculosis and esophageal cancer. However, there is limited scientific evidence to support its use. Therefore the objectives of this study were to perform phytochemical analysis, investigate the antioxidant, antimicrobial, anticancer, immunomodulatory activities and toxicity of crude acetone and water extracts from selected *Tulbaghia* species.

Standard methods were used for preliminary phytochemical analysis. The total phenolic content of the plant extracts was determined using the folin ciocalteu method whereas the total flavonoids were determined by using the aluminium chloride colorimetric method. DPPH and ABTS assays were used to evaluate the antioxidant activity. The antimicrobial activity was assessed by agar well diffusion, microtiter dilution and time kill assays.

For anticancer studies, the antiproliferative activity of the extracts was evaluated using the MTT assay on Hkesc-1 and KB cells. Morphological changes of the cancer cells treated with extracts were examined using light microscopy. Induction of apoptosis was assessed using fluorescence microscopy and acridine orange/ethidium bromide staining. Flow cytometry analysis was conducted to examine the multicaspase activity and cell cycle arrest. For immunomodulatory activity, the Greiss reagent and Luminex cytokine assays were used to determine the effect of the extracts on NO production and the concentration of the cytokines in the treated cells, respectively.

Toxicity of selected *Tulbaghia* species was examined by investigating the effect of the extracts on the metabolic activity and cell membrane integrity on the treated RAW264.7 cells using the MTT and LDH assays, respectively. The zebrafish assay was used to evaluate the embryotoxicity and teratogenic effects of crude acetone and water extracts of *T. violacea* at 24 h intervals for 96 h post fertilisation (hpf). The percentage mortality, hatchability and heart rate were examined.

Phytochemical screening of eight *Tulbaghia* species demonstrated the presence of flavonoids, glycosides, tannins, terpenoids, saponins and steroids. The amount of total phenol and flavonoid content varied in different plant extracts ranging from 4.50 to 11.10 milligrams gallic acid equivalent per gram (mg GAE/g) of fresh material and 3.04 to 9.65 milligrams quercetin equivalent per gram (mg QE/g) of fresh material respectively. The IC_{50} values based on DPPH and ABTS for *T. alliacea* (0.06 and 0.06 mg/mL) and *T. violacea* (0.08 and 0.03 mg/mL) were

generally lower showing potential antioxidant activities. For antimicrobial activity, the acetone extracts of *T. acutiloba, T. alliacea, T. leucantha, T. ludwigiana, T. natalensis* and *T. simmleri* showed moderate antimicrobial activity against all test organisms while the water extracts showed moderate to no activity. One species, *T. cernua,* showed poor activity against all the tested microbes. The acetone and water extracts of *T. violacea* showed the greatest antibacterial and antifungal activity against all the tested microorganisms with minimum inhibitory concentration ranging from 0.1 mg/mL to 3.13 mg/mL. The acetone extracts of *T. violacea* also exhibited both bacteriostatic/fungistatic and bactericidal/fungicidal activity depending on the incubation time and concentration of the extract. The bactericidal/fungicidal activity was observed at x2 MIC.

The results for anticancer activity showed that treatment of Hkesc-1 cells with acetone and water crude extracts had anti-proliferative activity with IC_{50} values of 0.4 mg/mL and 1.625 mg/mL, respectively while KB had 0.2 mg/mL and 1 mg/mL, respectively. Morphological changes such as blebbing, cell shrinkage and rounding were observed in the treated cells suggesting that apoptosis was taking place. AOEB staining showed that the level of apoptosis was dependent on the concentration of the extracts. The activation of multicaspase activity in both Hkesc-1 and KB treated cells was also concentration dependent leading to cell death by apoptosis and the induction of cell cycle arrest at the G_2/M phase.

Immunomodulatory activity results indicated that cell viability was above 80% when concentrations of 50 µg/mL or less of both acetone and water crude was used. Treatment with the acetone extract had no significant effect (p>0.05) on the LPS induced NO production in RAW264.7 cells except at 50 µg/mL where significant inhibition was observed. The water extract had no significant effect (p>0.05) on NO production at all the concentrations. Treatment of LPS–induced RAW264.7 cells with acetone extract stimulated the production of IL-1α, IL-6 and TNF-α, but had no significant effect (p > 0.05) on IL-1β. On the other hand, treatment with the water extracts stimulated the production of IL-1α, IL-6 but had no significant effect (p>0.05) on TNF-α and IL-1β. Treatment of LPS-induced RAW264.7 cells with the acetone extract had very little stimulatory effect on IL-4, IL-5 and IL-13 and no significant effect on IL-10 whereas for the water extract a significant stimulatory effect was only observed for IL-4 after 48 h of treatment. High concentrations (>10000 pg/mL) of MCP-1, MIP1-α, MIP1-β, MIP-2, GCSF, GM-CSF, RANTES and IP-10 were also observed in acetone and water extract treated RAW264.7 cells.

For toxicity studies, acetone and aqueous crude leaf extracts from *T. alliacea*, *T. simmleri*, and *T. violacea* had a significant inhibitory ($p<0.05$) effect on the RAW264.7 cells after 48h treatment. Acetone extracts from *T. alliacea, T. simmleri* and *T. violacea* resulted in IC_{50} values of 0.48 mg/mL, 0.72 mg/mL and 0.1 mg/mL, respectively. Treatment with water extracts showed minimal toxic effect indicated by higher IC_{50} values of 0.95 mg/mL, 2.49 mg/mL and 0.3 mg/mL for *T. alliacea, T. simmleri* and *T. violacea*, respectively. The LDH release by macrophages after 24 h treatment with acetone extracts was observed to be concentration dependent while treatment with water extracts did not induce LDH release. The zebra fish assay showed a lethal dose (LD_{50}) for the *T. violacea* acetone crude extract of 20 µg/mL whereas that for water extract was 85 µg/mL. The observed teratogenic effects included scoliosis, edema of the pericardial cavity, retarded yolk resorption, hook-like/bent tail and shorter body length.

In conclusion, the results from this study indicate that the extracts from the eight *Tulbaghia* species examined contain phytochemicals that may have the antioxidant, antimicrobial, anticancer and immunomodulatory properties. Extracts from *T. violacea* were observed to be the most potent. This study thus supports the use of *T. violacea* in treating bacterial and fungal infections in traditional medicine. The results of this study also confirm the anticancer potential of *T. violacea*. The immunomodulatory activity of the acetone and water extracts from *T. violacea* indicated a dominantly pro-inflammatory activity. Traditional medicine prepared form *T. violacea* may be of benefit to individuals with weak immune systems. The toxicity of selected *Tulbaghia* species was observed to be concentration, extract and time dependent. Therefore, traditional medicine prepared from *Tulbaghia* extracts should be taken with caution preferably in small doses over a short period of time. Future studies will focus on the identification of the bioactive compound(s) responsible for the antimicrobial, anticancer and immunomodulatory activities.

Key words: Antioxidant activities, Anticancer activity, Apoptosis, Bacteriostatic, Cytokines, Embryotoxicity, Esophageal cancer, Fungicidal, LDH, Lipopolysaccharide, Macrophages, Multicaspase, Immunomodulation, Phenolic compounds, zebrafish

ABBREVIATIONS

ABTS:	2, 2-azino-bis (3-ethyl-benzthioziline-6-sulfonic acid)
AOEB:	Acridine orange /Ethidium bromide
BEA:	Benzene: Ethanol: Ammonia
BHA:	Butylated hydroquinone
BHT:	Butylated hydroxyl toluene
CFU/mL:	Colony Forming Units per millilitre
COX-2:	Cyclo-oxygenase-2
DMEM:	Dulbecco's Modified Eagles' Medium
DMSO:	Dimethyl Sulfoxide
FBS:	Foetal Bovine Serum
DPPH:	2, 2 diphenyl-1-picrylhydrazyl
EAC:	Esophageal adenocarcinoma
EC:	Esophageal cancer
ESCC:	Esophageal squamous cell carcinoma
FET:	Fish embryo toxicity test
GAE:	Gallic acid equivalent
HKESC-1:	Esophageal cancer cell line
IC_{50}:	concentration of sample which is required to scavenge 50%
IL1-β:	Interleukin 1β
IL-6:	Interleukin 6
INT:	*p*-iodonitrotetrazolium chloride
KB:	Human oral cancer cell line
LDH:	Lactate dehydrogenase

$LD_{50:}$	Lethal dose
LPS:	Lipopolysaccharide
MCP-1:	Monocyte chemoattractant protein-1
MEA:	Malt Extract Agar
MHA:	Mueller Hinton Agar
MIC:	Minimum Inhibitory Concentration
MTT:	[3(4, 5 dimethylthiazol-2-yl-2, 5-diphenyltetrazolium bromide]
NO:	Nitric oxide
QE:	Quercetin equivalent
TLC:	Thin Layer Chromatography
TFC:	Total Flavonoid Content
TPC:	Total Phenolic Content
TNF-α:	Tumour necrosis factor-alpha
WHO:	World Health Organisation

PUBLICATIONS

1. **Takaidza, S**., Kumar, A.M., Ssemakalu, C.C., Natesh, N. S., Karanam, G., Pillay, M. (2018). Anticancer activity of crude acetone and water extracts of *Tulbaghia violacea* on human oral cancer cells. *Asian Pacific Journal of Tropical Biomedicine*, 8(9): 456-462.
2. **Takaidza, S**., Mtunzi, F., Pillay, M. (2018). Analysis of the phytochemical contents and antioxidant activities of crude extracts from *Tulbaghia* species. *Journal of Traditional Chinese Medicine*,38(2016):272-279.
3. **Takaidza, S**., Pillay, M., Mtunzi, F.M. (2015). Biological activities of species in the genus *Tulbaghia*: A review. *African Journal of Biotechnology*, 14(45): 3037-3043.

MANUSCRIPTS UNDER REVIEW

1. **Samkeliso Takaidza**, Cornelius Cano Ssemakalu, Michael Pillay.The immunomodulatory activity of acetone and water crude extracts from *T. violacea* on LPS stimulated RAW264.7 cells. Submitted in *Journal of integrative medicine*.

2. **Samkeliso Takaidza**, Cornelius Cano Ssemakalu, Fanyana Mtunzi and Michael Pillay. Anti-proliferative and apoptotic activity of acetone and water crude extracts of *T. violacea* on Hkesc-1 cell line. Submitted in the Journal *Advances in Pharmacological Sciences.*

3. **Takaidza, S**., Kumar, M., Natesh, N., Santosh, J., Karanam, G. and Pillay, M. Embryotoxicity and teratogenic effects of *Tulbaghia violacea* acetone and aqueous leaf crude extracts on *Danio rerio* (Zebrafish). Submitted in *African Journal of Traditional, Complementary and Alternative Medicines*

4. **Takaidza, S**., Mtunzi, F., Pillay, M. Antimicrobial activities of *Tulbaghia* species against potential human pathogens. Submitted in *Journal of Herbs, Spices & Medicinal Plants*

CONFERENCE ATTENDANCE

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1. The International Conference on Traditional Medicine, Phytochemistry and Medicinal plants. 15-17 October 2018.Hilton Narita, Tokyo, Japan.

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2. The International Conference on Environment, Genes, Health and Diseases 2017.22- 24th August 2017. Coimbatore, India.

   Takaidza, S., Kumar M, Natesh N, Santosh J, Karanam G, Pillay M. Embryotoxicity and teratogenic effects of *Tulbaghia violacea* acetone and aqueous leaf crude extracts on *Danio rerio* (Zebrafish).

3. The 2nd International Conference and Exhibition on Pharmacology and Ethnopharmacology. 02-04 May 2016. Chicago, USA.

   Takaidza S, Pillay M, Mtunzi F. 2016. Preliminary phytochemical analysis, total phenolics and antioxidant activity of selected species in the genus *Tulbaghia*.

4. The 17th International Conference on Medicinal and Aromatic Plants. 3-4th December 2015. Penang, Malayasia.

   Takaidza S, Pillay M, Mtunzi F. 2015. Biological activities of species in the genus *Tulbaghia*: a review.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1 GENERAL INTRODUCTION

1.0 INTRODUCTION

Medicinal plants have been used in traditional medicine for treatment of numerous human diseases for thousands of years in many parts of the world (Vindhya et al., 2014; Razafintsalana et al., 2017; Arulmozhi et al., 2018). In the developing world, especially in rural areas, traditional medicine continues to be a primary source of health care. Scientifically, medicinal plants have proven to be an abundant source of bioactive compounds, many of which have already been formulated into useful therapeutic substances or have provided a basis for the development of new lead molecules for pharmaceuticals (Anyanwu and Nwosu, 2014; Bouyahya et al., 2017).

Infectious and degenerative diseases are the major causes of death world-wide (Namita and Mukwesh, 2012; Ogbole and Fasinu, 2018). The treatment of infectious diseases is considered a major challenge at present with many microbes developing resistance to widely used antibiotics. Drug resistance of microbial pathogens is an on-going global problem (Yadav and Khan, 2012; Moussaoui and Alaoui, 2016) and the incidence of multidrug resistance in pathogenic and opportunistic bacteria is on the rise (Dubey et al., 2012; Mohamed et al., 2010; Arulmozhi et al., 2018).

Synthetic drugs currently used for the treatment of degenerative diseases like cancer and arthritis have side effects (Islam et al., 2013). Although treatments of cancer has enabled the disease to be manageable, chemotherapy and surgery treatments have side effects such as hair loss, nausea, vomiting, infections and fatigue (Breen et al., 2017). In addition, synthetic molecules like nonsteroidal anti-inflammatory drugs (NSAIDs) and selective *COX* 2 inhibitors are known for their side-effects such as increasing the incidence of adverse cardiovascular thrombosis (Damte et al., 2011).

These health-related challenges have, therefore, necessitated a continuous search of potential bioactive compounds with diverse chemical structures and novel mechanisms of action from alternative sources such as medicinal plants (Ahmad and Beg, 2001; VijayaBarathi 2014; Savithramma et al., 2011). The importance of medicinal plants lies in their ability to produce bioactive compounds which are mainly the products of secondary metabolic pathways. These secondary metabolites include alkaloids, flavonoids, tannins and phenolics (Thiyagarajan and Uriyavathana, 2010; Narendra et al., 2013). These phytocompounds are known to possess high antioxidant, antimicrobial, anti-inflammatory and anticancer activities (Ignat et al., 2011).

Bioactive compounds that can either inhibit the growth of pathogens or kill them with little or no toxicity to host cells can be considered potential candidates for developing new drugs. It is expected that plant extracts showing target sites other than those used by antibiotics will be active against drug-resistant microbial pathogens (Ahmad and Beg, 2001). It is believed that compounds from natural plant origin will have less side effects compared to the synthetic drugs in current use (Al-Tohamy 2018).

The genus *Tulbaghia* is reported to have nutritive, ornamental and medicinal value (Aremu and van Staden, 2013). This monocotyledonous group of herbaceous perennial bulbs is predominantly found in Southern Africa. For centuries, several *Tulbaghia* species have found diverse uses in traditional medicine (Lyantagaye 2011; Kubec et al., 2013). In this study, an analysis of the phytochemicals and biological activities of extracts from eight *Tulbaghia* species was carried out.

1.1PROBLEM STATEMENT

The misuse and over-use of antibiotics has produced many resistant microbes. Infections such as tuberculosis and septicaemia, diseases of earlier centuries, are once again major causes of death (Shute 2014; Elisha et al., 2017). Resistance to antibiotics such as norfloxacin, ciprofloxacin and amoxicillin/clavulanic acid by *Pseudomonas aeruginosa* and enterohemorrhagic *Escherichia coli* are on the rise (Mwitari et al., 2013). Multidrug resistance has made the treatment of diseases such as tuberculosis and pneumonia a huge challenge (de Oliveira-Júnior et al., 2018). The most common multidrug resistant bacteria include Gram positive (MR *S. aureus*, vancomycin resistant *Enterococci*) and Gram negative bacteria (members of *Enterobacteriaceae* producing plasmid-mediated extended spectrum β-lactamase (ESβL)) and others such as *P. aeruginosa* and *Mycobacterium tuberculosis* (Mambe et al., 2016).

Invasive fungal infections have also emerged as an important cause of morbidity and mortality in immune-compromised patients. These individuals are at high risk for contracting many opportunistic fungal diseases including candidiasis, aspergillosis, cryptococcosis and pneumocytosis. Although several antifungal drugs have been licenced in recent years, antifungal resistance has also become a major concern when treating immunecompromised patients (Chakrabarti 2011).

Apart from microbial infections which are responsible for the high rate of mortality globally, cancer is one of the most life-threatening diseases which is characterised by proliferation of abnormal cells that invade and disrupt surrounding tissues. It constitutes serious public health problems in both developed and developing countries. The limited success of clinical therapies including radiation, chemotherapy, immunomodulation and surgery in the treatment of cancer, indicates that there is an urgent need for alternative strategies in cancer management (Tagne et al., 2014).

Chronic inflammation is linked to a wide range of progressive diseases, including cancer, obesity, aging, diabetes, neurological diseases, metabolic disorder, and cardiovascular diseases (Islam et al., 2013). Non-steroidal anti-inflammatory drugs (NSAIDs) are commonly prescribed for treatment of pain and inflammatory conditions. However, many NSAIDs are associated with side effects such as gastrointestinal bleeding and suppressed function of the immune system.

Therefore challenges faced in the treatment of infectious and degenerative disease have triggered considerable interest in the search for new, efficient and cost effective ways for the control of diseases. New organic molecules with antioxidant, antimicrobial, anticancer and immunomodulatory activities derived from plants could be cost effective, with little or no side effects and readily available to the local population as a means of improving primary health care (Adwan et al., 2010; Ayed et al., 2016).

1.2 MOTIVATION

Medicinal plant-derived compounds have gained widespread interest in the search of alternative therapeutics because of the perception that they are safe and have a long history of use in traditional medicine for the treatment of various diseases (Chavanova et al., 2013; Teanpaisan et al., 2016). The use of phytochemicals for pharmaceutical purposes has also gradually increased in recent years in many countries.

According to the World Health Organisation (WHO 2002), medicinal plants would be the best source to obtain a variety of drugs. About 80% of individuals from developing countries use traditional medicine, which has compounds derived from medicinal plants (Al- Tohamy et al., 2018). Plants produce a wide variety of secondary metabolites which are used either directly as precursors or as lead compounds in the pharmaceutical industry (Shute 2014).

The genus *Tulbaghia* has great potential in terms of its pharmacology and phytochemistry (van Wyk 2011). This genus has received little attention when compared to the closely related genus *Allium* (Lyantagaye 2011; Kubec et al., 2013). Significant phytochemical and biological investigations have been done on only two species *Tulbaghia violaceae and T. alliaceae* despite the presence of approximately 30 species in the genus (Aremu and van Staden, 2013). The presence of sulphur compounds seem to be typical for the genus. Among these compounds kaempferol and other sulphur compounds are the most important and have received much attention because of their anticancer potential.

Given the potential of medicinal plants, it is of great importance to investigate their antimicrobial, anticancer, immunomodulatory and toxicity profiles since a major part of the South African population still relies on them as a first line treatment (Akhalwayo et al., 2018). Therefore, establishing the efficacy and safety of these plant extracts would be beneficial. The potential of the genus *Tulbaghia* in treating various ailments needs verification. This study

aimed to investigate the phytochemical composition and bioactivities of eight *Tulbaghia* species.

1.3 AIM

To investigate the phytochemistry and biological activities of crude extracts from eight *Tulbaghia* species namely *T. acutiloba, T. alliacea, T. cernua, T. ludwigiana, T.leucantha, T. natalensis, T. simmleri* and *T. violacea*.

1.4 OBJECTIVES

The research objectives of this study were to:

1. Prepare plant crude extracts and perform preliminary photochemical analysis of eight species of *Tulbaghia* according to the standard methods of Harborne (1973).
2. Investigate the *in vitro* anti-oxidant activity of crude extracts of the eight plant species, using 2, 2 diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay and the 2, 2-azino-bis (3-ethyl-benzthioziline-6-sulfonic acid) (ABTS assay).
3. Determine the antimicrobial activities by microdilution assay and death kinetics of the eight crude plant extracts against Gram positive (*S. aureus and E. faecaelis*) and Gram negative bacteria (*K. pneumoniae and P. aeruginosa*), and the fungal species (*C.albicans and C. neoformas*).
4. Investigate anticancer activity of the most potent plant extracts by assessing the morphology of treated KB and HKesc-1 cells using acridine orange/ ethidium bromide staining, caspase activity and cell cycle arrest through flow cytometry.
5. Evaluate the immunomodulatory activity of the crude extracts by examining their effect on the production of NO and cytokines on LPS induced RAW264.7 cells through Griess reagent assay and Luminex assay, respectively.
6. Investigate the toxicity of some crude extracts using the 3-[4, 5 –Dimethylthiazol-zyl)-2, 5 diphenyltetreazolium bromide (MTT) cellular viability and LDH on RAW264.7 cells and zebrafish assay.

CHAPTER 2 LITERATURE REVIEW

2.1 MEDICINAL PLANTS

South Africa is home to more than 30,000 species of higher plants of which at least 3000 species could possibly be used medicinally (van Wyk and Prinsloo, 2018). The importance of using medicinal plants can be attributed to a number of reasons, including affordability, limited availability of Western medicine and the trust placed in herbal medicine due to the positive results obtained from its use (Maregesi et al., 2008).

Natural products, either as pure compounds, or standardised plant extracts, provide unlimited opportunities for new drug leads because of the unmatched availability of chemical diversity in the plant kingdom (Cos et al., 2006; Mgbeahuruike et al., 2017). According to Suleiman et al. (2010) natural products and related drugs are used to treat 87% of all categorised human diseases including bacterial infections, cancer and immunological disorders.

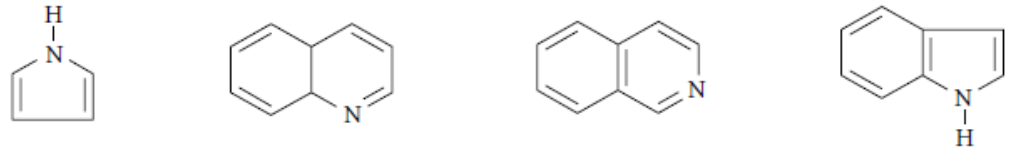
In South Africa as in other developing countries, a major portion of the population treats diseases by using plants with medicinal properties (Street and Prinsloo, 2013). Plants have an almost limitless ability to synthesise secondary chemical substances which play a pivotal role in their ecophysiology (Briskin 2000) including a defensive role against herbivores, pathogen attack, and an attractant role toward beneficial organisms such as pollinators or symbionts. Some of these secondary-derived compounds have beneficial effects in the treatment of microbial infections in animals and humans (Suleiman et al., 2010).

2.2 PHYTOCHEMICALS FROM MEDICINAL PLANTS

A brief outline of the pharmacological applications of some of these phytochemicals is as follows:

2.2.1 Alkaloids

These are the largest group of secondary chemical constituents made largely of ammonia compounds. Some examples are provided in Figure 2.1. Alkaloids have pharmacological applications such as anaesthetics (e.g. cocaine) and central nervous system stimulants (e.g. caffeine). Plant-derived alkaloids are used clinically as a muscle relaxant, as antibiotics and anticancer agents such as vincristine (Madziga et al., 2010).

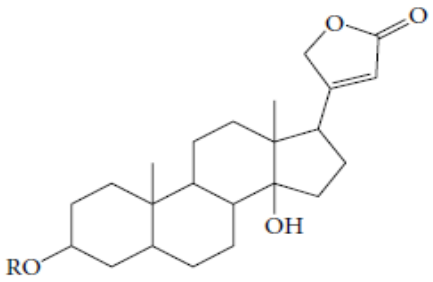


Pyrrole 11 Quinoline 12 Isoquinoline 13 Indole 14

Figure 2. 1 Typical basic structure of alkoloids (Achinolu and Umesiobi, 2015).

2.2.2 Glycosides

Glycosides, in general, are defined as the condensation products of sugars (including polysaccharides) with a host of different varieties of organic hydroxyl compounds (Kar 2007) Cardiac glycoside comprise of two main classes of compounds that differ in the structure of their aglycone (R) as shown in Figure 2.2.



Cardenolide

Figure 2. 2 Basic structure of cardiac glycosides (Calderon-Mantano et al., 2014).

Examples include cardiac glycosides (acts on the heart), anthracene glycosides (purgative, and for treatment of skin diseases) and chalcone glycoside which has anticancer activity (Firn 2010).

2.2.3 Flavonoids

Flavonoids are an important group of polyphenols widely distributed among the plant flora (Kar 2007). Chemically they are based upon fifteen-carbon skeleton consisting of two benzene rings (A and B), (Fig. 2.3) linked to a heterocyclic pyrane ring (C). They can be divided into a variety of classes such as flavones (flavone, apigenin and luteolin) and flavonols such as quercitin, kaempferol, myricetin and nariagenin (Kumar and Pandey, 2013).

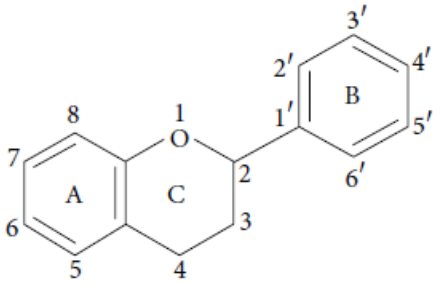


Figure 2. 3 Basic flavonoid structure (Kumar and Pandey, 2013).

Quercetin, kaempferol and quercitrin are common flavonoids present in nearly 70% of plants. Flavanols have several proven medicinal properties, such as anti-inflammatory, anti-oxidant, anti-allergic, antibacterial and antiviral effects (Wang et al., 2018).

2.2.4 Phenolics

Phenols are chemical components that occur in most plants as natural colour pigments responsible for colour. They are aromatic benzene ring compounds with one or more hydroxyl groups. Their most important role in plants may be defence against pathogens and herbivore predators (Subedi et al., 2014). Phenolics essentially represent a host of natural antioxidants with enormous ability to combat cancer and are also thought to prevent heart ailments to an appreciable degree and sometimes are anti-inflammatory agents (Cowan 1999).

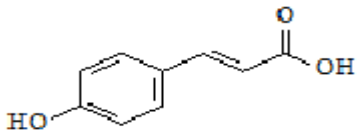


Figure 2.4 Structure of phenolic acids (Luthria et al., 2015).

2.2.5 Saponins

Saponins are a major family of secondary metabolites that occur in a wide range of plants. They are a complex and chemically varied group of compounds consisting of triterpenoid and steroidal glycosides (Fig. 2.5) linked to oligosaccharide moieties (Aremu and van Staden, 2013). The saponins in plants act as defense compounds against antifungal pathogens. In addition, many plant saponins have been isolated and present a broad spectrum of biological uses, such as anti-cancer, anti-inflammatory, ion channel blocking, immune stimulating and antifungal properties (Lanzotti et al., 2012; Wang et al., 2016).

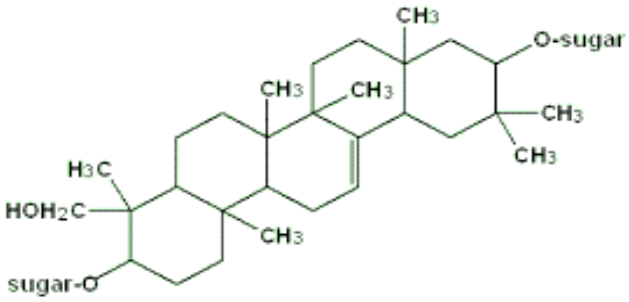


Figure 2.5 General structure of saponins (Moses et al., 2014).

2.2.6 Terpenoids

Terpernoids are the largest and most widespread class of secondary metabolites mainly in plants. Chemically all terpenoids can be considered to be derived from the basic branched C5 unit isoprene as shown in Figure 2.6 (Zhu et al., 2017). They have been found to be useful in the prevention and therapy of several diseases, including cancer. Terpenoids also possess antimicrobial, antifungal, antiparasitic, antiviral, antiallergenic, antispasmodic, anti-hyperglycemic, anti-inflammatory and immunomodulatory properties (Mbaveng et al., 2014).

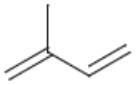


Figure 2.6 Basic stucture of terpenoids (de Las Heras and Bosca, 2003).

2.2.7 Steroids

Steroids are biologically active organic compounds with four rings arranged in a specific molecular configuration as shown in Figure 2.7.

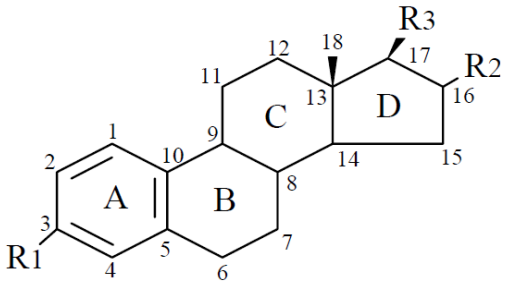


Figure 2.7 Basic structure of steroids (Gomes et al., 2015).

Steroids extracted from plants are known to have cardiotonic effects and also possess antibacterial and insecticidal properties (Sandjo and Kuete, 2013).

2.3 EXTRACTION OF PHYTOCHEMICALS

There are various phytochemical extraction techniques which include maceration, Soxhlet, and reflux extraction, ultrasound-assisted and microwave-assisted (Azmir et al., 2013).The maceration extraction is a solid–liquid extraction where the bioactive compound (solute) inside the plant material is extracted by soaking the plant material in a specific solvent for a period of time. The efficacy of maceration process is determined by two main factors, solubility and effective diffusion. The solubility is governed by basic rule of "like dissolves like" which indicated that polar compounds dissolve in polar solvents, and nonpolar compounds dissolve in nonpolar solvents (Castro-López et al., 2017). Various solvents have been used to extract different phytochemicals. Plant material is either used as fresh or dried material. The plant material is pulverised by mechanical grinders and extracted by soaking in water or organic solvent of choice. No complicated utensil and equipment are needed for the set-up of a maceration extraction system that has made it a popular choice for researchers (Cheok et al., 2014). The resulting extract is filtered, concentrated *in-vacuo* or by evaporation. This material can then be used to determine the presence of phytochemicals and analyses of bioactivities.

2.4 MECHANISM OF ACTION OF PHYTOCHEMICALS

There are different mechanisms of action of phytochemicals. They may inhibit microorganisms, interfere with some metabolic processes or may modulate gene expression and signal transduction pathways (Surh 2003). In general, the mechanism of action of phytochemicals is considered to be the disturbance of the cytoplasmic membrane, disrupting the proton motive force, electron flow, active transport, and coagulation of cell contents. Some specific biological activities are discussed below.

2.4.1 Antimicrobial activity

Medicinal plants are known to have phytochemicals that protect them against pathogenic insects, bacteria, fungi or protozoa (Elisha et al., 2017). Research studies have also shown that phytochemicals such as saponins, alkaloids, phenols and flavonoid exhibit antimicrobial activity against human pathogens (Boussoussa et al., 2016). Antimicrobial activity of many medicinal plant species has been investigated by various researchers with plants such as *Zanthoxylum chalbeum* exhibiting strong antimicrobial activity against bacteria associated with periondontal dieases (Ocheng et al., 2014), *Punica granatum* and *Indigofera daleoides* showed antimicrobial activity against bacteria known to cause diarrhoea (Mathabe et al., 2006), *Alcornea floribunda*, *Masanga cocropiodes*, *Tetracera potatoria* and *Xylopia aethiopica*

exhibited effectiveness against *mycobacteria* species thus supporting its traditional use for treating tuberculosis (Fomogne- fodjo et al., 2014).

Tulbaghia species have also shown great potential in inactivating a range of microorganisms affecting both humans and plants (Aremu and van Staden, 2013). Plant extracts may exhibit different modes of action against bacterial strains, such as interference with the phospholipids bilayer of the cell membrane which may result in a permeability increase and loss of cellular constituents (Kotzekidou et al., 2008).

2.4.2 Antioxidant activity

Antioxidants protect cells against the damaging effects of reactive oxygen species otherwise called free radicals such as singlet oxygen, super oxide, peroxyl radicals, hydroxyl radicals and peroxynite which results in oxidative stress leading to cellular damage (Mattson and Cheng, 2006). Natural antioxidants play a key role in maintaining health and the prevention of the chronic and degenerative diseases, such as atherosclerosis, carcinogenesis, neurodegenerative disorders and rheumatic disorder (Uddin et al., 2009; Jayasri et al., 2009).

Antioxidants exert their activity by scavenging the 'free-oxygen radicals' thereby giving rise to a fairly 'stable radical'. The free radicals are metastable chemical species, which tend to trap electrons from the molecules in the immediate surroundings. These radicals, if not scavenged effectively in time, may damage crucial bio molecules such as lipids, proteins including those present in all membranes, mitochondria and DNA resulting in abnormalities leading to disease conditions (Uddin et al., 2009).

The human body produces an insufficient amount of antioxidants which are essential for preventing oxidative stresses. Therefore this deficiency has to be compensated by making use of natural exogenous antioxidants, such as vitamin C, vitamin E, flavones, carotene and natural products from plants. Plants contain a wide variety of free radical scavenging molecules including phenols, flavonoids, vitamins, terpenoids that are rich in antioxidant activity (Anderson and Teuber, 2001; Cai et al., 2003). Zou et al. (2016) reported the presence of more than 170 antioxidants from citrus fruits including vitamins, minerals, phenolic compounds, terpenoids and pectin. The mechanism of action of these antioxidants has been suggested to be either through inhibition of oxidant enzymes, interaction with redox signalling pathways, direct reaction with reactive oxygen species (ROS) or a chelator with transitional metals (Adewusi et al., 2011).

2.4.3 Immunomodulatory activity

Inflammation is a normal biological process that occurs in response to tissue injury, microbial pathogen infection and chemical irritation (Islam et al., 2013; Dong et al., 2017). This biological process also involves the innate and adaptive immune systems. At a damaged site, inflammation is initiated by the migration of immune cells from blood vessels and the release of mediators, followed by recruitment of inflammatory cells and the release of reactive oxygen species (ROS), reactive nitrogen species (Stanković et al., 2016) and proinflammatory cytokines to eliminate foreign pathogens, resolving infection and repairing injured tissues (Min-Hsuing 2010). Thus, the main function of inflammation is beneficial for a host's defence system. In general, normal inflammation is rapid and self-limiting, but aberrant resolution and prolonged inflammation causes various chronic disorders. Chronic inflammation is associated with a wide range of progressive diseases, including cancer, neurological disease, metabolic disorder and cardiovascular disease (Phuong et al., 2014). Many studies suggest that the elimination of chronic inflammation is a major way to prevent various chronic diseases (Kim et al., 2016). On the other hand, immunosuppression caused by excess stress, drug-associated adverse effects, reductions in body temperature, and excess bleeding can lead to poor quality of life and increased susceptibility to infections and disease. Subsequent infections will further deteriorate the immune system, leading to aggravation of disease (Park et al., 2018).

Currently, nonsteroidal anti-inflammatory drugs (NSAIDs) are among the most widely used medication due to their efficacy in treating a wide range of pain and inflammatory conditions (Amessis- Ouchemoukh et al., 2014). However, the long-term administration of NSAID may induce gastro-intestinal ulcers, bleeding, and renal disorders due to their nonselective inhibition of both constitutive (COX-1) and inducible (COX-2) isoforms of the cyclooxygenases enzymes (Adebayo et al., 2015). Therefore, new immunomodulating and analgesic drugs lacking those side effects are being sought as alternatives to NSAIDs and opiates.

Medicinal plants are believed to be an important source of new chemical substances with potential therapeutic effects (Kaushik et al., 2012). A study by Kenny et al. (2013) showed that potato glycoalkoloids and potato peel extracts possess anti-inflammotory effects *in vitro* by inhibiting the production of the chronic inflammation cytokines interleukin -2 (IL- 2) and interleukin -8(IL-8). Another study by Dzoyem and Eloff (2015) on twelve South African medicinal plants used traditionally to alleviate pain and inflammation showed that all plants extracts had anti-inflammatory activity with *Burkea africana* showing high activity and high amounts of phenolic and flavonoid content. The plant extracts exhibited anti-15-lipoxygenase

activity and inhibited nitric oxide production. Therefore there is need to investigate plants with potential immunomodulatory activity which may be useful in the prevention of diseases associated with inflammation.

2.4.4 Anticancer activity

Cancer is a disease that severely affects the human population world-wide. There are many forms of cancer but they share similar characteristics such as insensitivity to signals which inhibit cell growth making their replication limitless. Apoptosis is evaded and never induced in cancer cells while angiogenesis is sustained within the tumour tissue allowing the survival of cancer cells. There is, therefore, a constant demand for new therapies to treat and prevent this life threatening disease (Greenwell and Rahman, 2015). Approximately, 60% of anticancer drugs are of natural origin with the plants being a significant source (Solowey et al., 2014). Examples of clinically useful antitumor agents derived from plants include paclitaxel, vincristine, and camptothecin. Many of these plant-derived anticancer agents have been discovered through large-scale screening programs (George et al., 2010). Anti- cancer agents act by inducing apoptosis and regulating other cancer characteristics (Yu et al., 2017).

2.5 BIOASSAY METHODS

2.5.1 Antimicrobial assays

Common methods used in the evaluation of the antibacterial and antifungal activities of plant extracts include the agar well diffusion assay, microtiter dilution assay and time kill studies. These methods are relatively easy to carry out under laboratory conditions.

2.5.1.1 Agar well diffusion assay

The agar well diffusion method is widely used to evaluate the antimicrobial activity of plants or microbial extracts (Balouiri et al., 2016). The inoculated agar is poured into the assay plate (9 cm in diameter), and allowed to cool down on a levelled surface. Once the medium has solidified, wells of 4 mm in diameter, are cut out of the agar using sterile 200 µL micropipette tips. The antimicrobial agent is placed into each well and the plates are incubated at 37°C for 24 hours. The diameters of the clear zone of inhibition are measured in mm using a ruler (Magaldi et al., 2003). The procedure is similar to the disc diffusion assay; the discs are supplemented with dilutions of the drug placed in wells which have been cut out in the agar. It is a cheap, simple and reliable method of antimicrobial drug susceptibility testing,

2.5.1.2. Microtiter dilution assay

The Minimum Inhibitory Concentration (MIC) assay is used to determine antimicrobial activity quantitatively based on the principle of contact of a test organism to a series of dilutions of test substance (van Vuuren 2008). In this method, susceptibility panels in 96-well microtiter plates containing various concentrations of antimicrobial agents are prepared. Then, standardized numbers of bacteria are inoculated into the wells of the microtiter plates and incubated overnight at 37°C. The MIC value is observed as the lowest concentration where no viability is observed in the wells after incubation. Compared with agar-based methods, broth microdilution can decrease much labour and time (Wikaningtyas and Sakandar, 2016).

2.5.1.3 Time kill studies

Assays for the rate of killing bacteria by crude drugs or extracts are carried out using a modified plating technique (Chen et al., 2016). Bacterial cells in the exponential growth phase are cultured and diluted to approximately 10^5 -10^6 CFU/mL. Solutions of test compounds with concentrations corresponding to 1/2 MIC, MIC and 2 MIC are added into the inoculum suspension. Mueller Hinton broth inoculated with each bacterial strain without the test compound acts as the control. The inoculum cultures are incubated at 37°C on an orbital shaker at 200 rpm. Samples are removed from each inoculum culture at different time points and ten-fold serially diluted in 0.9% normal saline and 100 µL sub-cultured from each dilution on Mueller Hinton agar. Viable counts are calculated in the units of CFU/mL and kill curves plotted with time (h) against logarithm of the viable count ($\log_{10}$ CFU). Bactericidal effects of a compound is taken as ≥ 3 $\log_{10}$ decrease in viable counts relative to initial inoculum (Chen et al., 2016).

2.5.2 Antioxidant assays

Numerous studies have been conducted in order to evaluate the antioxidant capability of certain compounds or plant materials. A wide variety of methods have been developed for the investigation of antioxidant activity including the 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH•) assay and 2, 2′-azino-bis (3- ethylbenzthiazoline-6-sulphonate) radical cation (ABTS•+) assay (Noipa et al., 2011).

2.5.2.1 DPPH

The DPPH method is one of the simplest assays and has commonly been used for assessing antioxidant activity of natural colorants (anthocyanins and carotenoids) and phenolic compounds (Cai et al., 2003). DPPH is a stable free radical which has an unpaired valence

electron at one atom of the Nitrogen Bridge. Scavenging of DPPH radical is the basis of the popular antioxidant assay (Sharma and Bhat 2009).

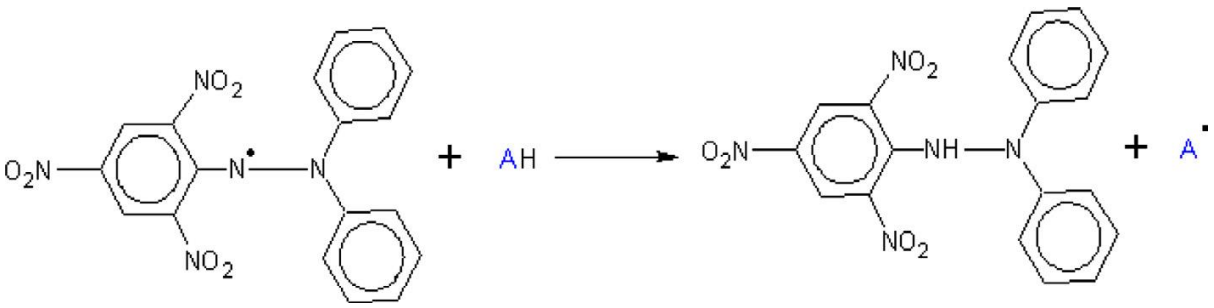


Figure 2.8 The reaction of DPPH free radical with antioxidant where AH is donor molecule and A· is free radical produced (Musa et al., 2016).

The DPPH alcohol solution is deep purple in colour with an absorption peak at 517 nm, which disappears with the presence of the radical scavenger in the reactive system and when the odd electron of the nitrogen in the DPPH are paired. The reactive rate and the ability of the radical scavenger depends on the rate and the peak value of disappearance of the DPPH. Compared with other methods, the DPPH assay has many advantages, such as good stability, credible sensitivity, simplicity and feasibility (Deng et al., 2011).

2.5.2.2. ABTS assay

The ABTS assay is a remarkable tool for determining the antioxidant activity of hydrogen-donating compounds and chain-breaking antioxidant (Huang et al., 2016).

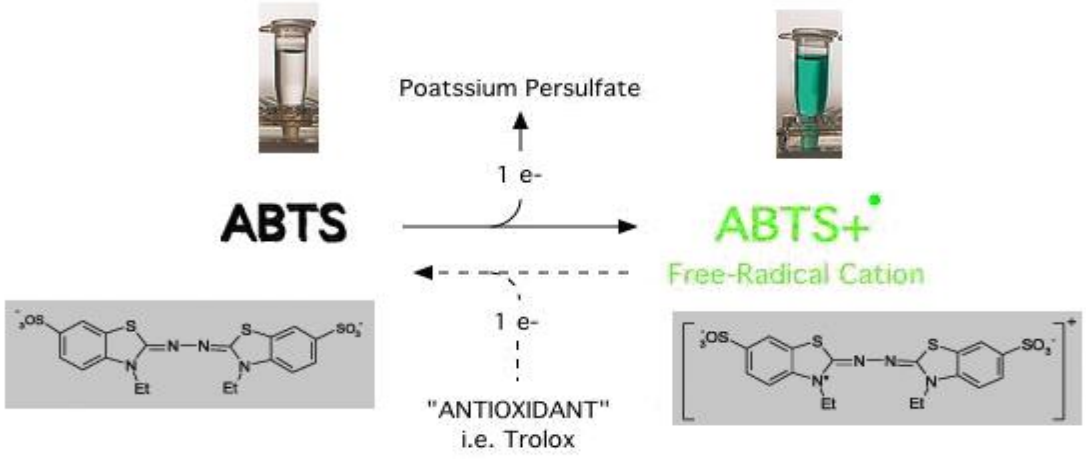


Figure 2.9 ABTS chemical reaction (Boligon et al., 2014).

The ABTS assay is based on the generation of a blue/green $ABTS^{-+}$ that can be reduced by antioxidants which is applicable to both hydrophilic and lipophilic antioxidant systems, (Floegel et al., 2011). The content of antioxidants in the sample is determined as a percentage decrease of colour intensity (% of inhibition) related to the negative control.

2.5.3 Immunomodulation Assays

2.5.3.1 Griess reagent assay

After pre-incubation of RAW 264.7 cells with LPS (1 µg/mL) and treatment with plant extracts for 24 h, the quantity of nitrite accumulated in the culture medium is measured as an indicator of NO production based on the Griess reaction. The quantity estimation of nitrite is based on a sodium nitrite standard calibration curve generated using serial dilutions of $NaNO_2$ in fresh culture medium (Moro et al., 2012).

2.5.3.2 Cytokine assays

Cytokines are bioactive proteins produced by many different cells of the immune system. Due to their role in different inflammatory disease states and maintaining homeostasis, there is enormous clinical interest in the quantitation of cytokines. The typical standard methods for quantitation of cytokines are immunoassay-based techniques including enzyme-linked immunosorbent assays (ELISA) and bead-based immunoassays read by either standard or modified flow cytometers.

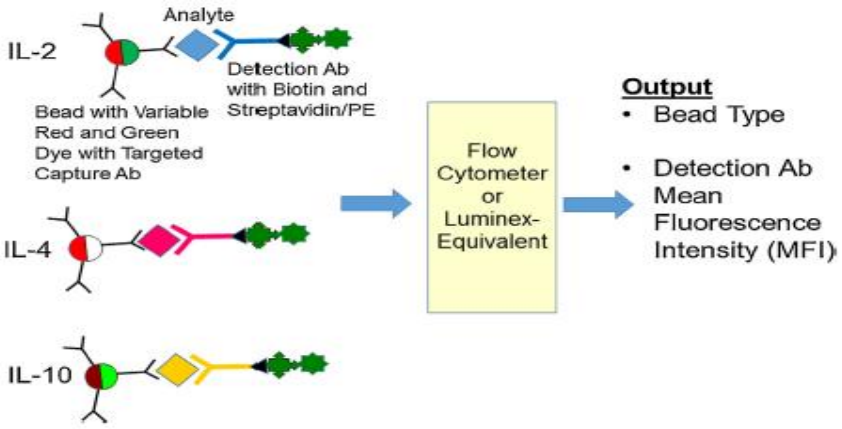


Figure 2.10 Overview of the bead-based immunoassays. Different color coded with dyes that fluoresce either red or green are used. The instrument measures the bead color intensity and the mean fluorescence intensity of the labelled detection antibody which is typically labelled with streptavidin/phycoerythrin (Skenken and Poschenrieder, 2015).

2.5.4 Anticancer Assays

Apoptosis is characterised by cell morphological changes, chromatin condensation, DNA cleavage and nuclear fragmentation. The most commonly used methods include morphological imaging (through light, fluorescent and electron microscopy), immunohistochemical (Caspase detection through flow cytometry), biochemical (DNA electrophoresis or flow cytometry based methods and immunological through Elisa assays (Ulukaya et al., 2011).

2.5.5 Toxicology studies

Although traditional plant medicine is considered generally safe and has been used for centuries, it cannot be taken for granted that these treatments are free of potentially toxic, mutagenic and/or carcinogenic properties (Taylor et al., 2001). For example, several studies have indicated that the use of plant extracts in high doses could lead to toxic injury to the kidneys and may interfere with renal tubular function and induce acute renal failure (Colson and De Broe 2005; Ijeh and Ukweni 2007). There is therefore a need to investigate safer concentrations of ethno-medicinal preparations.

2.5.5.1 *In vitro* toxicity evaluation

These methods are based on the extraction of active compounds from herbs and incubating different cell lines with these extracts at various concentrations. After exposure of the cells to the herbal extracts, cytotoxicity is assessed by different methods such as microscopic cell morphology evaluation, MTT (methyltetrazolium) assay (Chanda & Nagani, 2013) and lactate dehydrogenase activity (LDH) (Donmez et al., 2012). Such *in vitro* toxicological studies have the advantage of having relatively well controlled variables and they are widely accepted as the most effective safety and toxicity tests in pharmaceutical development **(**Lee et al., 2000).

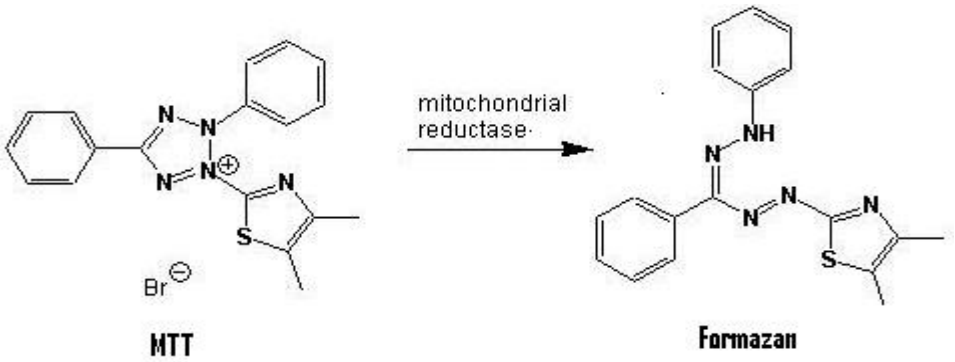


Figure 2.11 MTT (yellow) reduction in live cells by mitochondrial reductase results in the formation of soluble formazan (purple) which characterized by high absorptivity at 570 nm.

The MTT assay relies on the metabolic activity of a cell. It is used to assess how effectively the mitochondrial dehydrogenases of viable cells metabolically reduce pale yellow MTT salt (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) to insoluble purple formazan product [1-(4, 5-dimethylthiazol-2-yl)-3, 5 diphenylformazan] (Masson-Meyers et al., 2016). The production of formazan is directly proportional to the number of metabolically viable cells (Pozzolini et al., 2013; Stepanenko, 2015). Therefore absorption of dissolved formazan in the visible region correlates with the number of intact alive cells, since dead cells following toxic damage cannot metabolize MTT (Ulukaya 2008; Stockert 2012).

On the other hand, the LDH assay is centered on the activity of the intercellular enzyme lactate dehydrogenase. The enzyme lactate dehydrogenase is an exclusively intercellular enzyme. However, when cellular irritancy happens the cell membrane is compromised resulting in the leakage of cytosolic components including lactate dehydrogenase into the surrounding medium (Abe 2000; Lobner 2000; Fotakis 2006).

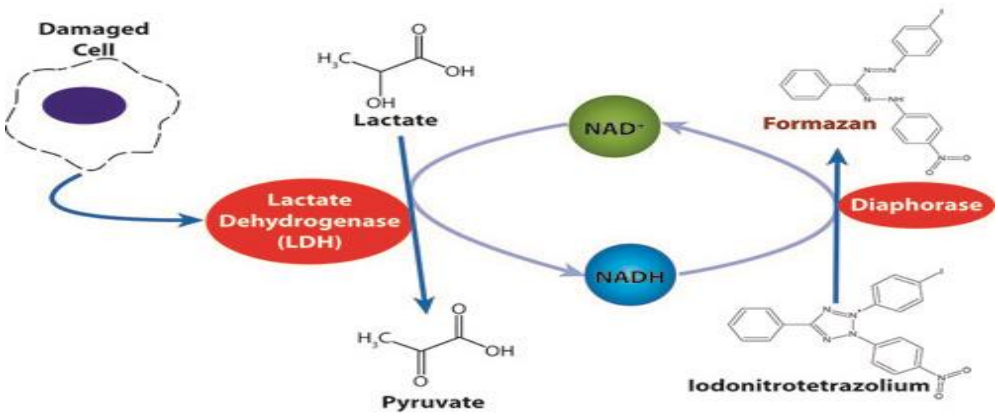


Figure 2.12 LDH assay quantitatively measures the stable, cytosolic, lactate dehydrogenase (LDH) enzyme, which is released from damaged cells.

This assay therefore detects the leaked lactate dehydrogenase and based on the amount of this enzyme in the surrounding environment, it is possible to deduce the level of cell membrane damage incurred by the cells. The released LDH is measured with a coupled enzymatic reaction that results in the conversion of a tetrazolium salt (iodonitrotetrazolium (INT)) into a red color

formazan by diaphorase. The resulting formazan absorbs maximally at 492 nm and can be measured quantitatively at 490 nm (Scherließ 2011).

2.5.5.2 *In vivo* toxicity evaluation

For all *in vivo* methods, samples to be tested are administered to the testing animals (rats, mice, etc.) at a definite dosage regimen, usually as described by the respective method. Animal models which use minimal number of animals being exposed to non-lethal doses are preferred (Ukelis et al., 2008). After a specified period of time, the tested animals are sacrificed and tissues or blood are used for the assay (Zou et al., 2016).

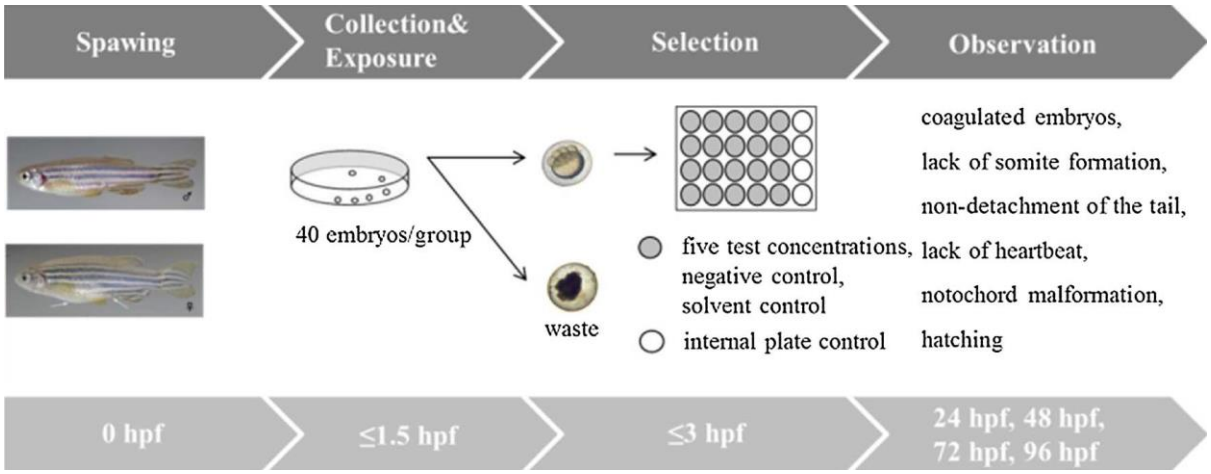


Figure 2.13 Scheme of the zebrafish acute toxicity test procedure (Yang et al., 2018).

Zebrafish embryo as an animal model is a very reliable and important tool due to its very rapid developmental processes, high fecundity, transparency, easy maintenance in the laboratory, accessible to experimental manipulation and similarity to the embryonic development of higher forms of vertebrates (Ramagosa et al., 2016). The use of zebrafish is, however, not only limited to toxicity screening (Ismail et al., 2017).

2.6 THE GENUS *TULBAGHIA*

2.6.1 Description of the *Tulbaghia* genus

Tulbaghia L. is one of the 30 genera in the family Amaryllidaceae with approximately 600 species. Taxonomically, the family is widely distributed in the Mediterranean, Europe, Asia, North and South America and South Africa (Benham 1993). *Tulbaghia* is commonly known as “wild garlic”, “sweet garlic” or “pink agapanthus” due to its close relationship to the genus *Agapanthus*. A prominent feature of the genus is the ‘garlic-like odour’ (alliaceous odour)

produced due to the cysteine-derived sulphur compounds released from wounded or decaying tissue and organs such as leaves and rhizomes (Kubec et al., 2013).

The genus *Tulbaghia* comprises approximately 63 species which are mostly rhizomatous plants with about 30 indigenous to the Southern Africa region (van Wyk and Gericke, 2000). For centuries members of the genus have found use in medicine, as ornamentals and in culinary practices. Researchers have highlighted the great potential of the genus *Tulbaghia* due to their nutritive, ornamental and medicinal value (van Wyk 2011). Some of the more important species are discussed below.

2.6.1.1 *Tulbaghia acutiloba*

Tulbaghia acutiloba has green recurved flowers with a fleshy orange to reddish brown ring on the stamens. The corona is half as long as the flowers and the tepals are often much reflexed. The leaves are up to 4-5 mm wide. The species is mostly summer growing and winter dormant. In very mild winters, the leaves will stay on the plant. It can flower several times a year. It takes 3 years to bloom this species from seed. *T. acutiloba* is found on dry rocky grassland in the Eastern Cape Province of South Africa (Archer and Victor 2005; Pooley 2015). *Tulbaghia acutiloba* leaves are used as culinary herb and snake repellent (Aremu and van Staden, 2013).

Figure 2.14 *T. acutiloba* (Pooley 2015 pg 511).

2.6.1.2 *T. alliacea*

Tulbaghia alliacea is a geophyte with rhizome up to 10 cm long. The leaves are 15-25 cm × 0.3-0.5 cm, strap shaped, smelling of onion when bruised. The flowers are borne in an umbellate cluster of 6-10 individuals on a scape 15-30 cm long. The perianth tube and segments are green; corona orange-brown; corona lobes fused into a 3-6 crenate fleshy collar, 4-8 mm long, on which the upper anthers are inserted.

Figure 2.15 *T. alliacea* (van Wyk et al., 2013 pg 299).

Tulbaghia alliacea is found in the Western and Eastern Cape Provinces, KwaZulu-Natal, Mpumalanga and Gauteng in clay or loam soils, in a variety of habitats (Archer and Victor 2005; van Wyk et al., 2013). *Tulbaghia alliacea* has the same common name as *T. violacea,* i.e., Wild garlic (English), Wildeknoffel (Afrikaans), Isihaqa (Zulu) and Moelela (Sotho). *Tulbaghia alliacea* has been reported to be used as a remedy for fevers, rheumatism and paralysis (Jäger and Stafford, 2012) in traditional medicine. Rhizome infusion is administered as anemas for fits in the Eastern Cape, (Aremu and van Staden, 2013).

2.6.1.3 *Tulbaghia cernua*

Tulbaghia cernua is a perennial that grows up to 30 cm. The leaves are strap-shaped and arranged like a fan. The brown flowers are unusual and the plant is highly aromatic. *T. cernua* is commonly found in the Gauteng, Limpopo and Northwest province (Archer and Victor, 2005). *Tulbaghia cernua* is used for ornamental purposes (Aremu and van Staden, 2013).

Figure 2.16 *T. cernua* flowers (Archer and Victor, 2015).

2.6.1.4 *Tulbaghia ludwigiana*

Tulbaghia ludwigiana has a branched rootstock, topped by a reduced corm-like structure. The flowering stems are 40-60 cm tall, rigid, bright green, distichously arranged. Flowers are in umbels of eight or nine, green with a yellow corona and about 1.2 cm long. It is found in Natal and Eastern Cape in grassland from sea level to 1400 m (Pooley 2005). *Tulbaghia ludwigia* is traditionally used as a love charm (Pooley 2005).

Figure 2.17 *T. ludwigiana* (Pooley 2015 pg 511).

2.6.1.5 *Tulbaghia leucantha*

Tulbaghia leucantha is about the only wild garlic that prefers a damp spot. They are quite common along seepage zones in the Drakensberg.

Figure 2.18 *Tulbaghia lecantha* (Pooley 2005 pg 93).

The Highland wild garlic is a perennial herb growing from small corms that arise from a rhizome. It is a very variable specie. Leaves are normally linear. Several green and white flowers occur on every inflorescence with conspicuous fleshy light red inner coronas. *Tulbaghia leucantha*, commonly known as fonteinknoffel in Afrikaans, smells of garlic, as do several of the other species of the genus (Archer and Victor, 2005). It is used as a culinary herb and a Protective charm (Pooley 2005).

2.6.1.6 *Tulbaghia natalensis*

Tulbaghia natalensis is normally found in rocky or marshy ground and can be up to 40 cm tall. The glaucous leaves are strap-shaped and upright. In spring it bears wonderfully fragrant flowers with creamy tepals and a greenish-yellow corolla in the centre which emits a spicy fragrance late afternoon (Archer and victor, 2005). *Tulbaghia natalensis*, also known as sweet garlic, is used as a culinary herb and snake repellent (Pooley 2005).

Figure 2.19 *T. natalensis* (Archer and Victor, 2005)

2.6.1.7 *Tulbaghia simmleri*

Tulbaghia simmleri is a clump-forming, evergreen perennial to 250 mm, with a bulb-like rootstock. Leaves are grey-green, flat and strap-like.

Figure 2.20 *T. simmleri* (van Wyk et al., 2013 pg 299).

The inflorescence is an umbel of pink to mauve-pink, occasionally white, strongly fragrant, tubular flowers. Each flower is made up of six, fleshy perianth segments, with a raised corona. In *T. simmleri* the corona is made up of 6 lobes which are united to form a tube for about one third of its length. *Tulbaghia simmleri* is endemic to a relatively small area in the northern Drakensberg of Mpumalanga and Limpopo, where it occurs as isolated plants on rocky ledges (Archer and 2005; van Wyk 2013). *Tulbaghia simmleri* is often used as a substitute for *Tulbaghia violacea (*van Wyk et al., 2002) when the latter is not available.

2.6.1.8 *T. violacea*

Tulbaghia violacea is a fast-growing, bulbous plant that reaches a height of 0.5 m. The leaves are long, narrow, strap-like, a bit fleshy and smell strongly of garlic when bruised. The pinkish-mauve tubular flowers, clustered into umbels of up to 20 flowers, are held above the leaves on a tall flower stalk, and appear over a long period in summer (January to April). The fruit, triangular capsules, are grouped into a head, and when ripe they split to release the flattened, hard black seeds. This drought-resistant plant stretches from the Eastern Cape, KwaZulu-Natal and Limpopo, to as far north as Zimbabwe (Archer and Victor, 2005; van Wyk et al., 2013).

Figure 2.21 *T. violacea* (van wyk et al., 2013 pg 299).

Tulbaghia violacea commonly known as wild garlic (English), Wildeknoffel (Afrikaans), Isihaqa (Zulu) and Moelela (Sotho) is used in traditional medicine for problems like fever, colds, asthma, tuberculosis, stomach-ache, and cancer of the oesophagus (Aremu and van Staden, 2013).

2.6.2 Bioactive components of *Tulbaghia*

There are many chemical constituents that have been identified in the family Amarylliadaceae. *Tulbaghia violacea* has been found to be rich in sulphur compounds that account for the characteristic odour and the medicinal properties of both the *Tulbaghia* and *Alluim* species (Lyantagaye 2011). The characteristic flavour of garlic (*Allium sativum*) and related alliums occurs when the enzyme Alliinase (EC.4.4.1.4) hydrolyses the S-alka (en)yl-L-Cys Sulfoxides (ACSOs) to form pyruvate, ammonia and sulphur containing volatiles. The enzyme *C-lyase* has been identified in the genus *Tulbaghia* and is associated with the formation of marasmicin (Lancanster et al., 2000; Kubec et al., 2002).

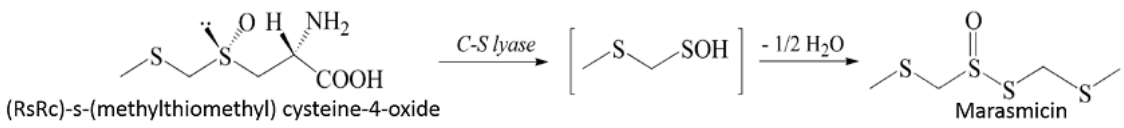


Figure 2.22 Formation of marasmicin in *Tulbaghia viloacea* Harv. (Kubec et al., 2002).

Many of these compounds have been shown to possess antimicrobial, antifungal and anticancer properties (Bungu et al., 2006; Jäger and Stafford, 2012) which supports the use of some *Tulbaghia* species in traditional medicine.

2.6.3 Antioxidant activity of *Tulbaghia*

There are several studies on the antioxidant activity of *T. violacea* (Aremu and van Staden, 2013). The antioxidant activities of *T. violacea* have been done mostly on the rhizomes and moderate activity was observed (Naidoo et al., 2008; Olorunnisola et al., 2012; Soyngibe et al., 2013). Given the potential antioxidant activity of the *Tulbaghia* there is need to further investigate different parts of the plant as well as unexplored *Tulbaghia* species.

2.6.4 Antimicrobial activity of *Tulbaghia*

Antibacterial activities of various extracts from *Tulbaghia violacea* have been tested against a wide range of microorganisms affecting both humans and plants. There is wide variation in the antimicrobial activity of *T. violacea* reported by different researchers. However, it has been established that crude extracts of *T. violacea* exhibits a broad spectrum antibacterial activity against organisms such as *Bacillus subtilis, Staphylococcus aureus* and *Escherichia coli* (Aremu and van Staden, 2013). *Tulbaghia violacea* and *T. alliacea* have also been shown to have antifungal activities (Lindsey and van Staden, 2004). The presence of antimicrobial activity in *T. violacea* makes it incumbent to examine the other species in the genus for similar properties.

2.6.5 Immunomodulatory activity of *Tulbaghia*

There are few studies that indicate the immunomodulatory potential of *Tulbaghia* species (Gaidamashvili and van Staden, 2006; Ncube et al., 2012). A study by Gaidamashvili and van Staden (2006) indicated low anti-inflammatory activity of lectin-like proteins from *T. violacea*. Further investigations of the immunomodulatory activities of the *Tulbaghia* species is necessary.

2.6.6 Anticancer activity of *Tulbaghia*

Tulbaghia is used in traditional medicine to treat oesophageal cancer (van Wyk and Gericke, 2000; Bungu et al., 2006; Aremu and van Staden, 2013). Oesophageal cancer is the ninth most common cancer in the world and the second most common cancer among South African men. It also has one of the lowest possibilities of cure, with the 5-year survival rate estimated to be only 10% overall (Skerman et al., 2011). Studies done so far indicate that *T. violacea* inhibits growth and induces apoptosis in cancer cells (Bungu et al., 2006; Thamburan et al., 2009;

Lyantagaye 2013; Saibu et al., 2015). However there are a limited number of studies done on the anticancer activity of the species. Therefore more studies are warranted to investigate the anticancer activity of the *Tulbaghia* species on oesophageal cancer.

2.6.7 Toxicity of *Tulbaghia*

The consumption of traditional medicine prepared from *T. violacea* has been associated with effects such as abdominal pain, gastro-enteritis, and cessation of gastro-intestinal peristalsis, contraction of the pupils and sloughing of the intestinal mucosa (Aremu and van Staden, 2013). Current studies show that *T. violacea* has a potential of being toxic (Resende et al., 2012; Olorunisola et al., 2013) whereas others reported no toxicity (Bamuamba et al., 2008; Soyngibe et al., 2013). There was a need for further investigations to clarify the safety and toxicity of species in the genus *Tulbaghia*.

CHAPTER 3 PHYTOCHEMICAL ANALYSIS AND ANTIOXIDANT ACTIVITY OF EIGHT *TULBAGHIA* SPECIES

ABSTRACT

Medicinal plants are known to produce a wide variety of bioactive compounds such as phenolics, vitamins and terpenoids which are known to have antioxidant properties. Antioxidants scavenge free radicals or reactive oxygen species and can be extremely important in inhibiting oxidative stress that leads to degenerative diseases. The aim of this study was to assess the phytochemicals and antioxidant activities of crude extracts from selected species in the genus *Tulbaghia*. Standard methods were used for preliminary phytochemical analysis. The total phenolic content of the plant extracts was determined using the folin ciocalteu method whereas the total flavonoids were determined by using the aluminium chloride colorimetric method. DPPH and ABTS assays were used to evaluate the antioxidant activity. Phytochemical screening of selected *Tulbaghia* species demonstrated the presence of flavonoids, glycosides, tannins, terpenoids, saponins and steroids. The amount of total phenol and flavonoid content varied in different plant extracts ranging from 4.50 to 11.10 miligrams gallic acid equivalent per gram (mg GAE/g) of fresh material and 3.04 to 9.65 milligrams quercetin equivalent per gram (mg QE/g) of fresh material respectively. The IC_{50} values based on DPPH (0.06 and 0.08 mg/mL) and ABTS (0.06 and 0.03 mg/mL) for *T. alliacea* and *T. violacea* respectively, were generally lower showing potential antioxidant activities. There is a need to isolate and examine individual compounds from *Tulbaghia* species for antioxidant activity as some compounds work best as single entities.

Keywords: *Tulbaghia* ssp., Phytochemicals, Antioxidant activities, Phenolic compounds.

3.1 INTRODUCTION

The human body has complex antioxidant defence systems that include the antioxidant enzymes: superoxide dismutase, glutathione peroxidase, catalase and non-enzymatic antioxidants such as glutathione, Vitamin E and C, thiol antioxidants, melatonin, carotenoids and other compounds (Subedi et al., 2014). Although almost all organisms possess antioxidant and repair systems to protect them against oxidative damage, these systems are insufficient to prevent the damage entirely (Magalhães et al., 2014; Ke et al., 2009) which therefore results in oxidative stress.

Oxidative stress caused by free radicals is related to the development of multiple diseases in which inflammation plays an important role, such as rheumatoid arthritis, asthma, chronic obstructive pulmonary disease, cardiovascular, neurodegenerative and autoimmune diseases, and some cancers (Cai et al., 2004; Deng et al., 2011; Huang et al., 2016). Hence, free radicals are important mediators that provoke or sustain inflammatory processes and, consequently, their neutralization by antioxidants can prevent inflammation. Currently available synthetic antioxidants, like butylated hydroxyl anisole (BHA), butylated hydroxyl toluene (BHT), tertiary butylated hydroquinone and gallic acid esters have been reported to cause several side effects (Adebayo et al. 2015).

Therefore, investigations are now focused on naturally occurring bioactive compounds from sources such as medicinal plants (Jimenez et al., 2005). Plant phytochemicals not only counteract free radical induced oxidative stress but also overcome the side effects of synthetic antioxidants (Subedi et al., 2014). Antioxidant compounds which are responsible for reducing cellular oxidative stress could be isolated and then can serve as leads for the development of novel drugs for the prevention and treatment of many human diseases. Medicinal plants contain a wide variety of phytochemicals, such as flavonoids, anthocyanins, cartenoids, dietary glutathionine, vitamins and endogenous metabolites and such natural products are rich in antioxidant activities (Atala et al., 2009).

These plant-derived antioxidants have been shown to function as singlet and triplet oxygen quenchers, peroxide decomposers, enzyme inhibitors and synergists (Choi et al., 2002).This study evaluates the phytochemical, total phenolics and antioxidant activities of selected *Tulbaghia* species. The genus *Tulbaghia* (family Amaryllidaceae) includes approximately 30 species of mostly rhizomatous plants endemic to southern Africa (Aremu and Van Staden, 2013). Their natural distribution covers southern Tanzania, Malawi, Botswana, Zimbabwe,

Mozambique, Lesotho and South Africa, with the majority of species growing in the Eastern Cape, southern KwaZulu-Natal, Gauteng, Limpopo and Mpumalanga provinces (Ranglová, et al., 2015 ; Zschocke and van Staden, 2000). The most popular member of the genus is *Tulbaghia violacea* which is known under a variety of common names, such as wild/sweet/society garlic, pink agapanthus, wilde knoffel (Afrikaans), itswele lomlambo (Xhosa), mothebe (Sotho) and isihaqa (Zulu) (Ranglová et al., 2015).

Tulbaghia plants have been widely used in traditional medicine for the treatment of numerous ailments. Uses include the treatment of fits, fevers, rheumatism, paralysis, headaches, high blood pressure, heart problems, chest complaints, stomach ailments and constipation/colic (Jäger and Stafford 2012 ; Ncube et al., 2012 ; Raji et al., 2012 ;Saibu et al., 2015). The plants are also extensively used to treat various microbial infections, including oral, ear and pulmonary infections (Ranglová et al., 2015; Moodley et al., 2014; Ncube et al., 2011).

3.2 MATERIALS AND METHODS

3.2.1 Plant material

The eight *Tulbaghia* plant samples (*T. acutiloba, T. alliacea, T. cernua, T.leucantha, T. ludwigiana, T. natalensis, T. simmleri and T. violacea*) were obtained from different indigenous plant nurseries in Gauteng, South Africa. Plants were kept in a greenhouse at Vaal University of Technology. The herbarium samples were authenticated by a botanist. Voucher specimens were deposited in the AP Goossens Herbarium, North West University, Potchefstroom, South Africa.

3.2.2 Preparation of plant powder material and acetone crude extracts

Fresh leaf material was freeze dried and then ground into a fine powder. The powdered material was stored in airtight bottles at 4°C till use. For crude extracts, ten grams of fresh leaves from eight samples were homogenized in 100 ml of absolute acetone in separate preparations. The homogenate from each plant was allowed to stand for 24 hours then filtered with No. 1 whatman filter paper. The acetone was evaporated under fume hood and the extract was kept at 4°C until use.

3.2.3 Phytochemical screening

Phytochemical constituents of *Tulbaghia* leaf extracts were determined according to the standard methods of Harbone (1973).

3.2.3.1 Alkaloids: Half a gram of the powdered leaf material was stirred in 5 mL of 1% aqueous hydrochloric acid on a water bath and filtered. The extract was divided into two portions, 1 mL

of the first portion was treated with a few drops of Mayer's reagent and the second portion was treated with Drangendorff's reagent. Turbidity or precipitation with either of those reagents was taken as preliminary evidence for the presence of alkaloids in the extract.

3.2.3.2 Tannins: Half a gram of powdered samples were boiled in 20 mL of water in a test tube and filtered. Few drops of 0.1% ferric chloride was added and observed for brownish green or a blue black coloration as indication of tannins.

3.2.3.3 Phlobatanins: Half gram of powdered samples were extracted in 10 mL of distilled water. 4 ml of the filtered aqueous extract was boiled with 1% aqueous hydrochloric acid and observed for deposition of red precipitate as indication of phlobatannins.

3.2.3.4 Saponins: Approximately 2 g of powdered samples were boiled in 20 mL of distilled water in a water bath and filtered. 10 mL of the filtrate was mixed with 5 mL of distilled water and shaken vigorously and observed for a stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken vigorously again and then observed for the formation of emulsion as indication of saponin.

3.2.3.5 Steroids: Two millilitres of acetic anhydride was added to 0.5 g of acetone extracts with 2 ml of suphuric acid. The colour change from violet to blue or green was an indication of steroids.

3.2.3.6 Terpenoids (Salkowski test): Five milliliter of extracts were mixed with 2 mL chloroform and 3 mL sulphuric acid was carefully added to form a layer. A reddish brown colouration of the interface was taken as an indication of terpenoids.

3.2.3.7 Cardiac glycosides (Keller-Killani test): Five milliliter of the extract was treated with 2 mL of glacial acetic acid containing one drop of ferric chloride solution. This was underlayed with 1 mL of concentrated H_2SO_4. A brown ring of the interface indicated presence of glycosides.

3.2.3.8 Flavonoids: One gram of the powdered material was heated with 10 ml of ethyl acetate in the water bath for 3 min. The mixture was filtered and 4 ml of the filtrate was shaken with 1 mL of dilute ammonia solution. Development of yellow/green coloration was taken as indication of flavonoids.

3.2.4 Quantitative phytochemical screening

3.2.4.1 Total phenol content

The total phenolic content of the extracts was determined by using the Folin ciocalteau reagent method (McDonald et al., 2001). Dilute acetone plant extracts (0.5 mL of 1mg mL^{-1}) or gallic acid (standard) were mixed with Folin Ciocalteau reagent (5 mL, 1:10 diluted with distilled water) and aqueous sodium carbonate (4 mL, 1M). The mixture was allowed to stand for 15 minutes and total phenols were determined spectrophotometrically at a wavelength of 760 nm. The standard curve was prepared by using 0, 50, 100, 150, 200, 250 mg L^{-1} of gallic acid in methanol: water (50:50, v/v). The total phenol values were expressed in terms of gallic acid equivalent (mg g^{-1} extracted compound) (Paumorad et al., 2006).

3.2.4.2 Total flavanoid content

The aluminium chloride colorimetric method (Chang et al., 2001) was used to determine the flavonoid content. Plant extracts (0.5 mL, 0.1 mg/mL) were diluted with acetone to 2 mL and then mixed with 0.5 mL of aluminium chloride (1.2 %) and potassium acetate (120 mM). The mixture was allowed to stand for 30 minutes at room temperature and then the absorbance was measured at 415 nm. Quercetin was used as a standard. Flavanoid content is expressed in terms of quercetin equivalent (mg g^{-1} of extracted compound) (Kallel et al., 2014).

3.2.5 Antioxidant activity

3.2.5.1 Analysis of extracts by TLC

Four microliters of 100 mg/mL plant extracts were loaded individually on the baseline of two thin layer chromatography plates (Fluka, silica gel F254 plates). Benzene: ethanol: ammonium hydroxide (BEA) (36: 4: 0.4), was used as the separating solvent. One TLC plate was sprayed with freshly prepared vanillin spray reagent (0.1 g vanillin, 28 mL methanol, and 1mL sulphuric acid) to visualise separated compounds and was heated at 110°C for optimal colour development. Anti-oxidant activities were determined by spraying the second developed TLC plate, with a solution of DPPH (1, 1-diphenyl-2-picrylhydrazyl) spray reagent. The plate was allowed to stand for approximately 5 minutes and the colour change was noted for compounds with the ability to scavenge the radical, reducing the DPPH, resulting in a colour change from a deep purple to a yellow-white color.

3.2.5.2 DPPH assay

A 1 mL aliquot of a suspension of the extracts (from 0.01 mg/mL to 0.5 mg/mL) were mixed with 1 mL of 0.12 mM DPPH solution. After shaking, the mixture was incubated at ambient temperature in the dark for 30 minutes, following which the absorbance was measured at 517

nm using a UV-160A spectrometer. Acetone was used as negative control while L-ascorbic acid was taken as a positive control. Radical scavenging activity was expressed as percentage inhibition and calculated using the formula:

$$\% \text{ scavenging activity} = [(A_{control} - A_{test})/A_{control}] \times 100$$

where $A_{control}$ is the absorbance of the control (DPPH solution without test sample) and A_{test} is the absorbance of the test sample (DPPH solution plus antioxidant). IC_{50} value denotes the concentration of sample which is required to scavenge 50% of DPPH free radicals.

3.2.5.3 ABTS assay

Serial dilutions of plant extracts were prepared, ranging from 0.01 mg/mL to 0.5 mg/mL. L-ascorbic acid was used as a positive control while acetone was used as a negative control. A 7 mM stock solution of ABTS was prepared in double distilled water. The ABTS radical cation was then prepared by addition of 88 µL of 140 mM potassium persulfate ($K_2S_2O_8$) to 5 mL of ABTS. This solution was stored in the dark for 12-16 hours in order to stabilize it before use. The concentrated $ABTS^+$ solution was diluted with cold ethanol shortly before conducting the assay to a final absorbance of 0.70 ± 0.02 at 734 nm at 37 °C, in a cuvette. The total scavenging capacity of the extracts was quantified through the addition of 1000 µL $ABTS^+$ to 50 µL of plant extract. The solutions were heated on a heating block to 37°C for 4 minutes, after which the absorbance was read at 734 nm on a spectrophotometer (Re et al., 1999). All assays were done in triplicates.

3.2.5.4. Statistical analysis

All experiments were performed in triplicates. The data were analyzed using Microsoft Excel and expressed as mean ± standard deviation (n=3). IC_{50} was calculated using the software program Graphpad Prism version 7.0. Differences were considered to be significant when *p*-values were below 0.05 ($p < 0.05$).

3.3. RESULTS

3.3.1 Preliminary phytochemical analysis

Preliminary phytochemical analysis was done on eight selected *Tulbaghia* species and the results showed the presence of flavonoids, glycosides, tannins, terpenoids, saponins and steroids (Table 3.1).

Table 3. 1 Preliminary phytochemical analysis of eight *Tulbaghia* species

	Plant species							
	T. acutiloba	*T. alliacea*	*T. cernua*	*T. leucantha*	*T. natalensis*	*T. ludwigiana*	*T. simmleri*	*T. violacea*
Alkaloids	--	--	--	--	--	--	--	--
Flavonoids	++	++	++	++	++	++	++	++
Glycosides	++	++	++	++	++	++	++	++
Phlobatanins	--	--	--	--	--	--	--	--
Tannins	++	++	++	++	++	++	++	++
Terpenoids	++	++	++	++	++	++	++	++
Saponins	++	++	++	++	++	++	++	++
Steroids	++	++	++	++	++	++	++	++

*++ present; – absent

3.3.2 Total phenolic and flavonoid content

The amount of total phenolic acid content varied in different plant extracts, ranging between 4.50 and 11.10 mg GAE/g of fresh material. There was no significant difference ($p > 0.05$) in the total phenolic compounds between all extracts except *T. violacea*. *T. violacea* exhibited a significantly higher ($p < 0.05$) total phenolic content compared to all other extracts (Table 3. 2). On the other hand, the total flavonoid content of the plant extracts ranged between 3.05 and 9.65 mg QE/g of fresh material. Total flavonoids exhibited significant differences ($p < 0.05$) among all extracts except between *T. simmleri* and *T. natalensis*. *T. violacea* had the highest total flavonoid content at 9.65 mg QE/ g of fresh material.

Table 3.2 Total phenol and flavonoid content of selected *Tulbaghia* species.

Sample	Total phenolic content (mg GAE/g fresh wt of Sample)	Total flavonoid content (mg QE/ g fresh weight of sample)
T. acutiloba	5.12 ± 1.96^{a}	4.56 ± 0.79^{c}
T. alliacea	9.21 ± 2.88^{a}	7.99 ± 0.41^{d}
T. cernua	4.50 ± 1.44^{a}	3.04 ± 0.15^{e}
T. leucantha	6.70 ± 1.63^{a}	6.56 ± 0.34^{f}
T. ludwigiana	7.64 ± 0.94^{a}	7.14 ± 0.23^{g}
T. natalensis	7.01 ± 1.44^{a}	6.22 ± 0.43^{h}
T. simmleri	6.70 ± 0.94^{a}	6.06 ± 0.58^{h}
T. violacea	11.10 ± 1.44^{b}	9.65 ± 1.32^{i}

Results are presented in fresh weight as means ± SD, n=3. Means within a column with identical superscripts do not differ significantly ($p \geq 0.05$) while means with different superscripts show a significant difference ($p < 0.05$).

3.3.3 TLC of phytochemicals

For the qualitative detection of the antioxidant capacity of the plant extracts, TLC plates spotted with plant extracts were developed in BEA. Figure 3.1A shows the extracted compounds. The TLC plate was sprayed with DPPH in methanol (Fig. 3.1B) to enable visualisation of separated spots exhibiting antioxidant activities (Glod et al., 2015).

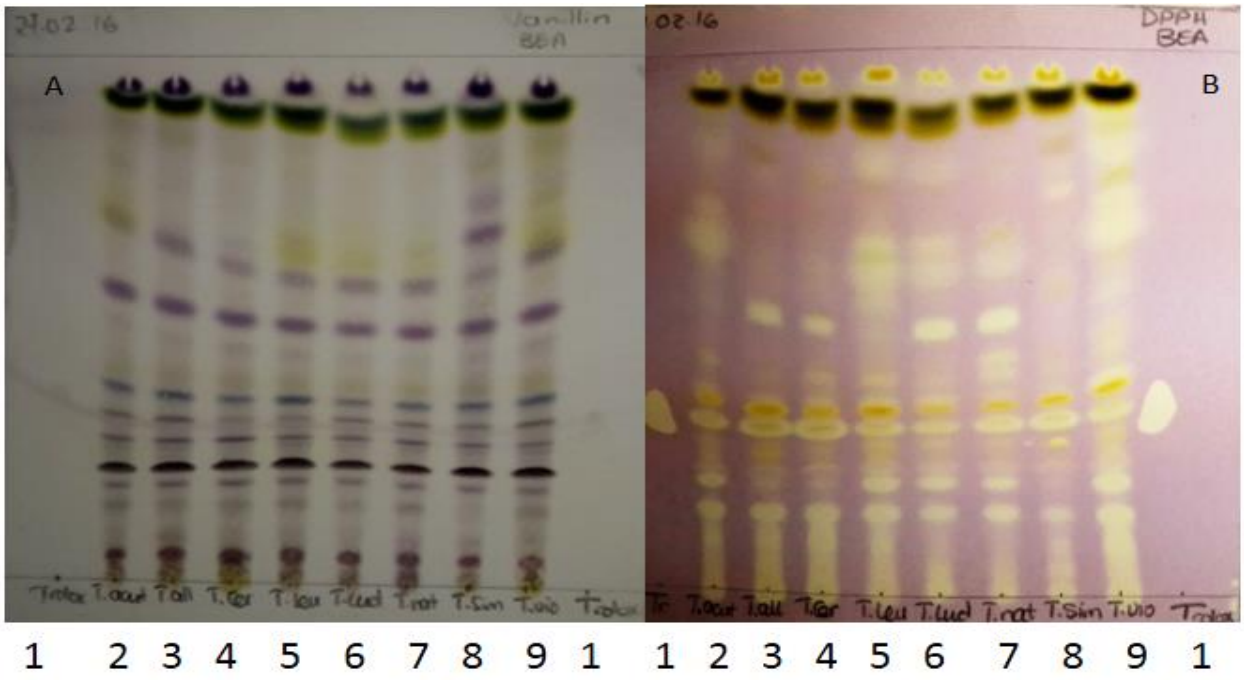


Figure 3.1 Chromatograms of crude extracts sprayed with vanillin/sulphuric acid reagent (A) 1.Trolox, 2. *T. acutiloba,* 3. *T. alliacea,* 4. *T. cernua,* 5. *T. leucantha,* 6. *T. ludwigiana,* 7. *T. natalensis,* 8. *T. simmleri* 9. *T. violacea* to show compounds extracted with acetone and 0.2 % DPPH (B) to indicate extracts with antioxidant activity.

All the samples showed numerous migrated spots having different intensities (Figure 3.1B), indicating the potential of radical scavenging capacity of the extracts.

3.3.4 DPPH assay and ABTS assay

In vitro antioxidant activity of the crude extracts was examined using DPPH and ABTS assays. The degree of colour change in the assays is proportional to the concentration and potency of the antioxidants. A large decrease in the absorbance of the reaction mixture indicates significant free radical scavenging activity of the compound under test (Saeed et al., 2012). Figure 3.2 and 3.3 illustrate the scavenging effects of samples on DPPH and ABTS radicals.

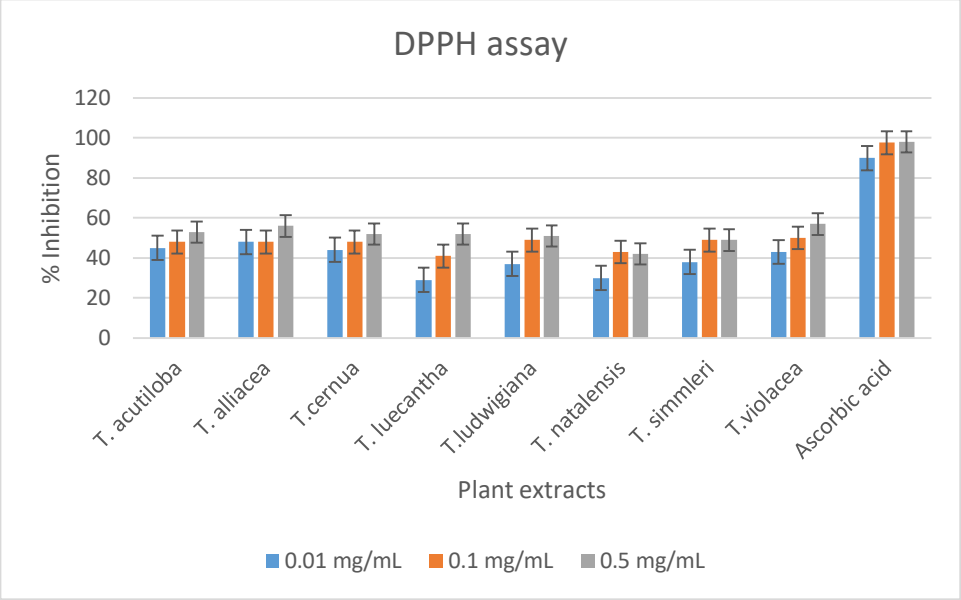


Figure 3.2 Percentage scavenging activity of acetone extracts. Values are expressed as mean ± standard deviation (n=3). Ascorbic acid was used as a standard.

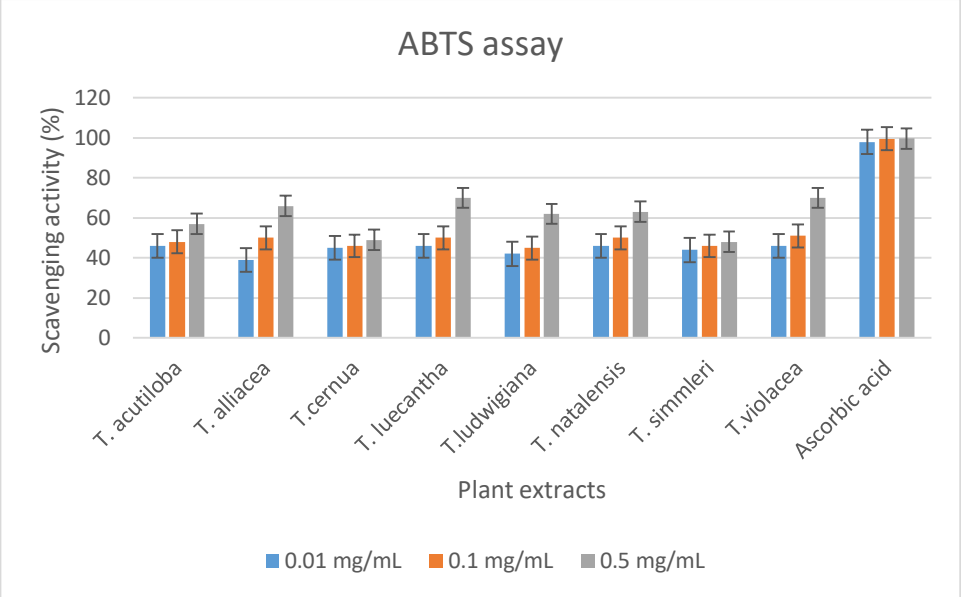


Figure 3.3 ABTS scavenging activity of acetone extracts of selected *Tulbaghia* species.

Values are expressed as mean ± standard deviation (n=3). Ascorbic acid was used as a standard.

Results of this study suggest that the plant extracts contain phytochemical constituents that are capable of donating hydrogen to a free radical to scavenge the potential damage. *T. violacea* showed a higher scavenging activity for both DPPH and ABTS from 0.01 mg/mL (43%) to 0.5 mg/mL (57%) and 0.01 mg/mL (46%) to 0.5 mg/mL (70%) respectively compared to the other extracts.

Table 3. 3 IC_{50} values of the acetone crude extracts of *Tulbaghia* species.

Samples	**DPPH** (mg /mL)		**ABTS** (mg/mL)	
	IC 50	1/IC 50	IC 50	1/IC50
T. acutiloba	0.16	6.25	0.07	14.29
T. alliacea	0.06	16.67	0.06	16.67
T. cernua	0.21	4.76	2.34	0.43
T. leucantha	0.39	2.56	0.03	33.33
T. ludwigiana	0.26	3.85	0.09	11.11
T. natalensis	2.70	0.37	0.04	25.00
T. simmleri	0.39	2.56	4.06	0.25
T. violacea	0.08	12.5	0.03	33.33
Ascorbicacid (µg/mL)	0.0029	344	0.0009	1111

IC_{50} values were determined by using the Graphpad Prism 7 software. IC_{50} is defined as the efficient concentration of antioxidant necessary to decrease the initial DPPH radical concentration by 50% (Table 3). The lowest IC_{50} indicates the strongest ability of the extracts to act as DPPH radicals scavengers. Out of the all extracts, *T. alliacea* and *T. violacea* showed the lowest IC_{50} at 0.06 and 0.08 mg/mL respectively for DPPH assay, whereas for ABTS assay T. *leucantha* and *T. violacea* showed the lowest IC_{50} values both at 0.03 mg/mL (Table 3.3). Ascorbic acid showed highest DPPH radicals scavenging with IC_{50} of 0.0009 mg/mL. Therefore *T. alliacea and T. violacea* exhibited the best antioxidant activity compared to other extracts.

3.4 DISCUSSION AND CONCLUSION

This study investigated the presence of phytochemicals and antioxidant activity of crude extracts from eight species in the genus *Tulbaghia*. Phytochemical analysis revealed the presence of flavonoids, glycosides, tannins, terpenoids, saponins and steroids (Table 3.1). The presence of these phytochemicals support the utilisation of some of the *Tulbaghia* species in various parts of South Africa such as Eastern Cape, KwaZulu Natal and Limpopo where they are used to prepare traditional medications for treatment of various ailments (Bungu et al., 2006; Street et al., 2008; Lanzotti et al., 2012; Jäger and Stafford 2012; Saxena et al., 2013; Abioye et al., 2013; Wintola and Afoloyan, 2015; Alhassan et al., 2016). These compounds are biologically active and may contribute to the antioxidant activities of the *Tulbaghia* species. Presence of flavonoids and terpenoids indicates the potential of the plant extracts to have antioxidant activities. Flavonoids are known to exert antioxidant activity through scavenging or chelating process (Suhartono et al., 2012; Abioye et al., 2013). Scavenging reactive oxygen species can counteract lipid oxidation *in vitro* and improve the body's antioxidant enzyme activity, and decrease peroxide formation *in vivo* (Zou et al., 2016). Tepernoids have been shown to act as primary antioxidants by donating hydrogen to radicals thereby slowing down lipid oxidations (Grassmann 2005). The presence of a phytochemicals of interest (Flavanoid and Terpenoid- antioxidants) may lead their further isolation, purification and characterization. Then they can be used as the basis for new pharmaceutical products.

Phenolic compounds constitute one of the major groups of compounds known to act as primary antioxidants (Fawole et al., 2009). Phenolics are composed of one or more aromatic rings bearing one or more hydroxyl groups and are potentially able to quench free radicals by forming stabilized phenoxyl radicals (Lu et al., 2014). Flavonoids are especially important antioxidants due to their high redox potential, which allows them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers. In addition, they have a metal chelating potential. Typically, flavonoids help to protect plants against UV light, fungal parasites, herbivores, pathogens and oxidative cell injury. When consumed regularly by humans, flavonoids have been associated with a reduction in the incidence of diseases such as cancer and heart disease (Ignat et al., 2010).

Phenolic compounds have thus strong antioxidant activities associated with their abilities to scavenge free radicals, donate hydrogen, chelate metals, break radical chain reactions, and quench singlet oxygen *in vitro* and *in vivo* (Zhang et al., 2013). As their free radical scavenging ability is facilitated by their hydroxyl groups, the total phenolic concentration could be used as

a basis for rapid screening of antioxidant activity. Many studies have reported that phenolic compounds possess other biological activities such as anti-inflammatory, antiulcer, antispasmodic, antiviral, antidiarrheal, and anti-tumoral properties, among others (López-Cobo et al., 2015; Torres Carro et al., 2016). Scavenging of reactive oxygen species (ROS) by plant phenolics may be the basis of the purported human health benefits of plants (Alarco´n et al., 2008). Thus, quantification and subsequently identification of phenolic compounds can provide vital information related to antioxidant function and potential health benefits of *Tulbaghia* ssp. Antioxidant activity denotes the ability of a bioactive compound to maintain cell structure and function by effectively clearing free radicals, inhibiting lipid peroxidation reactions, and preventing other oxidative damage (Atala et al., 2009). It is also a foundation of many other biological functions, such as anti-cancers, anti-inflammation and anti-aging. More importantly, the prevention of many chronic diseases such as cancer, diabetes and cardiovascular disease, has been suggested to be associated with the antioxidant activity. Therefore, studies of natural antioxidants, such as those from medicinal plants, is of great importance to human health (Zou et al., 2016).

DPPH is a free radical stable at room temperature, which produces a violet solution in methanol. When the free radical reacts with an antioxidant, its free radical property is lost due to chain breakage and its colour changes to light yellow. The extracts that produced yellow spots in the purple background were considered to have antioxidant properties (Fig 3.1B). The appearance of yellow-white colour is typically based on the inhibition of the accumulation of oxidised products, since the generation of free radicals is inhibited by the presence of antioxidants (Choi et al., 2002; Chandra and Arora, 2009).

Antioxidant activity of the plant extracts are often associated with the phenolic compounds present in them. The high amounts of total phenol and total flavonoid (Table 3.2) of *T. violacea* supports its higher antioxidant activity in both DPPH and ABTS assays (Fig 3.2 & 3.3). Plant phenols constitute the major group of compounds that act as primary antioxidant. They can react with active oxygen radicals, such as hydroxyl radicals, superoxide anion radicals and lipid peroxyl radicals and inhibit the lipid peroxidation at an early stage. This is because of their scavenging ability due to their hydroxyl groups.

Although the Figures 3.2 and 3.3 illustrate a dose dependant antioxidant activity, it is clear that the plant extracts exhibited low radical scavenging capacity compared to the control ascorbic acid. These findings correlate with results obtained by Soyngibe et al 2013, in the study of

essential oil of *T. violacea*. Soyingbe et al., (2013) concluded that *T. violacea's* oil may not necessarily scavenge pre-existing free radical, but does show the potential to prevent the generation of free radicals through Fe^{2+} chelating.

Given the many known medicinal properties of *Tulbaghia* plants, results from the present work that show relatively low values of antioxidant do not imply low medicinal value. Emerging trends in antioxidant research point to the fact that low levels of phenolics (and other phytochemicals) and low values of antioxidant indices in plants do not translate to poor medicinal properties (Doss et al., 2008). This may be due to the fact that some plant-derived compounds are effective as single entities (Saeed et al., 2012; Mokgotho et al., 2013) and crude extracts were used in this study.

Bioactivites of *Tulbaghia* species may be attributed to the phytocompounds observed to be present in the extracts in this study. Total polyphenolic and flavonoid content in the extracts indicate that *Tulbaghia* species could be a source of antioxidants. The extracts from the eight *Tulbaghia* species tested exhibited potential antioxidant activity and are capable of scavenging reactive oxygen species. *In vitro* antioxidant activity exhibited by the plant extracts indicate that they may be utilised to prevent oxidative stress and related disorders. However, further investigations need to be undertaken to isolate and identify compounds responsible for the antioxidant activity. Antioxidant activity of the pure compounds can then be validated *in vivo* prior to clinical use.

CHAPTER 4 ANTIMICROBIAL ACTIVITY OF EIGHT SPECIES IN THE GENUS *TULBAGHIA*

ABSTRACT

The genus *Tulbaghia* has been used in traditional medicine to treat various ailments such as fever, earache and tuberculosis. However, only a few species in this genus have been investigated for their antimicrobial activity. The increasing prevalence of antibiotic resistant pathogens, world-wide, has necessitated a continuous search for alternative treatments from sources like plants as antibacterial and antifungal agents. The aim of this study was to evaluate the antimicrobial activity of aqueous and acetone crude extracts from eight species in the genus *Tulbaghia*. The antimicrobial activity was assessed by agar well diffusion, microtiter dilution and time kill assays. The acetone extracts of *T. acutiloba, T. alliacea, T. leucantha, T. ludwigiana, T. natalensis* and *T. simmleri* showed moderate antimicrobial activity against all test organisms while the water extracts showed moderate to no activity. One species, *T. cernua*, showed poor activity against all the tested microbes. The acetone and water extracts of *T. violacea* showed the greatest antibacterial and antifungal activity against all the tested microorganism with minimum inhibitory concentration ranging from 0.1 mg/mL to 3.13 mg/mL. The acetone extracts of *T. violacea* also exhibited both bacteriostatic/fungistatic and bactericidal/fungicidal activity depending on the incubation time and concentration of the extract. The bactericidal/fungicidal activity was observed at x2 MIC. The results from this study support the use of *T. violacea* in treating bacterial and fungal infections in traditional medicine.

Keywords: Antimicrobial, Bacteriostatic, Fungicidal, Time kill assay, *Tulbaghia*.

4.1INTRODUCTION

In many countries, a large percentage of the population continues to use medicinal plants to treat different ailments including diseases of the respiratory, gastrointestinal, urinary and nervous systems (Yasunaka et al., 2005; Sharma et al., 2017). Many plants have been screened for their antibacterial activity because of their great medicinal relevance in the recent years. Infections have increased to a great extent and resistance against antibiotics has become a major concern world-wide (Fomogne-Fodjo et al., 2014; Kusuma et al., 2014; Mambe et al., 2016; Moussaoui and Alaoui, 2016).

Most of the infectious microorganisms have developed resistance to a large number of commercial antibiotics (Chah et al., 2006; Aswathanarayan and Vittal, 2013; Vijaya Lakshmi et al., 2013; Christensen et al., 2015). This coupled with the dangerous side effects of some commercial antibiotics, has motivated scientists to search for alternative therapies from other sources such as medicinal plants (Abraham and Thomas, 2012; Stanković et al., 2016; Mambe et al., 2016).

The World Health Organization (WHO) report on antimicrobial resistance indicates that overcoming the antibiotic resistance will be a major issue in the next millennium (WHO 2014). Screening of plants for antimicrobial agents has thus gained much importance because WHO is encouraging the development and utilization of medicinal plant resources in the traditional system of medicine (Ocheng et al., 2014; Omwenga et al., 2015). The presence of antibacterial, antifungal and other biological activities in extracts of different plant species used in traditional medicine practices has provided a source of inspiration for the search of novel drug compounds (Wikaningtyas and Sukandar, 2016; Mambe et al., 2016).

Plant derived medicines have made significant contributions towards human health. However, the potential of medicinal plants as a source for new drugs is still largely unexplored, and among the estimated 250 000-500 000 plant species, only a small fraction has been submitted to biological or pharmacological screening. The use of plant-derived antimicrobial agents may be effective in reducing the dependence on antibiotics thereby minimizing the chances of antibiotic resistance in pathogenic microorganisms (Essawi and Srour, 2000; Mickymary et al., 2016; Stanković et al., 2016; Wikaningtyas and Sukandar, 2016). Many indigenous plants has been investigated for their antimicrobial activity in South Africa (Nyila et al., 2012) including the species in the genus *Tulbaghia*.

Tulbaghia, a member of the family *Alliaceae* is indigenous to KwaZulu Natal and the Eastern Cape region in South Africa (Raji et al., 2012; Moodley et al., 2014). Some of the species are used in traditional medicines to treat a variety of infections (Thamburan et al., 2006) such as type-1 diabetes, fever and colds, paralysis, hypertension, asthma, rheumatism, sinus headaches, tuberculosis, oesophageal cancer, inflammation and gastrointestinal ailments and intestinal worms (Netshiluvhi and Eloff, 2016). Sulphur compounds in *Tulbaghia* are responsible for its garlic-like odour and medicinal properties (Lyantagaye 2011).

Although information of the ethnobotanical uses of many *Tulbaghia* species in available, only *Tulbaghia violacea* and *Tulbaghia alliacea* have been the subject to various pharmacological studies (Motsei et al., 2003; Ncube et al., 2012; Olorunnisola et al., 2012; Aremu and van Staden 2013). Therefore, this study assessed the antimicrobial activity of crude acetone and water extracts from fresh leaves of eight species of *Tulbaghia* against selected bacterial and fungal strains. Water was chosen as an extracting solvent since it is the main solvent used to prepare concoctions in traditional medicine (Ngouana et al., 2015) while acetone was selected based on its ability to extract compounds with a wide range of polarities and its ability to mix well with other solvents (Eloff, 1998b).

4.2 MATERIALS AND METHODOLOGY

4.2.1 Plant material

The eight *Tulbaghia* plant samples (*T. acutiloba, T. alliacea, T. cernua, T.leucantha, T. ludwigiana, T. natalensis, T. simmleri and T. violacea*) were obtained from different indigenous plant nurseries in Gauteng, South Africa. Plants were kept in a greenhouse at Vaal University of Technology. The herbarium samples were authenticated by a botanist. Voucher specimens were deposited in the AP Goossens Herbarium, North West University, Potchefstroom, South Africa.

4.2.2 Preparation of the leaf aqueous crude extract

Ten grams of fresh plant leaf samples were homogenized in 100 mL of distilled water in separate preparations. The homogenate from each plant was boiled for 10 minutes in a water bath at 100°C. Thereafter the homogenate was allowed to cool down and then filtered through a No. 1 whatman filter paper. Afterwards the filtrate was kept at -20°C, lyophilized and then the power stored at 4°C in an airtight container until it was used.

4.2.3 Preparation of the leaf acetone crude extract

Ten grams of fresh plant leaf samples were homogenized in 100 mL of absolute acetone in separate preparations. The homogenate from each plant was allowed to stand for 24 hours then filtered with No. 1 Whatman filter paper. The solvent was evaporated under the fumehood and the extract was kept at 4°c until use.

4.2.4 Test microorganisms

The following strains were used in this study**.** *Staphylococcus aureus* (ATCC 25923) (Gram-positive bacteria) • *Enterococcus faecalis* (ATCC 29212) (Gram-positive bacteria) • *Klebsiella pneumoniae* (NCTC 9633) (Gram-negative bacteria) • *Pseudomonas aeruginosa* (ATCC 15442) (Gram-negative bacteria) • *Candida albicans* (ATCC 14053) (yeast) • *Cryptococcus neoformans* (ATCC 14116) (yeast).

4.2.5 Well diffusion assay

A modified well diffusion test (Harris et al., 1989) was used as a preliminary screening method for the antimicrobial potential of the plant extracts. Wells (made from the bottom parts of 200 µl pipette tips, 5 mm in diameter) were created on the Mueller Hinton agar and malt extract agar (MEA) which were previously inoculated uniformly with the appropriate microorganism to obtain a lawn growth. Twenty microliters of each plant extract (100 mg/mL) was added to

the wells. As a negative control, 20 µL of acetone was used. Neomycin and Amphotericin B were used as positive controls for bacterial and fungal strains, respectively. The petri dishes were incubated for 24 h at 37°C. The level of bacterial susceptibility was determined according to the size of the zones of inhibition which were measured in mm.

4.2.6 Microtiter assay for bacteria

Minimum inhibitory concentrations (MIC) of extracts for antibacterial activity were determined using the microdilution bioassay as described by (Eloff, 1998a). Overnight cultures incubated at 37°C in a water bath with an orbital shaker of two Gram-positive (*S. aureus* ATCC 25923 and *E. faecalis ATCC* 29212) and two Gram-negative (*K. pneumoniae* ATCC 13883 and *P. aeruginosa* ATCC 15442) were diluted with sterile Mueller-Hinton broth to give final inoculums of approximately 10^6 CFU/mL (colony forming units). The crude acetone plant extracts were suspended in acetone to a concentration of 50 mg/mL while the crude water extracts were dissolved in distilled water to the same concentration. One hundred microliters of each extract were serially diluted two- fold with sterile distilled water in a 96-well microtiter plate for each of the four bacterial strains. A two-fold dilution of neomycin (Sigma-Aldrich, Darmstadt, Germany) at 0.1 mg/mL was used as a positive control against each bacterium. One hundred microliters of each bacterial culture was added to each well. Water and acetone were included as the negative and solvent controls, respectively. The plates were covered and incubated at 37°C for 24h. Bacterial growth was indicated by adding 50 µl of 0.2 mg/mL *p*-iodonitrotetrazolium chloride (INT) (Sigma-Aldrich, Germany) and a further incubation at 37°C for 24 h. Since the colourless tetrazolium salt is biologically reduced to a red product in the presence of active organisms, the MIC values were determined as the concentrations in the last wells in which no colour change was observed after adding the INT indicator. Bacterial growth in the wells was indicated by a reddish-pink colour. The assay were repeated twice with triplicates per assay (Ncube et al., 2011).

4.2.7 Microtiter assay for fungi

A microdilution method as described by Eloff (Eloff, 1998a) was used to determine the antifungal activity of extracts against *C. albicans* (ATCC 14053) and *C. neoformans* (ATCC 14116). An overnight fungal culture was prepared in yeast malt (YM) broth. Four hundred microliters of the overnight culture was added to 4 mL of sterile saline and absorbance was read at 530 nm. The absorbance was adjusted with sterile saline to match that of a 0.5 M McFarland standard solution. From this standardised fungal stock, a 1:1000 dilution with sterile YM broth was prepared to give a final inoculum of approximately 10^3 CFU/mL. Crude acetone

extracts were suspended in acetone to obtain concentration of 50 mg/mL and water extracts dissolved in water to the same concentration. One hundred microliters of each extract was serially diluted two-fold with sterile water in a 96-well microliter plate. A similar two-fold dilution of amphotericin B-(Sigma-Aldrich) at 2.5 mg/mL was used as the positive control while water and acetone were used as negative and solvent controls, respectively. One hundred microliters of the diluted fungal culture was added to each well. The plates were covered with lids and incubated at 37°C for 24 h, after which 50 µL of 0.2 mg/mL INT was added and the plates were incubated for a further 24 h at 37°C.The where there was inhibition of fungal growth remained clear. The MIC value was recorded as the lowest concentrations that inhibited fungal growth after 48h. The assay was repeated twice with triplicates per assay (Ncube et al., 2011).

4.2.8 Time kill assay

For further evaluation of antimicrobial activity, a time kill assay was performed as described in (Akinpelu et al., 2009). Bacterial cells in the exponential growth phase were cultured and diluted to approximately 10^6 CFU/mL inoculum. Solutions (100 µL) of the most potent test extract with concentrations corresponding to 1/2 MIC, MIC and 2 MIC were added to the inoculum suspension. Mueller Hinton broth inoculated with each bacterial strain without test extracts acted as the control in this experiment. The inoculated cultures were incubated at 37°C in a water bath at 60 rpm. Samples (100 µL) were removed from each inoculum culture at time points 0, 3, 6 and 24 h, serially diluted to 10^{-6} in 0.9% normal saline then 100 µL was used for obtaining colony forming units (CFU) using the spread plate technique. Viable counts were calculated in the units of CFU/mL and kill curves were plotted with time (h) against logarithm of the viable count (log10 CFU). Each experiment was carried out in triplicate and analysed with mean variables. The bactericidal effects of a compound were seen when there was a ≥3 log10 decrease in viable colony count relative to initial inoculum. The calculations were done as follows:

$$\% \text{ Reduction} = \frac{Initial\ count - count\ at\ \ x\ interval}{Initial\ count}\ X\ 100$$

$$\text{Log reduction} = \text{Log}_{10}\ (\text{Initial count}) - \text{Log}_{10}(\text{x interval time})$$

4.2.9 Statistical analysis

The experimental results were expressed as mean ± standard deviation of three replicates. Where applicable the data was subjected to ANOVA two factor without replication. *P* values less than 0.05 were considered statistically significant. Microsoft Excel 2010 statistical package was used.

4.3RESULTS

The antimicrobial activity of the eight *Tulbaghia* species was evaluated using agar well diffusion, microtiter dilution and time kill assays.

4.3.1 Agar well diffusion assay

With the exception of *T. cernua,* the acetone extracts of all the other species of *Tulbaghia* showed antibacterial/antifungal activity for the tested organisms as indicated by the zones of inhibition (Table 4.1). On the other hand, water extracts of all the species, except for *T. violacea* showed no activity against all the tested organisms. The zones of inhibition ranged between 6 mm and 27 mm in diameter. The biggest zones of inhibition for both the bacterial (10 mm -14 mm) and fungal strains (22 mm- 27mm) was shown by extracts of *T. violacea*.

Table 4.1 Zones of inhibition for selected bacterial and fungal species due to aqueous and acetone extracts from Tulbaghia

	E. faecalis		*S. aureus*		*K. pneumoniae*		*P. aeruginosa*		*C. albicans*		*C. neoformans*	
Plant extracts	Aq	Ac	Aq	Ac	Aq	Ac	Aq	Ac	Aq	Ac	Aq	Ac
T. acutiloba	0	9± 0.58	0	9±1.15	0	8±1.00	0	8±3.06	0	16±0.58	0	22 ± 1.00
T. alliacea	0	9±0.58	0	12±0.58	0	7±2.00	0	10±1.00	0	7±1.15	0	6±0.58
T. cernua	0	0	0	0	0	0	0	0	0	0	0	0
T. leucantha	0	12±0.58	0	11±1.00	0	9±1.53	0	8±3.79	0	17±1.73	0	21 ±1.00
T. ludwigiana	0	8±0.58	0	11±0.58	0	9±1.53	0	11±0.58	0	18±1.00	0	21±2.00
T. natalensis	0	6±0.58	0	12±0.58	0	9±0.58	0	10±1.15	0	18±1.53	0	22 ±2.00
T. simmleri	0	9±1.15	0	12±1.00	0	6±1.15	0	8±1.15	0	6±0.58	0	6 ±0.58
T. violacea	12±0.58	13±1.00	11±1.00	14±1.00	10±0.58	12±3.06	11±±1.00	12±1.00	22±0.58	27±0.58	26±0.58	27±1.00

Aq- aqeous extract Ac- acetone extract; mean zones of inhibition (mm) ±SD

4.3.2 Microtiter dilution assay

The MIC values obtained in this study for both the acetone and aqueous extracts ranged from 0.1 mg/mL to 50 mg/mL (Table 4.2). The aqueous extracts for all the species, except *T. violacea*, had MIC values greater than 10 mg/mL in all the bacterial and fungal strains.

Table 4.2 Minimum inhibitory concentration (MIC) of acetone and aqueous plant extracts (mg/mL) for selected bacterial and fungal species

Plant species	Acetone extracts						Aqueous extracts					
	E. f	S. a	K. p	P. a	C. a	C. n	E. f	S. a	K. p	P. a	C. a	C. n
T. acutiloba	6.25	1.56	6.25	3.13	0.39	0.39	50	50	50	50	25	12.5
T. alliacea	1.56	1.56	1.56	3.13	6.25	6.25	50	50	50	50	50	25
T. cernua	50	50	50	50	50	50	50	50	50	50	50	50
T. leucantha	3.13	3.13	3.13	1.56	0.39	0.39	50	50	50	50	12.5	12.5
T. ludwigiana	6.25	1.56	12.5	3.13	3.13	6.25	25	25	25	25	12.5	12.5
T.natalensis	3.13	3.13	6.25	1.56	0.39	0.39	50	50	50	50	25	12.5
T. simmleri	3.13	1.56	1.56	6.25	6.25	3.13	50	50	50	50	25	50
T. violacea	0.78	0.78	3.13	1.56	0.39	0.1	3.13	0.78	6.25	3.13	1.56	0.2
Neomycin (µg/mL)	6.25	1.56	12.5	12.5								
Amphotericin B (µg/mL)											0.1	0.05

E. f = *Enterococcus faecalis*; S. a = Staphylococcus aureus; K. p = *Klebsiella pneumoniae*;

P. a = *Pseudomonas aeruginosa*; C. a = *Candida albicans*; C.n= *Cryptococcus neoformans*

The results also indicate that there is a significant difference ($p < 0.05$) in the sensitivity of the tested bacterial and fungal species for the acetone extracts. The mean MIC values for the different acetone extracts ranged from 1.9 mg/mL to 4.91 mg/mL with the exception of *T. cernua* which did not show activity (Fig.4.1).

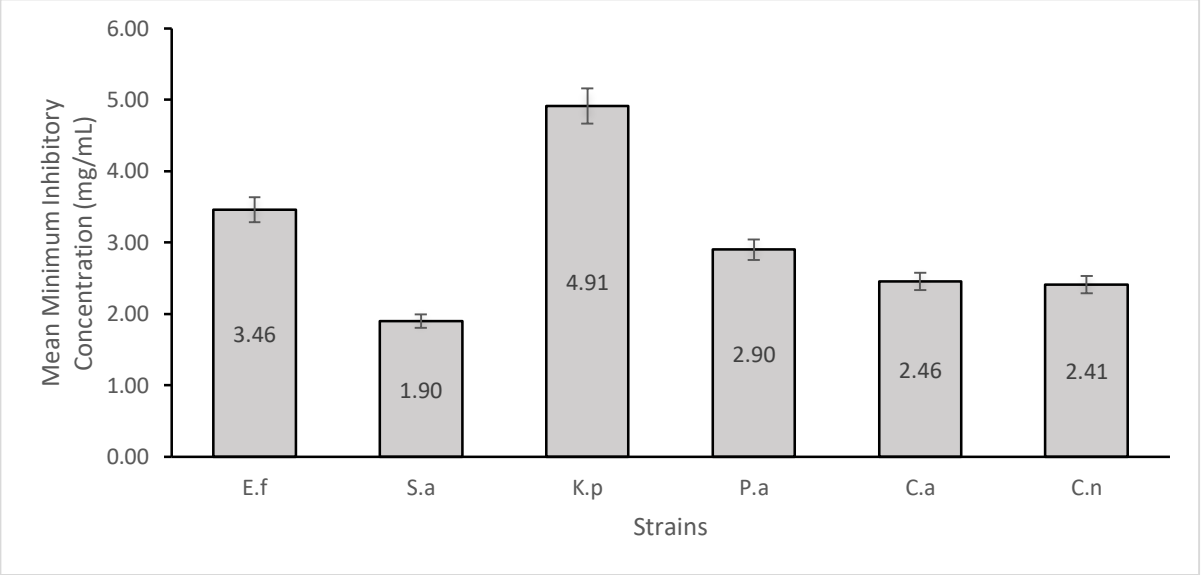


Figure 4.1 The mean MIC (mg/mL) of the acetone leaf extracts from seven *Tulbaghia* species against 4 bacterial and two fungal strains. *E.f = Enterococcus faecalis, S.a= Staphylococcus aureus, K.p = Klebsiella pnemoniae, P.a = Pseudomonas aeruginosa, C.a = Candida albicans, C.n = Cryptococccus neoformans*

4.3.3 Time kill assay

The results of time kill are presented in Tables 4.3-4.8. The acetone extract of *T. violacea* showed both bacteriostatic/ fungistatic and bactericidal/fungicidal effects that was dependent on time and concentration. The percentage reduction in viable cell count (99.9%) at 1x MIC and 2 x MIC indicated a very highly significant decrease (p<0.05) as compared to 0.5 x MIC (50-80%). Bactericidal/fungicidal effects were noted only after 6h exposure in all microorganisms exposed to 1x MIC while rapid cidal activity was observed at 3 h for the 2x MIC. Consistent with the MIC values, *S. aureus* was the most susceptible bacteria

Table 4.3 Time kill kinetics of *T. violacea* against *S. aureus*

Initial time	Population of microorganisms (cfu/mL)			% Reduction			Log reduction		
(h)	0.5 MIC	MIC	2MIC	0.5MIC	MIC	2MIC	0.5MIC	MIC	2MIC
0	$3x10^9$	$2.8x10^9$	$2.6x10^9$	N/A	N/A	N/A	N/A	N/A	N/A
3	$5.8x10^8$	$4.8x10^8$	0	81	83	99	0.72	0.75	9.41
6	$4x10^8$	0	0	87	99	99	0.88	9.45	9.41
24	$4x10^8$	0	0	87	99	99	0.88	9.45	9.41

Table 4.4 Time kill kinetics of *T. violacea* against *E. faecalis*

Initial time	Population of microorganisms (cfu/mL)			% Reduction			Log reduction		
(h)	0.5 MIC	MIC	2MIC	0.5MIC	MIC	2MIC	0.5MIC	MIC	2MIC
0	$3x10^9$	$1.96x10^9$	$1.25x10^9$	N/A	N/A	N/A	N/A	N/A	N/A
3	$9x10^8$	$1.1\ x10^8$	0	70	94	99	0.53	2.25	9.1
6	$86\ x10^8$	0	0	71	99	99	0.46	9.29	9.1
24	$5.8\ x10^8$	0	0	81	99	99	0.71	9.29	9.1

Table 4.5 Time kill kinetics of *T. violacea* against *P. aeruginosa*

Initial time	Population of microorganisms (cfu/mL)			% Reduction			Log reduction		
(h)	**0.5 MIC**	**MIC**	**2MIC**	**0.5MIC**	**MIC**	**2MIC**	**0.5MIC**	**MIC**	**2MIC**
0	$3.1x10^9$	$2.56x10^9$	$2.7x10^9$	N/A	N/A	N/A	N/A	N/A	N/A
3	$6x10^7$	$2x10^7$	0	58	69	99	2.7	2.11	9.4
6	$2x10^7$	0	0	59	99	99	2.19	9.4	9.4
24	$1x10^7$	0	0	60	99	99	2.49	9.4	9.4

Table 4.6 Time kill kinetics of *T. violacea* against *K. pneumoniae*

Initial time	Population of microorganisms (cfu/mL)			% Reduction			Log reduction		
(h)	**0.5 MIC**	**MIC**	**2MIC**	**0.5MIC**	**MIC**	**2MIC**	**0.5MIC**	**MIC**	**2MIC**
0	$1.55x10^9$	$1.5x10^9$	$1.2x10^9$	N/A	N/A	N/A	N/A	N/A	N/A
3	$4.6x10^8$	$8x10^7$	0	70	95	99	1.53	1.27	9.08
6	$4.3x10^8$	$6x10^6$	0	72	96	99	2.53	2.40	9.08
24	$4.8x10^8$	0	0	69	99	99	0.51	9.18	9.08

Table 4.7 Time kill kinetics of *T. violacea* against *C. albicans*

Initial time	Population of microorganisms (cfu/mL)			% Reduction			Log reduction		
(h)	0.5 MIC	MIC	2MIC	0.5MIC	MIC	2MIC	0.5MIC	MIC	2MIC
0	$1.09x10^9$	$1.35x10^9$	$2.5x10^9$	N/A	N/A	N/A	N/A	N/A	N/A
3	$1.7x10^8$	$1x10^7$	0	84	93	99	0.801	2.13	9.40
6	$1.6x10^8$	0	0	85	99	99	0.833	9.11	9.40
24	$1.3x10^8$	0	0	88	99	99	0.92	9.11	9.40

Table 4.8 Time kill kinetics of *T. violacea* against *C. neoformans*

Initial time	Population of microorganisms (cfu/mL)			% Reduction			Log reduction		
(h)	0.5 MIC	MIC	2MIC	0.5MIC	MIC	2MIC	0.5MIC	MIC	2MIC
0	$2.4x10^9$	$2.20x10^9$	$2.24x10^9$	N/A	N/A	N/A	N/A	N/A	N/A
3	$4x10^8$	$2x10^7$	0	83	99	99	0.78	1.91	9.40
6	$3x10^7$	0	0	88	99	99	1.90	9.34	9.40
24	$1x10^7$	0	0	96	99	99	9.38	9.34	9.40

4.4 DISCUSSION AND DISCUSSION

Medicinal plants have become the focus of intense studies in terms of validation of their traditional uses through the determination of their actual biological activities (de Lima et al., 2006). The antimicrobial potential of plant extracts in this study was initially examined against bacterial and fungal strains using the agar well diffusion assay. Among the species of *Tulbaghia* used in this study the acetone and water extracts of *T. violacea* appear to have contained the most active compounds especially against the proliferation of the fungal species *C. albicans* and *C. neoformans*. Both extracts of *T. violacea* also outperformed those of other species in inhibiting the growth of the bacterial species as evidenced by the zones of inhibition (Table 4.1).

The results also showed that acetone extracts were significantly ($p < 0.05$) more active than water extracts, as indicated by the larger zones of inhibition (Table 4.1). The differential activity of the acetone and aqueous extracts can be attributed to phytochemicals present in the extracts (Madike et al., 2017). Acetone is known to extract compounds of a wider polarity compared to water (Eloff, 1998b). According to van Vuuren (2008) an MIC of 1mg/mL or below is regarded as good, greater than 1mg/mL < 10 mg/mL as moderate and > 10 mg/mL as poor activity. This definition was adopted for the purpose of this study. The MIC is defined as the lowest concentration of the extract that shows no visible microbial growth after incubation (Lima et al., 2015).

The aqueous extracts for all the species of *Tulbaghia,* except that of *T. violacea*, produced MIC values of greater than 12.5 mg/mL and up to a maximum of 50 (Table 4.2). This observation is important since water is the solvent that is mostly used by traditional medical practitioners in preparing remedies in the form of infusions, decoctions, concoctions or poultices (Kelmanson et al., 2000). However, considering that the dosage dispensed by traditional healers is usually very high, for instance three to four cupfuls per day for an adult, water can still be considered as an appropriate extracting solvent for traditional medicine (Matu and van Staden, 2003).

The sensitivity of the strains to the acetone extracts from the most to the least sensitive were as follows: *S. aureus* (1.90 mg/mL) > *C. neoformans* (2.41 mg/mL) > *C. albicans* (2.46 mg/mL) > *P. aeruginosa* (2.90 mg/mL) > *E. facaelis* (3.46 mg/mL) > *K. pneumonia* (4.91 mg/mL).

The mean MIC value of the acetone extracts (Fig 4.1) against *S. aureus* was significantly ($p<0.05$) lower than that of *E. faecalis* and *K. pneumonia* while no significant difference ($p > 0.05$) was observed with *P. aeruginosa* and the fungal strains. The sensitivity of *S. aureus*

has been observed in extracts from different medicinal plants in various studies (Yasunaka et al., 2005, Mathabe et al., 2006, Maregesi et al., 2008, Fomogne-Fodjo et al., 2014, Biva et al., 2016) corroborating the results obtained in this study.

Staphylococcus aureus infection is a major cause of skin, respiratory, bone, joint and endovascular disorders. Many strains of *S. aureus* are developing resistance to the ß-lactam antibiotics which are the drugs of choice for the treatment of *S. aureus* infections (Aumeeruddy-Elalfi et al., 2015). Resistance to ß-lactam compounds has been reported for methicillin, oxacillin, nafcillin, cloxacillin, and dicloxacillin. Methicillin-resistant *S. aureus* (MRSA) infections can cause a broad range of symptoms depending on the parts of the body that are infected. *Staphylococcus aureus* is also resistant to other commonly used antimicrobial agents including aminoglycosides, macrolides, chloramphenicol, tetracycline, and fluoroquinolones (Chomnawang et al., 2009). Therefore medicinal plants can be a good alternative therapy for *S. aureus* related infections given their potent antimicrobial activity against the bacteria used in this study.

This study, showed no significant difference ($p > 0.05$) between the mean MIC values of Gram negative and that of Gram positive bacteria. This suggests that the *Tulbaghia* extracts may have a broad spectrum activity. It was also observed that *P. aeruginosa* a Gram negative bacteria showed a mean MIC average lower than that of *E. faecalis* a Gram positive bacteria. Gram-negative bacteria are generally more resistant to antibiotics compared to the Gram-positive ones (Cos et al., 2006). The difference in the sensitivity between the two is alluded to the variation in their cell wall structure or other complex mechanisms. For example, the resistance from Gram-negative bacteria against antibiotics like penicillin originates from the secretion of the lactamase enzyme in the periplasmic space between the thin outer membrane and the cytoplasmic membrane (Elisha et al., 2017; Maregesi et al., 2008).

This study also showed that the sensitivity of bacterial strains depended on the species from which the extract was obtained. Extracts from *T. violacea* were the most potent while those from *T. cernua* showed no activity. The acetone extract of *T. violacea* showed the most potent antibacterial and antifungal activity thus its bactericidal and fungicidal activity was further evaluated in more detail. The bactericidal/ fungicidal activity is defined as being equal to $3\log_{10}$ cfu/mL or greater reduction in the viable colony count relative to the initial inoculum (Chen et al., 2016). In contrast to MIC determination, which investigates the antimicrobial effect at one concentration after a specific time, the time kill assay involves evaluating the effect of different

concentrations i.e. 2 x, 1x and 0.5 x MIC, over a 24 h incubation period (Aiyegoro and Okoh, 2010; Teanpaisan et al., 2017). The time kill assay allows for the determination of the rate of cidal activity of the extract (Oladosu et al., 2013).The time kill assay of the extract of *T. violacea* gave variable results against tested microorganisms as seen in Table 4.3 to 4.8. The extract demonstrated both bacteriostatic/fungistatic and bactericidal/fungicidal effects as shown by concentration and time dependant killing. The bacteriostatic/fungistatic effect was observed at MIC values for each organism tested while the bactericidal/fungicidal effect was observed at higher concentration of 2MIC.

The time kill assay results of the acetone extracts of *T. violacea* acetone extract further substantiated the result of the well diffusion and microtiter assays. The effectiveness of acetone extract of *T. violacea* may be due to a higher amount of phytochemicals present in the extract compared to the other *Tulbaghia* species. The well diffusion, MIC and time kill assays are in agreement with the reported efficacies of *T. violacea* extracts in antimicrobial studies as reported by Lindsey and van Staden (2004), Thamburan et al. (2006), Buwa and Afolayan (2009) and Ncube et al. (2011).

In general, the acetone extracts of all the *Tulbaghia* species showed moderate activity against all the test microorganisms while *T. violacea* showed the strongest activity against the fungi *C. neoformans* and *C. albicans* with MIC values equal to 0.1 mg/mL and 0.2 mg/mL, respectively and Gram positive bacteria with an MIC of 0.78 mg/ml. The acetone extracts of some of the other species such as *T. acutiloba*, *T. leucantha* and *T. natalensis* had moderate activity against bacteria strains and higher activity against the fungal strains. The results of this study are congruent with the findings of Motsei et al. (2003) and Thamburan et al. (2006) which indicated that *T. violacea* and *T. alliacea* had fungicidal activity. *Tulbaghia* species contain sulphur compounds which are known to have anti-Candida activity (Jäger and Stafford, 2012).

The potent antifungal activity of the *Tulbaghia* species is of great significance as it has potential to provide an effective, easily accessible and cheaper treatment against problematic fungal infections. The antimicrobial activity of the *Tulbaghia* plant extracts is supported by the presence of phytochemicals such as saponins, terpenoids, tannins, and glycosides (Madike et al., 2017) which are known to have antifungal and antibacterial activities. Saponins, for example, are known to possess antifungal activities (Liu et al., 2004).

Different solvent extracts of *T. violacea* have been tested against a range of microorganisms affecting both humans and plants. Among such numerous studies, the current findings clearly

indicate a wide variation in the antimicrobial activity recorded among researchers (Aremu and Van Staden, 2013).The differences in the antimicrobial activities between the extracts could be due to the different chemical composition and the distinct mechanisms of action of their bioactive components (Mambe et al., 2016) or maybe different concentrations of the active ingredient in each plant (Ulloa-Urizar et al., 2015).

In this study, the acetone extract of plants under study exhibited antimicrobial activity with *T. violacea* extract being the most potent. The findings of antimicrobial activity studies of *Tulbaghia species* support the traditional use of these plants in traditional medicine (Motsei et al., 2003; Thamburan et al., 2006; Aremu et al., 2014). The strong antimicrobial activity of *T. violacea* supports its wide ethno-pharmacological use. *T. violacea* may contain potential antimicrobial compounds that may be of great use for the development of pharmaceutical products which can be therapeutic agents against various diseases.

CHAPTER 5 ANTI-CANCER ACTIVITY OF *T. VIOLACEA* ACETONE AND WATER EXTRACTS AGAINST OESOPHAGEAL AND ORAL CANCER CELL LINES

A. Anti-proliferative and apoptotic activity of acetone and water crude extracts of *T. violacea* on Hkesc-1 cell line.

ABSTRACT

Tulbaghia violacea is a medicinal plant with therapeutic properties for the treatment of various diseases and in particular esophageal cancer in traditional medicine. The aim of this study was to investigate the anti-proliferative and apoptotic activity of acetone and water crude extracts of *T. violacea* on Hkesc-1 cell line. The anti-proliferative activity of the extracts was evaluated using the MTT assay. Morphological changes of Hkesc-1 cells treated with extracts were examined using light microscopy. Induction of apoptosis was assessed using fluorescence microscopy and acridine orange/ethidium bromide staining. Flow cytometry analysis was conducted to examine the multicaspase activity and cell cycle arrest. The treatment of Hkesc-1 with acetone and water crude extracts showed anti-proliferative activity with IC_{50} values of 0.4 mg/mL and 1.625 mg/mL, respectively. Morphological changes such as blebbing, cell shrinkage and rounding were observed in the treated cells suggesting that apoptosis was taking place. AOEB staining showed that the level of apoptosis was dependent on the concentration of the extracts. The activation of multicaspase activity in the Hkesc-1 treated cells was also concentration dependent leading to cell death by apoptosis and the induction of cell cycle arrest at the G_2/M phase. The results of these study confirm the anticancer potential of *T. violacea*. Future studies will focus on the identification of the bioactive compound(s) responsible for the anticancer activity.

Keywords: Anti-proliferative, Apoptosis, Esophageal cancer, Multicaspase, *Tulbaghia*.

5.1 INTRODUCTION

Esophageal cancer (EC) is a malignant tumor that develops in the inner mucosal layers of the esophagus. It is the eighth most common cancer worldwide and is known for its marked variation by geographic region, ethnicity, and gender (Ying et al., 2018). Currently, more than 80% of cases and deaths from EC occur within developing countries. Esophageal cancer is prevalent in northern China, North-eastern Iran, Eastern South America and Southern Africa (Zhang et al., 2013; Cheng et al., 2015; Wua et al., 2015). The two main types of esophageal cancer, squamous cell carcinoma (ESCC) and adenocarcinoma (EAC), share a poor prognosis but have distinct histopathologic and epidemiologic profiles (Gatenby and Preston, 2014). Worldwide, ESCC is the most common cancer subtype representing 87% of all cases of this type of cancer in 2012 (Dandara et al., 2005; Thrift 2016).

Esophageal squamous cell carcinoma occurs in the squamous cells in the upper third of the esophagus, while EAC appears in glandular cells and ordinarily develops in the lower one third of the esophagus near the stomach. The most prevalent risk factor for EAC is chronic gastro-esophageal reflux disease, which triggers inflammation in the distal esophagus, resulting in progression of the pre-malignant lesion known as Barrett's esophagus (BE) or intestinal metaplasia. The development of BE to EAC progresses through established histological changes: intestinal metaplasia (BE) to low-grade dysplasia (LGD) to high grade dysplasia (HGD) to EAC (Hemmatzadeh et al., 2016).

Squamous cell carcinoma (SCC) of the esophagus presents a significant health problem because development of the disease is asymptomatic, resulting in late diagnosis and poor prognosis. The burden of esophageal cancer is huge and the 5-year survival rate is less than 10% (Dandara et al., 2005).Several predisposing factors such as (i) deficiency of trace minerals, (ii) N-nitroso-compounds, (iii) consumption of food containing mycotoxins, (iv) spicy food, (v) tobacco smoking, (vi) consumption of alcohol, and (vii) low socioeconomic status (Patel et al., 2013) have been reported to play a role in the etiology of EC. Nonetheless, the consumption of alcohol and smoking of tobacco have been reported to be the major risk factors for EC in North and South America, Europe, Asia and Africa (Thrift 2016).

The risk of developing EC is greater among smokers of black tobacco than those who smoke blond tobacco (Vos et al., 2003). Black tobacco contains a higher content of *N*-nitroso compounds that are carcinogenic when compared to the blonde tobacco (Patel et al., 2013). It has been suggested that tobacco plays a role in both early and late stages of carcinogenesis

(cancer initiation and promotion), whereas alcohol may play a more important role in the late stages (Vos et al., 2003). The Eastern Cape Province of South Africa remains one of the world's hotspot regions for ESCC, with age-standardized incidence rates of 23.2 and 14.5 per 100 000 in males and females, respectively (Sewram et al., 2016). Although approved anticancer drugs are available for the treatment of more than 200 different tumors, effective therapies for most of them are lacking. Treatment options of EC vary depending on the type and stage of cancer. Treatment options include surgery, endoscopic therapy, radiation therapy, chemotherapy and targeted therapy. Multimodality neoadjuvant concurrent chemo-radiotherapy (CCRT) has been increasingly administered in the treatment of esophageal cancer (Huang and Yu, 2016).

Limitations in the application of chemotherapeutic agents include toxicity and manifestation of deleterious side-effects. Currently, renewed and concerted efforts are geared towards the discovery and development of newer and better tolerated anticancer drugs, especially from natural products, mainly plants (Akindele et al., 2015). Many medicinal plants have been shown to exert significant anti-tumour effects by blocking the cell cycle, inducing cell apoptosis or regulating other characteristics of cancer (Skerman et al., 2011; Monga et al., 2013).

The ability to inhibit cancer cell proliferation is considered as an indicator of anticancer potential because the balance of tumour cell proliferation over cell death has been proposed to be one of the key factors in cancer evolution and progression (Ravi et al., 2012). *Tulbaghia violacea*, which belongs to the family Amarylliadiae, has been used in traditional medicine for the treatment of esophageal cancer and other ailments in Southern Africa (Bungu et al., 2006). However, there is little scientific data on the anticancer activity of *T. violacea.* This study aimed at assessing the anti-proliferative and apoptotic inducing effect of the crude acetone and water extracts of the leaves *T. violacea* on an esophageal cancer cell line (Hkesc-1).

5.2 MATERIALS AND METHODS

5.2.1 Plant material

Samples of *T. violacea* were obtained from an indigenous plant nursery in Gauteng, South Africa and maintained in a greenhouse at Vaal University of Technology. The plant was authenticated by a botanist, Professor Stefan Seibert at North West University (Potchefstroom, South Africa) where a voucher specimen (ST0008) was deposited in the AP Goossens Herbarium.

5.2.2 Preparation of *T. violacea* acetone and water crude extracts

The preparation of acetone and water extracts of *T. violacea* was done following the protocol previously in section 4.2.2 and 4.2.3.

5.2.3 Culturing of the Hkesc-1 cancer cell line

The esophageal cell line (Hkesc-1) was obtained from Separations (Hkesc-1, Cellonex, Johannesburg, South Africa). The cells were grown in complete culture medium consisting of high glucose DMEM (GE Health Life Sciences, Logan, UT) supplemented with 10% FBS (ThermoScientific, Cramlington, ND) and 1 x penicillin, streptomycin and neomycin at 37^{o}C in a humidified atmosphere at 5% CO_2.

5.2.4 Viability of Hkesc-1

The Hkesc-1 cells were seeded in 96-well plates and incubated for 24 h at 37°C in a humidified incubator with 5% CO_2. After 24 h the cells were washed three times with PBS buffer. The cells were then treated with acetone and water extracts prepared in complete cell culture media consisting of high glucose DMEM (GE Health Life Sciences, Logan, UT) supplemented with 10% FBS (ThermoScientific, Cramlington, Northumberland) and 1 x penicillin at concentrations ranging between 25 µg/mL and 1000 µg/mL. Complete cell culture media was used as a negative control. The cells were incubated for 24 h after which the media was aspirated and the cells washed twice with PBS buffer. Thereafter, 100 µL of the cell culture media was added into each well followed by addition of 10 µL of MTT solution consisting of 5 mg/ml in PBS. The plates were then incubated for 4 h after which 85 µL of the media was removed from each well and 100 µL of DMSO was added. The plates were then gently shaken to solubilize the formazan. The amount of formazan produced was then measured at 570 nm using a microplate reader (Epoch 2, BioTek, Winooski, VT). The percentage viability of Kb cells was calculated as follows:

Cell Viability (%) = [(absorbance of untreated control – absorbance of treated

Sample)/absorbance of untreated control] × 100.

The Inhibitory concentration (IC_{50}) was calculated from the straight line graph plotted in Excel software using percentage viability.

5.2.5 Morphological Observations

The morphological features of the treated Hkesc-1 cells were examined using microscopy. The Hkesc-1 cells were seeded in 12-well plates and incubated at 37ºC in a humidified 5% CO2 incubator. After 24 h the cells were treated, separately, with *T. violacea* acetone and water crude leaf extracts at 0.5 x IC_{50}, 1 x IC_{50} and 2 x IC_{50}. The 12-well plates were then incubated at 37ºC in a humidified 5% CO2 incubator. After 24 h images of the cells were taken at 20x magnification using an Olympus IX53 microscope (Tokyo, Japan).

5.2.6 Acridine orange / Ethidium bromide (AO/EB) staining of cells

Dual AO/EB staining was used to visualise cells undergoing apoptosis. The Hkesc-1 cells were treated as explained in section 2.5. After 24 h the cells were washed with PBS twice and 100 µL of PBS was then added to all the wells. Thereafter, 5 µL of acridine orange (100 µg/mL) was added to each well and after 5 min 5 µL ethidium bromide (100 µg/mL) was added. The plates were covered with foil and incubated for 10 min at room temperature before visualising and imaging the cells with a fluorescent microscope (Evos-FL- AMG, Life Technologies, Bothwell, WA).

5.2.7 Multicaspase activity

The multicaspase activity in the Hkesc-1 cells stimulated with either acetone or water extracts of *T. violacea* as in section 2.5 was evaluated using the Multicaspase SR kit (Cat. # 4500-0500, Merck) following the manufacturer's instructions. In brief, 10 µL of the caspase working solution was added to 100 µL of 1 x 10^6 cells/mL and incubated for 1 h at 37ºC in a CO_2 incubator. Thereafter, 100 µL of 1x apoptosis wash buffer was added to each tube. The samples were then centrifuged for 5 min at 300 x g and the supernatant was discarded. Exactly 200 µL of 1x apoptosis wash buffer was added to the tubes, the sample was homogenised by vortexing briefly, then centrifuged for 5 min at 300 x g and the supernatant was discarded. The cells were then suspended in 200 µL of the caspase 7- aminoactinomycin D (7–AAD) working solution and incubated for 10 min at room temperature. Each sample was analysed with the Guava Easy Cyte 12HT flow cytometer (EMD Millipore Corp, Bellerica, MA) using 10 000 events.

5.2.8 Cell cycle analysis

The cell cycle analysis was conducted to determine the percentage of cells in G_0/G_1, S and

G_2/M phases based on DNA content. The Guava cell cycle reagent (Teiten et al. 4500- 0220, MERCK) was used following the manufactures' instructions. Briefly, about 200 KB cells per microliter were treated with either acetone or water extracts of *T. violacea* as in section 2.5 for 24 and 48 h. Untreated cells were considered as the negative control while positive control cells were treated with Melphalan. After each 24 and 48 h time points, both the treated and untreated cells were harvested and washed with 1x PBS twice. The cells were fixed with ice-cold ethanol and kept at 4ºC for at least 12 h before staining. The cells were stained with propidium iodide. The samples were transferred to 96 well plates and then incubated at room temperature for 30 min before acquiring 10,000 events using the Guava Easy Cyte 12HT system. The experiment was done in triplicate.

5.3 RESULTS

5.3.1The anti-proliferative activity of acetone and water crude extracts of *T. violacea*

The acetone crude extract showed a pronounced anti-proliferative activity against Hkesc-1 cells (Fig. 5.1A). The anti-proliferative effect increased as the concentration of the crude acetone extracts was raised from 25 µg/mL to 1000 µg/mL. The viability of the Hkesc-1 cells treated with acetone extract was significantly (p <0.05) reduced from the lowest concentration (25 µg/mL) to the highest concentration (1000 µg/mL) compared to the untreated control. The IC_{50} value of the acetone crude extract was 0.4 mg/mL.

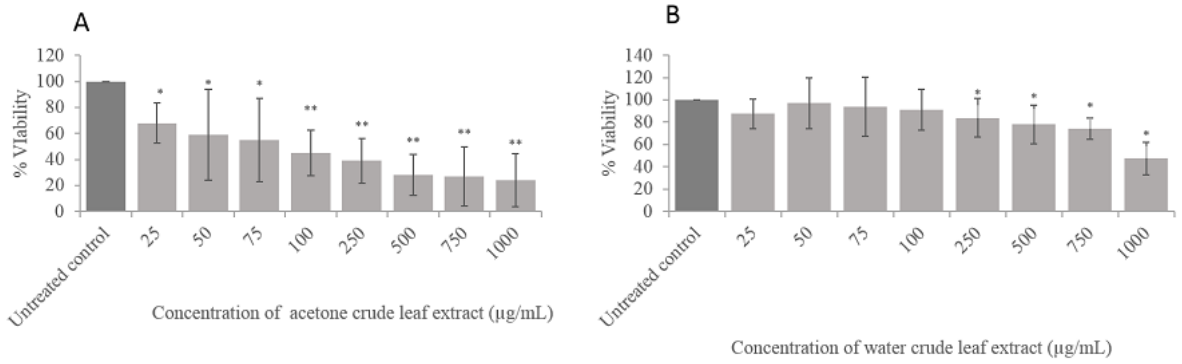


Figure 5.1 Cell viability of Hkesc-1 cells assessed using the MTT assay. Hkesc-1 cells were treated with various concentrations of crude acetone (A) and water (B) extracts from the leaves of *T. violacea*. Error bars indicate the standard error of the mean of three biological replicates.* P < 0.05,** P<0.01 treatment vs untreated control.

An anti-proliferative effect was also observed when the cells were treated with the crude water extract at similar concentrations (Fig. 5.1B). However, the decrease in cell viability was not as pronounced as that observed in treatments involving acetone (Fig. 5.1A and B). There was no significant (p<0.05) difference in cell viability at concentrations between 25 µg/mL and 100 µg/mL in comparison to the untreated cells. A significant anti-proliferative activity (p<0.05) was observed when the concentration of the water extracts was increased from 250 µg/mL to 1000 µg/mL. The IC_{50} value of the crude water extracts was 1.25 mg/mL.

5.3.2 Morphological observation

Hkesc-1 cells treated with acetone extracts showed morphological changes such as cell shrinkage, cell rounding and membrane blebbing at all concentrations of 0.5 x IC_{50} (0.2 mg/mL), 1 x IC_{50} (0.4 mg/mL) and 2 x IC_{50} (0.8 mg/mL) (Fig. 5.2). There was a decrease in the number of Hkesc-1 cells as the concentration of the acetone extract increased. A relatively similar trend was observed in Hkesc-1 cells treated with water crude extracts. However, there

were no morphological changes at 0.5 x IC_{50} (0.625 mg/mL) with water extracts while cell shrinkage and rounding of the cells was observed at 1 x IC_{50} (1.25 mg/mL) and 2 x IC_{50} (2.5 mg/mL) as illustrated in Figure 5.2.

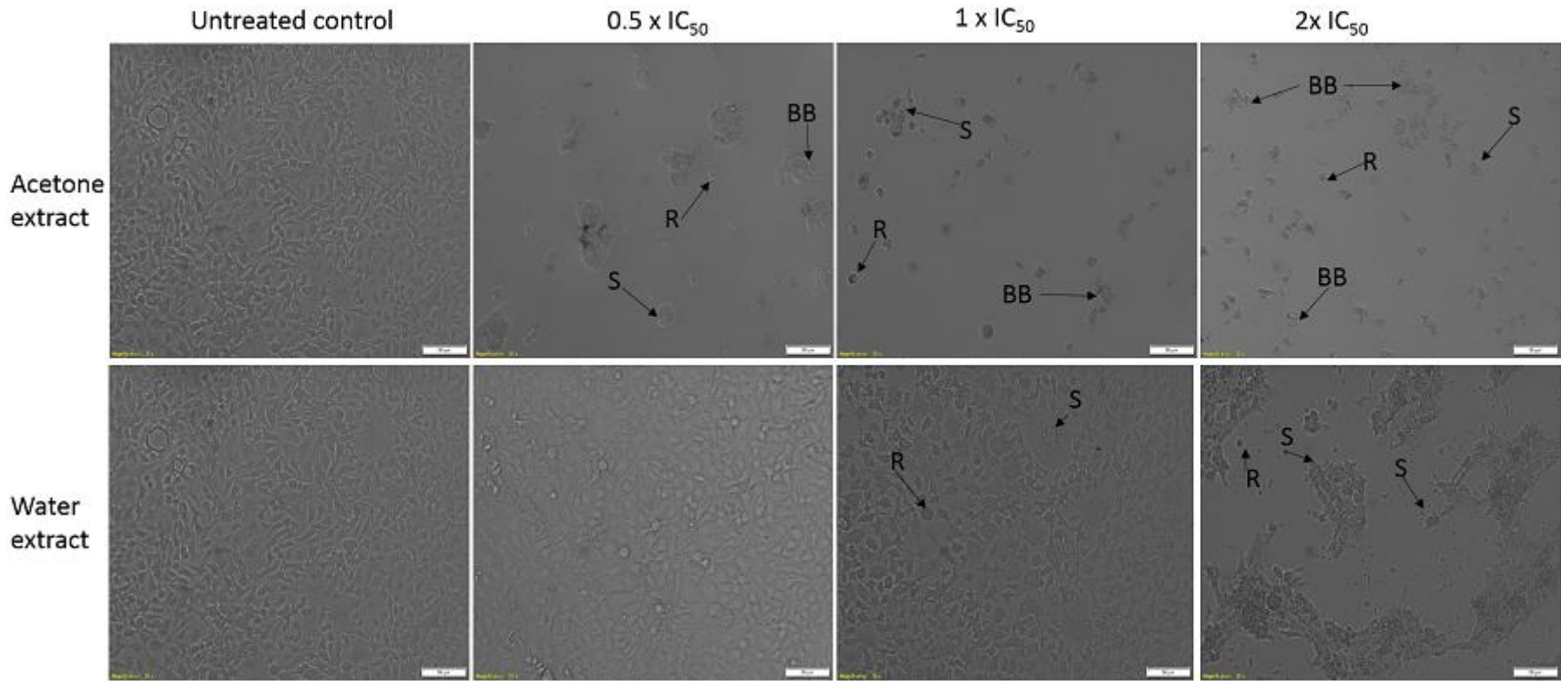


Figure 5.2 Microscopy images of Hkesc-1 cells treated with acetone and water extracts at concentrations 0.5 x IC_{50} (0.2mg/mL), 1x IC_{50} (0.4mg/mL), 2 x IC_{50} (0.8mg/mL) and 0.5 x IC_{50} (0.625 mg/mL), 1x IC_{50} (1.25 mg/mL), 2 x IC_{50} (2.5 mg/mL), respectively. Images were taken at 20x magnification. Scale bars represent 50 µm. Morphological cell changes after treatment with acetone and water extract: BB: Blebbing, R: rounding, S: Shrinkage

5.3.3 AOEB staining

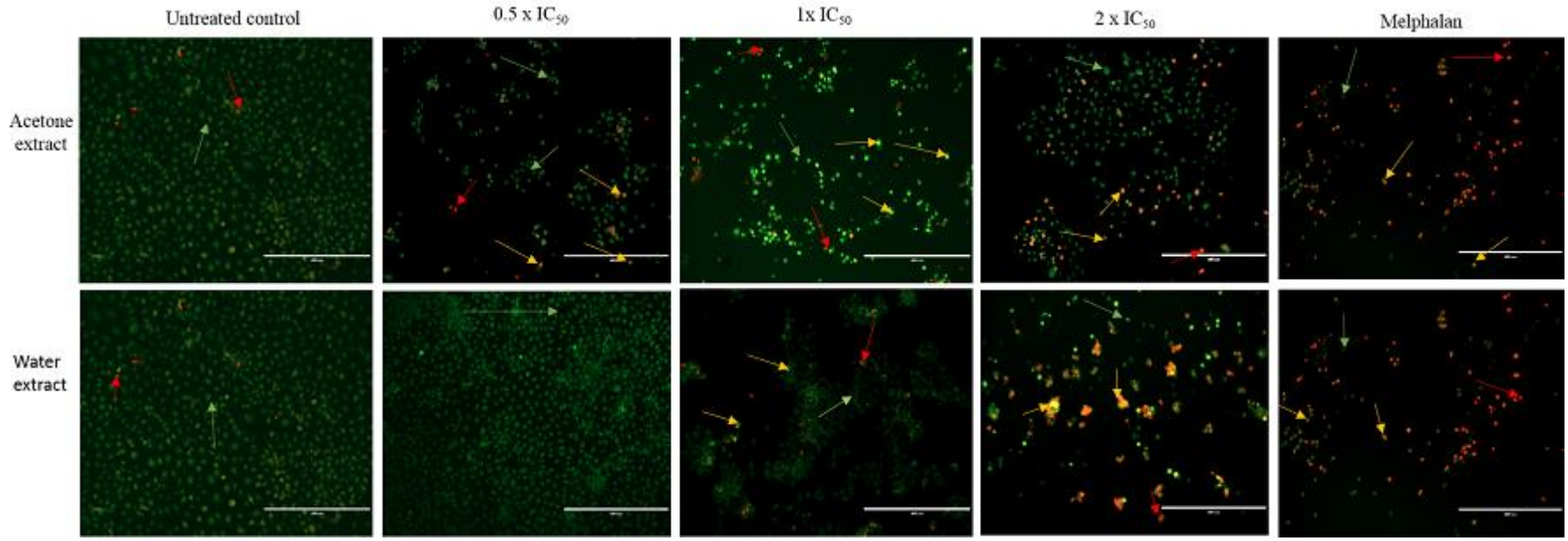


Figure 5.3 Illustration of dual AOEB staining images of Hkesc-1 cells treated with crude acetone [0.5 x IC_{50} (0.2 mg/mL), 1x IC_{50} (0.4 mg/mL) and 2 x IC_{50} (0.8 mg/mL)] and water [IC_{50} (0.625 mg/mL), 1 x IC_{50} (1.25 mg/mL) and 2 x IC_{50} (2.5 mg/mL)] leaf extracts from *T. violacea*. Images were captured at 10x magnification. Melphalan was used as a positive control. Scale bars represent 400 µm. Viable cells (green arrow) excluded ethidium bromide and their intact nuclei stained bright green, while apoptotic cells (yellow arrow) with condensed nuclei stained yellow. Necrotic cells (red arrow) stained bright red.

When the Hkesc-1 cells treated with acetone and water crude extracts were stained with AO/EB viable cells fluoresced green, apoptotic cells appeared yellow, while necrotic cells were red (Fig 5.3). The number of apoptotic cells increased as the concentration of the both extracts increased (Fig. 5.3). The cells treated with acetone induced apoptosis at a concentration starting at 0.5 x $IC_{50.}$ There were more necrotic cells at 2 x IC_{50} as shown by the bright red staining (Fig. 5.3). For the water extract, apoptotic cells were observed at 1 x IC_{50} and 2 x IC_{50} (Fig.5.3). The results showed that the acetone extract was able to induce apoptosis at a lower concentration IC_{50} compared to the water extract. The Hkesc-1 cells treated with the positive control, Melphalan, showed a higher number of apoptotic and necrotic cells.

5.3.4 Multicaspase activity

Multicaspase activity showed that there was an increase in the percentage of apoptotic cells as the concentration of either the acetone or water extract was increased (Fig. 5.4A). The acetone extract significantly ($p<0.05$) induced apoptosis gradually from 10.07% (0.2 mg/mL) to 15.7 (0.4 mg/mL) and up to 32.25% (0.8 mg/mL) compared to 1.8% observed in the untreated control within 24 h of treatment. In a similar manner the water extract increased the levels of apoptotic cells from 5% at 0.625 mg/mL to 6.28% at 1.25 mg/mL and finally to 18.30 % at 2.5 mg/mL. The positive control, Melphalan, also showed increased levels of apoptotic cells at 25.95% compared to the control. Both extracts significantly ($p<0.05$) induced apoptosis in a concentration dependent manner compared to the control (Fig. 5.4 B).

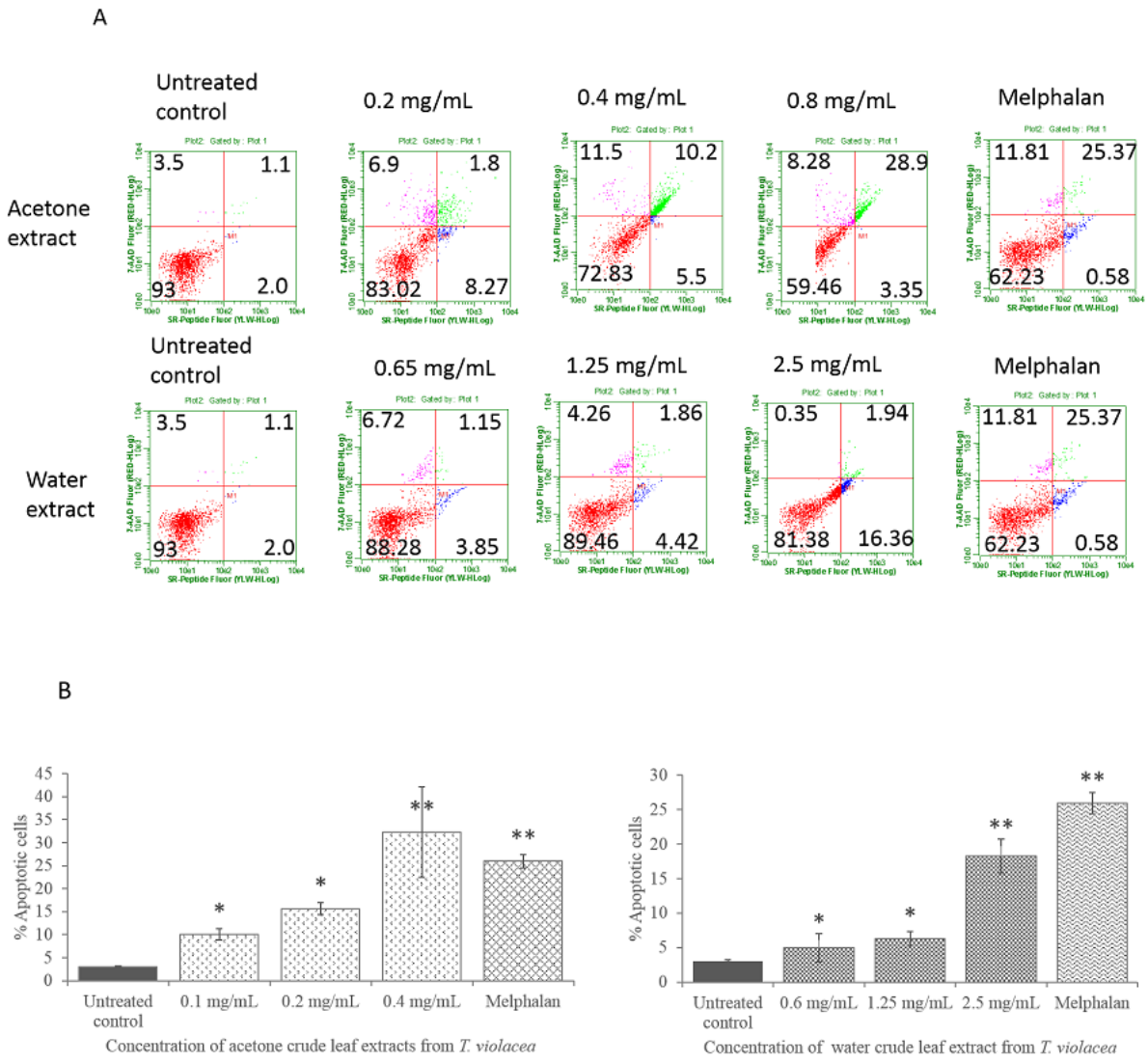


Figure 5.4 A: Dot plot analysis depicting Hkesc-1 cells treated with crude acetone and water leaf extracts of *T. violacea* for 24 hr, stained with 7-AAD and acquired using the Guava Caspase Software module. Events in each of the four quadrants are as follows: LL quadrant: Viable cells, not undergoing detectable apoptosis; LR: Cells in the middle stages of apoptosis; UR quadrant: cells in the late stages of apoptosis or dead and UL quadrant: necrotic cells. B: Bar graphs represent the percent apoptotic cells (±SEM). *$p < 0.05$ and **$p < 0.01$ treatment vs untreated control.

5.3.5 Cell cycle analysis

Hkesc-1 cells treated for 24 h with the acetone crude extract at concentrations of 0.2, 0.4 and 0.8 mg/mL, showed a decrease in the percentage of the cells in the G_0/G_1 phase of the cell cycle in a concentration dependent manner while there was in increase in the number of cells in the G_2/M phase (Fig. 5.5A).

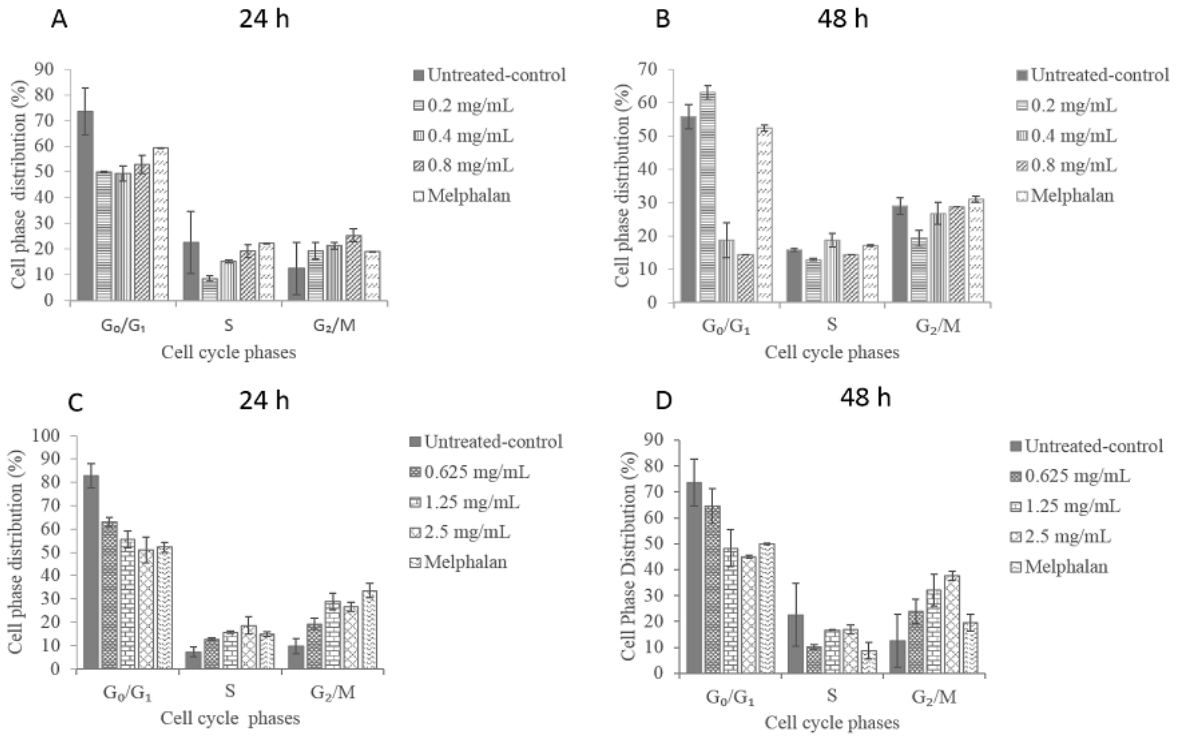


Figure 5.5 The effect of *T. violacea* acetone and water extracts on the cell cycle in the Hkesc-1 cells. Cells were treated for 24 h (A and C) and 48 h (B and D). **A** and **B** represents acetone extracts at different concentrations (0.2 mg/mL, 0.4 mg/mL and 0.8 mg/mL). **C** and **D** represents water extracts at different concentrations (0.625 mg/mL, 1.25 mg/mL and 2.5 mg/mL).

On the other hand, Hkesc-1 cells treated for 48 h with the same concentrations of the acetone extract showed a significant decrease ($p<0.05$) in the G_0/G_1 and an increase in the G_2/M phase (Fig. 5.5B). In a similar manner, Hkesc-1 cells treated with water extracts at concentrations of 0.625 mg/mL, 1.25 mg/mL and 2.5 mg/mL showed a decreased percentage of cells in the G_0/G_1 phase and an increase in the G_2/M phase (Fig. 5.5C). After a 48 h treatment a more pronounced decrease in the G_0/G_1 and an increase in the G_2/M phase of the cell cycle was observed (Fig. 5.5D). Similarly, the positive control Melphalan showed an increase in the percentage of the cells in the G_2/M phase.

5.4 DISCUSSION AND CONCLUSION

The incidence of squamous cell carcinoma of the oesophagus remains to be a significant threat in the Eastern Cape Province of South Africa (van der Merwe et al., 2010; Loots et al., 2017). *Tulbaghia violacea* is one of the medicinal plants that is used to manage esophageal cancer in this region (Saibu et al., 2015). However, there is little scientific evidence for the anticancer properties of this plant. Therefore, this study examined the anti-proliferative and apoptotic activity of acetone and water crude extracts of *T. violacea* on a typical cancer cell line, Hkesc-1.

The crude extracts obtained with both acetone and water inhibited the proliferation of the Heksc-1 effectively. The anti-proliferative effect of the extracts on Hkesc-1 was dose dependent with IC_{50} values of 0.4 mg/mL and 1. 25 mg/mL for acetone and water, respectively (Fig. 5.1). The relatively greater potency of the acetone extract is perhaps due to the greater number of extracted phytochemicals (Madike et al., 2017) since acetone is known to extract compounds of wider polarity compared to water (Eloff, 1998). The morphological changes such as shrinkage, blebbling and rounding (Fig. 5.2) of the acetone and water treated Hkesc-1 cells indicated that the cells were undergoing apoptosis (Ravi et al., 2012). Cell death can be achieved via apoptosis as well as necrosis.

In the search for active compounds with anticancer activity, extracts that induce cell death via apoptosis are preferred as low apoptosis is one of the characteristics of cancer cells (Farha, 2012; Turan et al., 2017). The AO/EB staining confirmed that the treated cells were becoming apoptotic (Fig. 5.3). The number of apoptotic cells indicated by the yellow fluorescence (Fig.5.3) suggests that apoptosis became more pronounced as the concentration of both acetone and water extracts increased. Therefore, the apoptotic nuclei demonstrated that acetone and water extracts induced apoptosis in Hkesc-1 cells providing evidence that *T. violacea* has anticancer properties.

To further confirm the apoptotic activity of the extracts the multicaspase activity and cell cycle arrest were evaluated using flow cytometry. The multicaspase assay simultaneously determines the count and percentage of cells with caspase activity in combination with dead cell dye (7-AAD). As an apoptosis marker, the multicaspase assay in this study showed that the acetone and water extracts of *T. violacea* induced a significant increase ($p<0.05$) in the percentage of Hkesc-1 cells undergoing apoptosis, with a greater increase observed for the acetone extract (Fig. 5.4A & B). These results indicate that treatment of Hkesc-1 cells with acetone and water

crude extracts caused cell death via induction of multicaspase activity. Caspase is one apoptosis pathway which is deregulated in cancer cells thus favouring their survival (Nair and Van Staden, 2018). The ability of acetone and water crude extracts to activate these enzymes indicate their potential in anti-cancer drug discovery. Furthermore, this study showed that the anti- proliferative activity of acetone and water crude extracts is due to cell cycle arrest at the G_2/M phase (Fig. 5.5).

The cell cycle is controlled by specific cyclins and cyclin-dependent kinases (CDKs) in an orderly manner (Eymin and Gazzeri, 2010). The precise regulation of the cell cycle plays an important role in the control of cell proliferation and dysregulation of the cell cycle is associated with the formation of various cancers (Zhou et al., 2016). Consequently, inhibition of the cell cycle of cancerous cells can be an effective strategy for the development of anticancer therapeutics (An et al., 2018). The anticancer activity of *T. violacea* implies that it has some active compounds that function against the Hkesc-1 cancer cell line. Previous studies have shown that *T. violacea* possesses compounds such as flavonoids, polyphenols and saponins (Aremu and Van Staden, 2013; Takaidza et al., 2018) which have been reported to have anticancer activity.

Flavonoids have the ability to induce apoptosis, block the cell cycle by breaking down the structure of the spindle fiber and inhibiting angiogenesis (Zhang et al., 2018). Polyphenols have been reported to have the ability to prevent the proliferation of cancer cells by activating cell cycle arrest, apoptosis, and cell signalling (Bouyahya et al., 2018; Demir et al., 2018) while saponins are natural glycosides which have been previously proposed as anti-inflammatory and anti-cancer agents (Ncube et al., 2011).

In conclusion, the acetone and water crude leaf extracts of *T. violacea* had an anti-proliferative activity against Hkesc-1 cell. The extracts resulted in cell death due to induction of apoptosis and cell cycle arrest at G2/M phase. This study is one of the few that provides empirical evidence of the anticancer potential of *T. violacea*. Future studies will focus on the identification of the bioactive compound(s) responsible for the anticancer activity. Immune toxicity studies should also be investigated to ensure that the extracts have no adverse immunological consequences.

B. The anticancer activity of crude acetone and water leaf extracts of T. violacea on human oral cancer cells.

ABSTRACT

The objective of this study was to evaluate the anticancer activity of crude acetone and water leaf extracts of *T. violacea* on a human oral cancer cell line (KB). The antioxidant activity of the leaf extracts was evaluated by using the DPPH assay while the anti-proliferative activity was assessed by using the MTT assay. The morphological characteristics of apoptotic cells were examined by using the dual acridine orange/ethidium bromide staining. Flow cytometry was used to evaluate the induction of multi-caspase activity and changes in the cell cycle. The acetone and water extracts exhibited antioxidant activity in a concentration dependent manner. The extracts inhibited the growth of the KB cell line with IC_{50} values of 0.2 mg/mL and 1 mg/mL, respectively for acetone and water. Morphological changes such as cell shrinkage, rounding and formation of membrane blebs were observed in the treated cells. In acridine orange/ethidium bromide staining the number of apoptotic cells increased as the concentration of the extracts increased. The activation of multicaspase activity in KB cells treated with *T. violacea* extracts was concentration dependent leading to cell death by apoptosis and cell cycle arrest at the G_2/M phase. The acetone and water extracts of *T. violacea* appears to have anti-cancer activity against human oral cancer cells and needs to be investigated further.

Keywords: Anticancer activity, antioxidant, apoptosis, caspase, cell cycle, *Tulbaghia*.

5.5 INTRODUCTION

Many types of cancer are known to affect the human population (Akindele et al., 2015). Oral cancer is the 8th most common cancer worldwide, with a high prevalence in South Asia (Shah et al.2013) and Eastern and Southern Africa (Warnakulasuriya 2009) and with a higher occurrence in males than in females (WHO 2014). Oral cancer can result from poor life style choices such as smoking tobacco and consumption of alcohol that are considered as major risk factors in the development of this type of cancer (Sewram et al., 2016, Huang et al., 2018). Tobacco contains known carcinogens such as N'-nitrosonornicotine and aromatic hydrocarbon benzo-pyrene, N-nitrosomine (4-nitrosomethylamino)-1-(3-pyridyl)-1-butanone (NNK). These compounds have been linked to oncogenesis capable of inducing tumours of the oral and nasal cavities, lungs, oesophagus and pancreas (Kumar et al., 2016).

Biological agents are also causes of oral cancer. Herrero et al. (2003) showed that the human papilloma virus was able to cause oral carcinoma. Genetic predisposition is another important risk factor in the development of oral cancer. Evidence shows that certain individuals inherit genetic dispositions that result in the inability to metabolize carcinogens as well as repair DNA damage thus resulting in cancer (Kumar et al., 2016). Treatment of oral cancer has been achieved through the use of either surgery or ionization radiation or both depending on the severity of the disease. These treatment options result in side effects such as nausea, vomiting, hair loss, fatigue, mouth sores and complications like mucositis (Huang et al., 2018).

In addition, these cancer treatment options are expensive and inaccessible to people living in resource limited or poor communities. Due to the inaccessibility and cost of cancer treatment many communities rely on medicinal plants to treat the disease. For example, anecdotal evidence reveals that many people in the rural parts of the Eastern Cape Province in South Africa rely on *Tulbaghia* species to treat cancer with various degrees of success(Bungu et al., 2006, Aremu and van Staden, 2013). Therefore it is imperative that empirical evidence to substantiate such claims be established.

Tulbaghia is a genus of herbaceous perennial bulbs in the family Amaryllidaceae. It is predominantly found in Southern Africa (Aremu and van Staden 2013). One of the species *T. violacea* known as wild garlic, wilde knoffel (Afrikaans), isihaqa (Zulu), or itswele lomlambo (Xhosa) is widely distributed and commonly used to treat oesophageal cancer and other ailments in traditional medicine (Bungu et al., 2006). The plant is rich in sulphur-containing compounds (Kubec et al., 2002; Aremu and van Staden, 2013) which may be contributing to

its characteristic odour and medicinal properties. Nonetheless, the mechanism of action through which *Tulbaghia* extracts are able to slow down cancerous growth remains unclear. To understand the role of *T. violacea* in inhibiting cancerous growth, one needs to understand cancer at the cellular level.

Cancer development is due to the inability of cells to undergo apoptosis (Lyantagaye 2011). One of the ways of treating cancer is to restore apoptosis. Strategies aimed at inhibiting cell proliferation through the induction of apoptosis, suppression of angiogenesis and metastasis are key strategies towards the discovery of anticancer drugs (Pereira et al., 2016). Of these, apoptosis has been extensively studied and has been recognised as an ideal way to eliminate malignant cells (Monga et al., 2013). The key process in apoptosis is the stimulation of a caspase cascade of signalling events, which is controlled via both the intrinsic and extrinsic apoptosis pathways (Suzuki et al., 2015; Wang et al., 2017). Apoptotic cell death is marked by morphological changes such as cell shrinkage, chromatin condensation, membrane blebbing and fragmentation of DNA (Skerman et al., 2011; Farha 2012).

These morphological and biochemical markers of apoptosis enables its distinction from other forms of cell death (Krysko et al., 2008). The induction of apoptosis in tumour cells is aforethought to be useful in the management, therapy and prevention of cancer. Screening apoptotic inducers, either in the form of crude extracts or as purified bioactive compounds is a crucial step toward cancer treatment (Mbele et al., 2017). This study investigated the ability of crude acetone and water leaf extracts of *T. violacea* to suppress the proliferation, activate apoptosis and initiate cell cycle arrest in a human oral cancer cell line.

5.6 MATERIALS AND METHODS

5.6.1 Plant material

Samples of *T. violacea* were purchased from an indigenous plant nursery in Gauteng, South Africa and kept in the greenhouse at the Vaal University of Technology, Vanderbijlpark, South Africa. The plant was authenticated by a botanist, Professor Stefan Seibert at North West University (Potchefstroom, South Africa) where a voucher specimen (ST0008) was deposited in the AP Goossens Herbarium.

5.6.2 Preparation of *T. violacea* acetone and water crude extracts

The preparation of acetone and water extracts of *T. violacea* was done following the protocol previously described in section 4.3.1 and 4.3.2.

5.6.3 Antioxidant activity

One ml of either acetone or water crude leaf extract at concentration ranging between 50 µg/mL to 500 µg/mL, was mixed with 1 mL of 0.12 mM DPPH solution . Thereafter 300 µL of the mixture was dispensed in triplicate into 96 well plates. The plates were incubated at room temperature in the dark for 30 min, following which the absorbance was measured at 517 nm using a microplate reader (Perklin Elmer, Walthman, MA). L-ascorbic acid was used as a positive control. The radical scavenging activity was expressed as percentage inhibition and calculated using the formula:

$$\% \text{ scavenging activity} = [(A_{control} - A_{test})/A_{control}] \times 100$$

where $A_{control}$ is the absorbance of the control (DPPH solution without test sample) and A_{test} is the absorbance of the test sample (DPPH solution plus antioxidant). The inhibitory concentration (IC_{50}) value denotes the concentration of sample which is required to scavenge 50% of DPPH free radicals.

5.6.4 Establishment of the human oral cancer cell line

A human oral cancer cell line was maintained at a cancer biology laboratory at Sathyabama University, Chennai, India. This cell line was grown in complete culture medium consisting of high glucose DMEM (GE Health Life Sciences, Logan, UT) supplemented with 10% FBS (ThermoScientific, Cramlington, Northumberland) and 1 X penicillin at 37^{o}C in a humidified atmosphere at 5% CO_2 (Galaxy 170 S-CO_2 Incubator, Eppendorf, Hamburg, Germany).

5.6.5 Inhibition of the KB cell growth

The MTT assay was used to assess the inhibitory effects of the crude acetone and water leaf extracts of *T. violacea* on the oral cancer cells. The KB cells were seeded in 96-well plates and incubated for 24 h at 37°C in a humidified 5% CO_2 incubator. After 24 h the cells were washed three times with PBS buffer. The cells were then treated with acetone and water extracts prepared in culture media at concentrations ranging between 0.003 mg/mL and 2 mg/mL. Culture media was used as a negative control. The cells were incubated for 24 h after which the media was aspirated and the cells washed twice with PBS buffer. Thereafter, 100 µL of the culture media was added into each well followed by addition of 10 µL of MTT solution (5 mg/mL in phosphate-buffered saline). The plates were then incubated for 4 h after which 85 µL of the media was removed from each well and 100 µL of DMSO was added. The plates were then gently shaken to solubilize the formazan. The amount of formazan produced was then measured at 570 nm using a microplate reader (Perklin Elmer). The percentage viability of Kb cells was calculated as follows:

Cell Viability (%) = [(absorbance of untreated control – absorbance of treated Sample)/absorbance of untreated control] × 100.

The Inhibitory concentration (IC_{50}) was calculated from the straight line graph plotted in the Excel software using percentage viability.

5.6.6 Morphological Observations

The morphological features of the treated KB cells were examined using microscopy. The KB cells were seeded in 12-well plates and incubated for 24 h at 37 ºC. After 24 h the cells were treated with *T. violacea* acetone and water crude leaf extracts at 0.5x IC_{50}, 1x IC_{50} and 2x IC_{50}. The 12-well plates were then incubated at 37ºC in a humidified 5% CO2 incubator. After 24 h images were taken using a microscope (Olympus IX53, Tokyo, Japan) at 20 x magnification.

5.6.7 Acridine orange / Ethidium bromide (AO/EB)

Dual AO/EB staining was used to visualise cells undergoing apoptosis. The KB cells were treated as explained in section 2.6. Untreated cells were taken as negative control while positive control cells were treated with Melphalan. After 24 h the cells were washed with PBS twice and 100 µL of PBS was then added to all the wells. Thereafter, 5 µL of acridine orange (100 µg/ml) was added into each well followed by the addition of 5 µl ethidium bromide (100 µg/ mL) after 5 min. The plates were covered with foil and incubated for 10 min at room temperature before visualising and imaging under a fluorescence microscope (Evos-FL- AMG,

Life Technologies, Bothwell, WA).

5.6.8 Multicaspase activity

The multicaspase activity in the KB cells stimulated with either acetone or water *T. violacea* as in section 2.6 was evaluated using the Multicasapase SR kit (Cat. No. 4500-0500, Merck KGaA, Darmstadt, Germany) following the manufacturer's instructions. In brief, 10 µl of the caspase working solution was added to 100 µl of 1 x 10^6 cells/ml and incubated for 1 h at 37ºC in a CO_2 incubator. Thereafter, 100 µl of 1x apoptosis wash buffer was added to each tube. The samples were then centrifuged for 5 min at 300 x g and the supernatant was discarded. Exactly 200 µl of 1x apoptosis wash buffer was added to the tubes, the sample was homogenised, then centrifuged for 5 min at 300 x g and the supernatant was discarded. The cells were then suspended in 200 µl of the caspase 7- aminoactinomycin D (7–AAD) working solution and incubated for 10 min at room temperature. Each sample was analysed with the Guava Easy Cyte 12HT flow cytometer (EMD Millipore Corp, Bellerica, MA) using 10 000 events.

5.6.9 Cell cycle analysis

The cell cycle analysis was conducted to determine the percentage of cells in G0/G1, S and G2/M phases based on DNA content. The Guava cell cycle reagent (Cat. No.4500-0220, MERCK) was used following the manufactures' instructions. Briefly, about 200 KB cells per microliter were treated with either *T. violacea* acetone or water leaf as in section 2.6 for 24 and 48 h. Untreated cells were taken as negative control while positive control cells were treated with Melphalan. After each time point, both the treated and controls were harvested and washed with 1x PBS twice. The cells were fixed with ice-cold ethanol and kept at 4ºC for at least 12 h before staining. The cells were stained with propidium iodide. The samples were transferred to 96 well plates and then incubated at room temperature for 30 min before acquiring 10,000 events using the Guava Easy Cyte 12HT system. The experiment was done in triplicate.

3.0 Statistical analysis

The experimental results were expressed as mean ± Standard Error (SE) of three replicates. *P* values less than 0.05 were considered statistically significant. Microsoft Excel 2010 statistical package was used.

5.7 RESULTS

5.7.1 The antioxidant activity of crude acetone and water extracts of *T. violacea*

The free radical scavenging activity of the crude acetone and water extracts of *T. violacea* were assessed using the DPPH assay. Figure 5.6 shows that there was a concentration dependent free radical scavenging activity by both extracts that ranged between 49% and 73%. The acetone crude extract exhibited a radical scavenging activity which increased as the concentration of the extract increased from 51% to 68% while for the water extract it increased from 49% to 73%. Although the water extract showed a slightly greater ability to scavenge free radicals (Figure 5.6) than that of acetone, there was no significant difference ($p > 0.05$) between the scavenging activities of the two extracts. The free radical scavenging ability of ascorbic acid which was approximately 100%, remained significantly higher ($p< 0.05$) than that of both the extracts irrespective of the concentration (Figure 5.6). The IC_{50} values for ascorbic acid, acetone extract and water extract were found to be 0.002 µg/mL, 207.33 µg/mL and 168.88 µg/mL respectively.

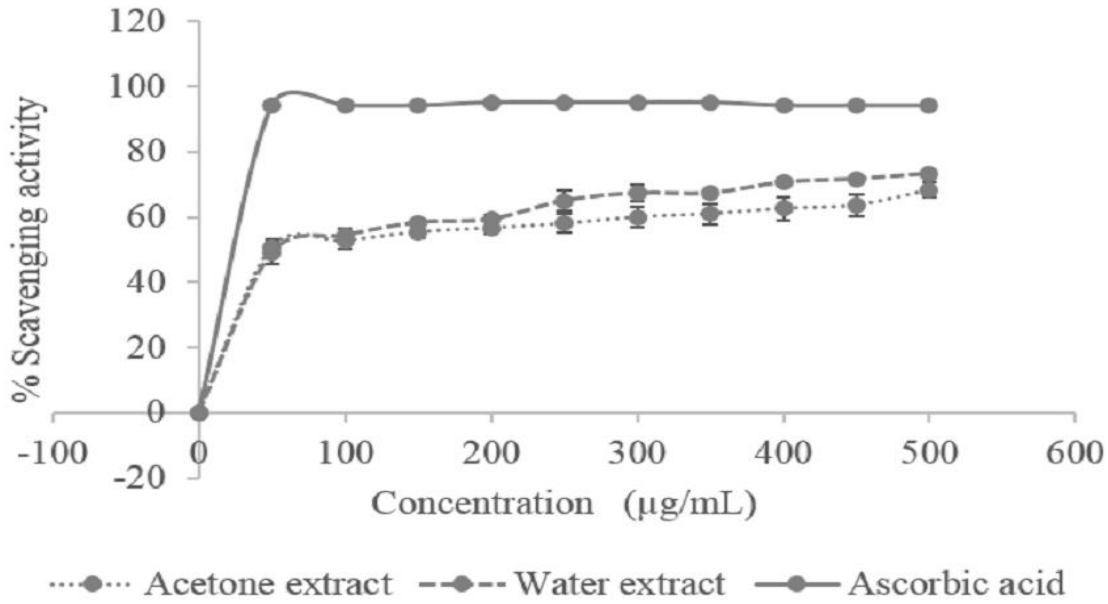


Figure 5.6: Percentage free radical scavenging activity of acetone and water crude leaf extracts from *T. violacea* examined using the DPPH assay. Ascorbic acid was used as a positive control. Error bars indicate the standard error of the mean of three biological replicates.

5.7.2 The effect of acetone and water leaf extracts on the viability of the KB cells

The inhibitory effect of crude acetone and water leaf extracts of *T. violacea* was evaluated using the MTT assay. The acetone extract exhibited a dose dependent inhibitory effect on the treated KB cells as shown in Figure 5.7. At concentrations 0.003 mg/mL to 0.1 mg/mL there was no significant difference ($p > 0.05$) in the viability of the control and the treated cells while for concentrations ranging from 0.25 mg/mL to 2 mg/mL a significant difference was observed ($p < 0.01$). In a similar manner, there was no significant difference ($p > 0.05$) in the cells treated with water extracts at concentrations between 0.003 mg/mL and 0.5 mg/mL compared to the control. A highly significant difference ($p < 0.01$) was observed between the control and the cells treated when the concentration of the water extracts was increased from 0.75 mg/mL to 2 mg/mL. Treatment with acetone showed a higher inhibitory effect compared to water extract. The IC_{50} values for acetone and aqueous extracts were 0.2 mg/mL and 1 mg/mL, respectively. These IC_{50} values were used in subsequent experiments.

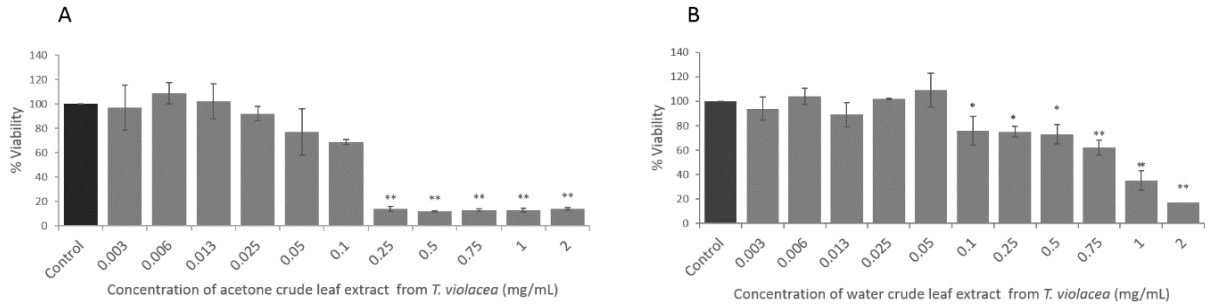


Figure 5.7 Cell viability of KB cells assessed using the MTT assay. KB cells were treated with various concentrations of crude acetone (A) and water (B) leaf extracts from *T. violacea.* Error bars indicate the standard error of the mean of three biological replicates. *p < 0.05 and **p < 0.01 untreated control vs treatment.

5.7.3 Morphological observations

When the KB cells were treated with acetone extracts at 0.5 x IC_{50} (0.1 mg/mL), 1x IC_{50} (0.2 mg/mL) and 2 x IC_{50} (0.4 mg/mL) a decrease was observed in the number of cells as the concentration of the extract increased (Fig. 5.8). Cell shrinkage, formation of membrane blebs and rounding of the cells was observed in all treatments with acetone extracts at 0.5 x, 1x and 2 x IC_{50} (Fig. 5.8). Similar observations were made in treatments involving the water extracts at 0.5 x IC_{50} (0.5 mg/mL), 1 x IC_{50} (1 mg/mL) and 2 x IC_{50} (2 mg/mL). A concentration dependent reduction in the number of cells was observed. At 0.5 x IC_{50} only cell shrinkage was observed. Cell shrinkage, formation of membrane blebs and rounding of the cells was observed at concentrations of 1x and 2 x IC_{50} (Fig. 5.8).

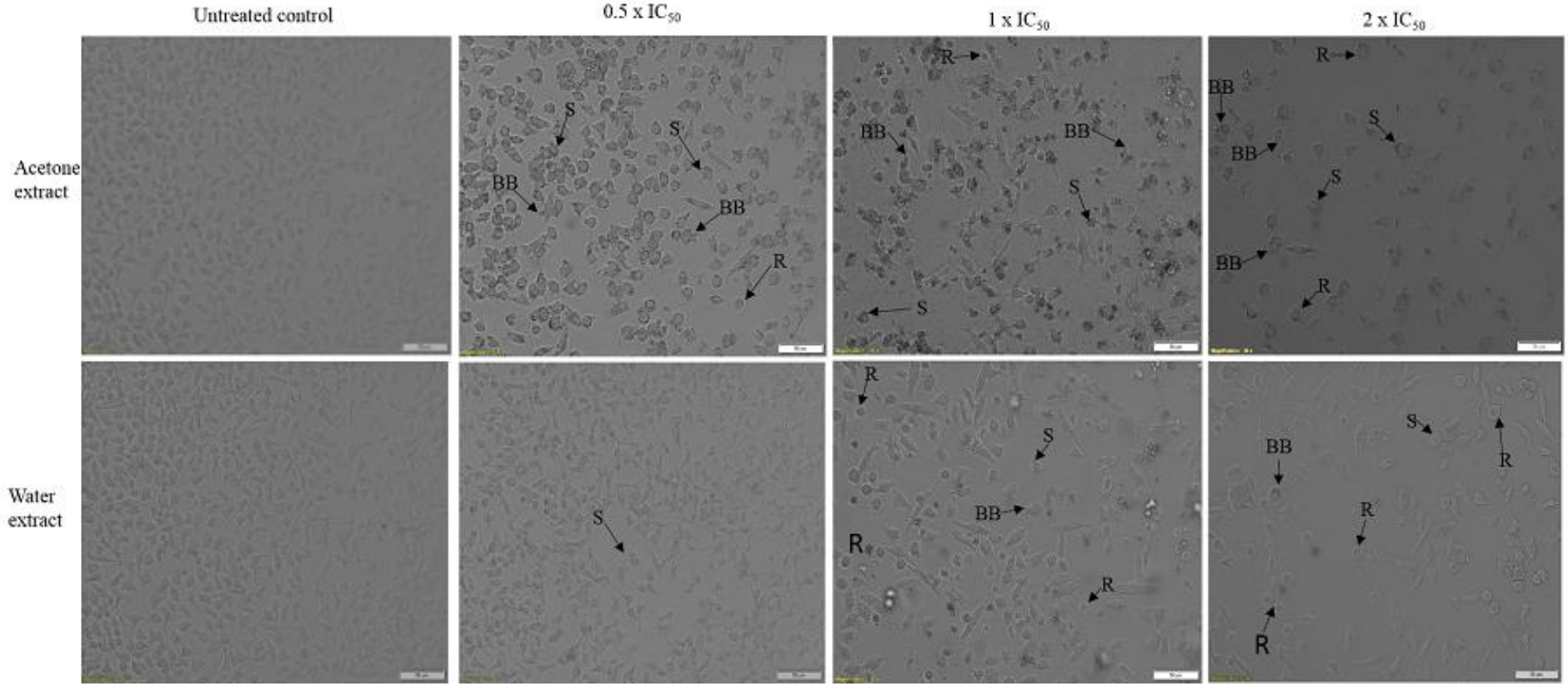


Figure 5.8 Microscopy images of KB cells treated with acetone and water extracts at inhibitory concentrations (IC_{50}), 0.5xIC_{50} (0.1mg/mL), 1xIC_{50} (02mg/mL), 2 x IC_{50} (0.4mg/mL) and 0.5 x IC_{50} (0.5 mg/mL), 1 x IC_{50} (1mg/mL), 2 x IC_{50} (2mg/ml), respectively. Images were taken at 20x magnification. Scale bars represent 50µm. Morphological cell changes after treatment with acetone and water extract: BB: Blebbing, R: rounding, S: Shrinkage.

5.7.4 AOEB staining

Microscopic images of the KB cells treated at 0.5 x IC_{50} of acetone (0.1 mg/mL) and water (0.5 mg/ml) extracts showed yellow/green staining indicating the induction of apoptosis (Figure 5.9). The number of apoptotic cells increased, as indicated by yellow staining of cells, as the concentration of the extracts increased (Fig. 5.9). At concentrations of 1 x IC_{50} for acetone (0.4 mg/mL) and water (1 mg/mL) extracts there was a higher number of cells staining bright yellow compared to those at lower concentrations. At a higher concentration of 2 x IC_{50} the number of cells that stained red increased indicating necrosis of the cells. In general, there was a higher number of apoptotic cells in the acetone treatment compared to those treated with water.

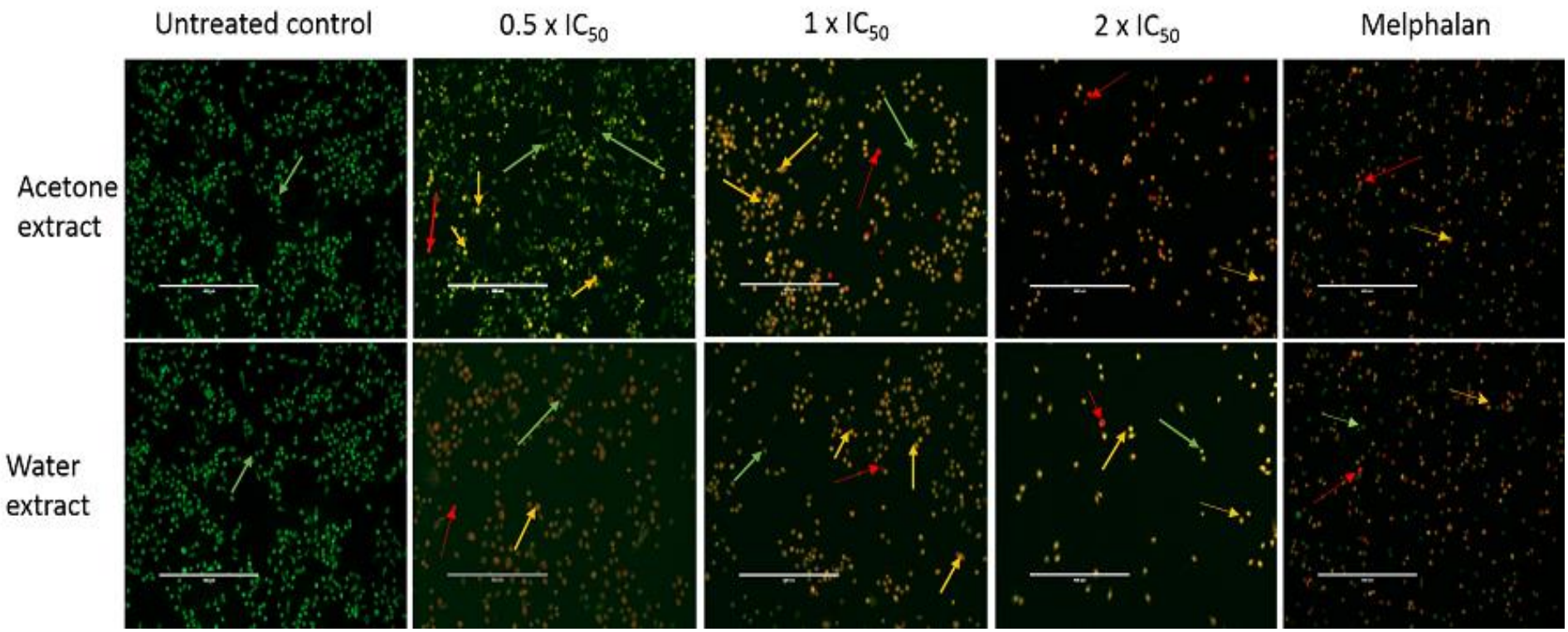


Figure 5.9 Illustration of dual AOEB staining images of Kb cells treated with crude acetone [0.5 x IC_{50} (0.1 mg/mL), 1 x IC_{50} (0.2 mg/mL) and 2 x IC_{50} (0.4 mg/mL)] and water [IC_{50} (0.5 mg/mL), 1 x IC50 (1 mg/mL) and 2 x IC_{50} (2 mg/mL)] leaf extracts from T. violacea. Images were captured at 10 x magnification. Melphalan was used as a positive control. Scale bars represent 400 µm. Viable cells (green arrow) excluded ethidium bromide and their intact nuclei stained bright green, while apoptotic cells (yellow arrow) with

5.7.5 Multicaspase activity

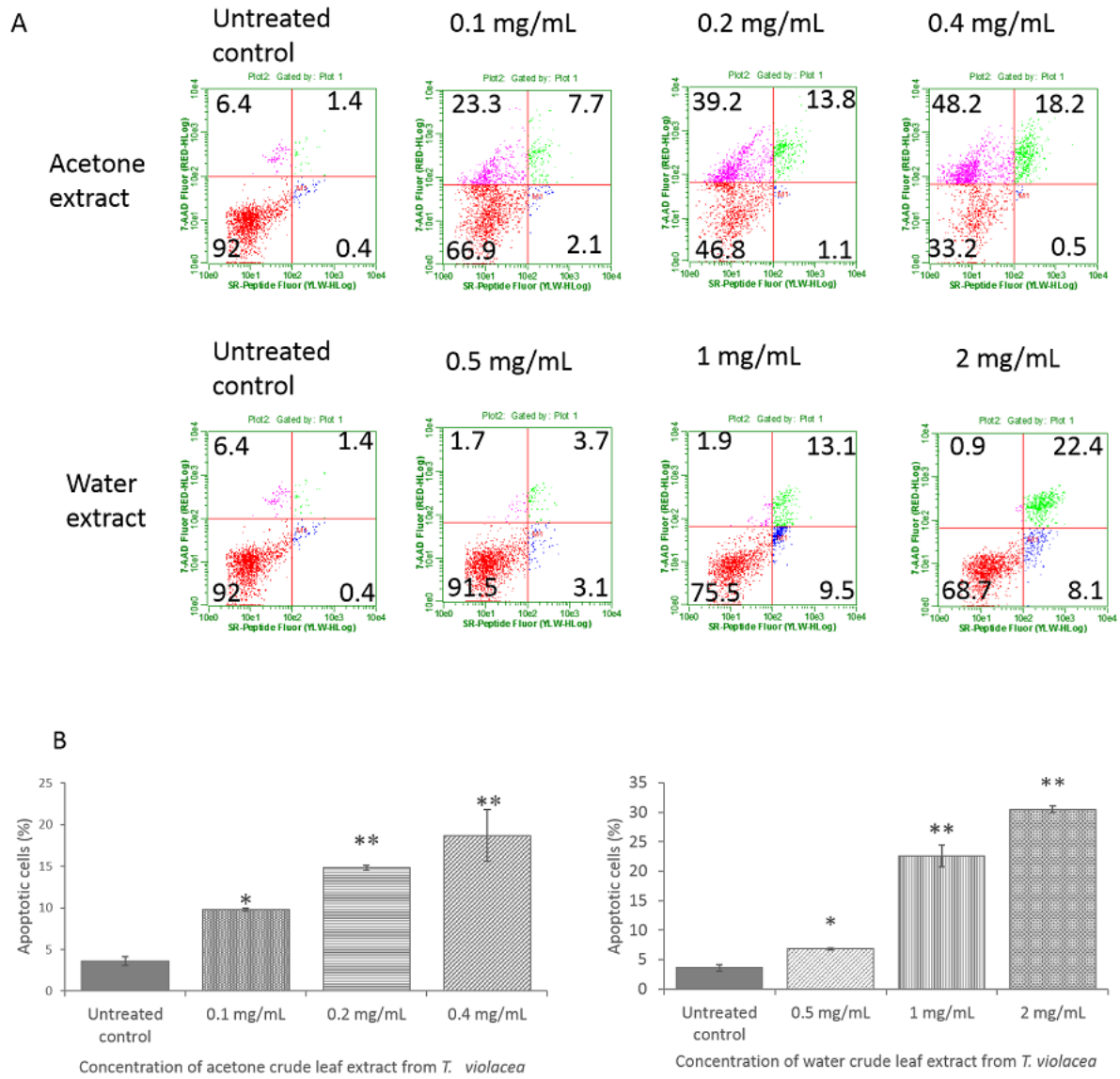


Figure 5.10. A: Dot plot analysis depicting KB cells treated with crude acetone and water leaf extracts of *T. violacea*, stained with 7-AAD and acquired using the Guava Caspase Software module. Events in each of the four quadrants are as follows: LL quadrant: Viable cells, not undergoing detectable apoptosis; LR: Cells in the middle stages of apoptosis; UR quadrant: cells in the late stages of apoptosis or dead and UL quadrant: necrotic cells. B: Bar graphs represent the % apoptotic cells (±SEM). *p < 0.05 and **p < 0.01 untreated control vs treatment.

The apoptotic effect of the acetone and water extracts was examined on KB cells using the Multicaspase SR kit. Flow cytometry analysis showed that the acetone and water extracts induced apoptosis in Kb cells (Figure 5.10A). Although, the acetone extract induced apoptosis

in a concentration dependent manner (9.8 % at 0.1 mg/mL, 14.9 % at 0.2 mg/mL and 18.7 % at 0.4 mg/mL), it also induced necrosis in a similar manner at higher percentages (23.3% at 0.1 mg/mL, 39.2 % at 0.2 mg/mL and 48.2% at 0.4 mg/mL). On the other hand, treatment of Kb cells with water extract at 0.5mg/mL induced greater levels of apoptosis (6.8 %) compared to necrosis (1.7%). The percentage of apoptosis was 22.6 % and 30.5% at concentrations of 1 mg/mL and 2 mg/mL, respectively whereas the necrosis levels were 1.9 % and 0.9 % for the same extract concentrations, respectively. Both extracts significantly (p <0.05) induced apoptosis in a dose dependent manner compared to the untreated control (Figure 5.10B).

5.7.6 Cell cycle analysis

Treatment of the KB cells with crude acetone extracts at concentrations of 0.1 mg/mL, 0.2 mg/mL and 0.4 mg/mL for 24 h resulted in a decrease in the percentage of cells in the G0/G1 phase of the cell cycle and a dose dependent increase in the G_2 phase (Fig. 5.11A). After 48 h treatment with the same concentrations of the extract a significant (p<0.05) decrease in the G_1/G_0 and an increase in the G_2 phase was observed (Figure 5.11B). In a similar manner, 24 h treatment of the KB cells with crude water extract at concentrations of 0.5 mg/mL, 1 mg/mL and 2 mg/mL resulted in a decrease in the percentage of cells in the G_0/G_1 phase and an increase in the cell population in the G_2 phase as the concentration of the extract increased. After 48 h treatment a more pronounced decrease in the G_1/G_0 and an increase in the G_2 phase of the cell cycle was observed. The 24 h and 48 h treatment of the KB cell line with Melphalan, a positive control, showed an increase in S phase cell population.

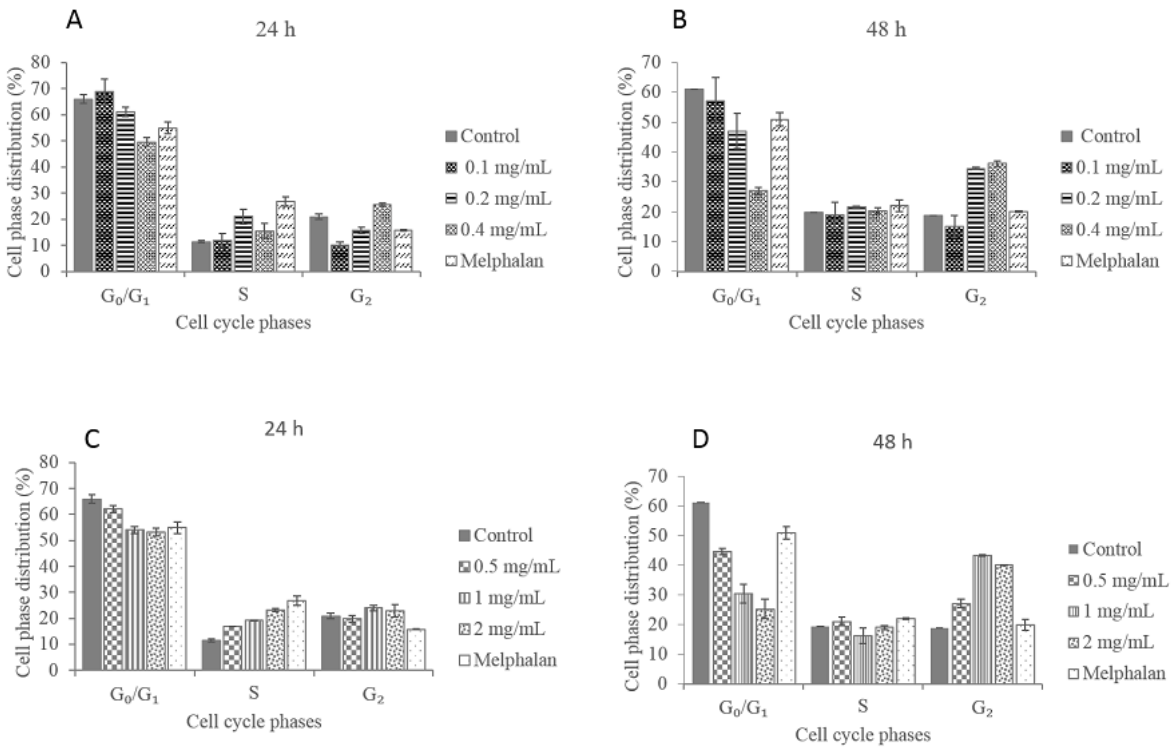


Figure 5.11 The effect of *T. violacea* acetone and water extracts on the cell cycle in the KB cells. Cells were treated for 24 h (A and C) and 48 h (B and D). **A** and **B** represents acetone extract at different concentrations (0.1 mg/mL, 0.2 mg/mL and 0.4 mg/mL). **C** and **D** represents water extracts at different concentrations (0.5 mg/mL, 1 mg/mL and 2 mg/mL).

5.8 DISCUSSION AND CONCLUSION

Bioactive compounds such as phenolic acids, terpenoids, lignans, tannins, flavonoids, coumarins, quinones, and alkaloids which exhibit significant antioxidant and anti-inflammatory activities from certain medicinal plants, have played an important role in treatment of cancer (Tagne et al., 2014). Although *T. violacea*, is widely used in traditional medicine little research on the anticancer activity of the plant is available. In this study, the antioxidant activity of crude acetone and water extracts of *T. violacea* at a concentration of 500 µg/mL was approximately 68% and 73%, respectively (Fig. 5.6).

Phyto-active compounds such as phenols, flavonoids and saponins are present in *T. violacea* (Madike et al., 2017) and are probably responsible for the level of antioxidant activity observed in this study. Secondary metabolites such as phenolic components are potential antioxidants and free radical terminators (Takaidza et al., 2018). Free radicals such as reactive oxygen species (ROS) contribute to the initiation of oncogenesis (Twilley et al.,2017). Therefore, inhibition of these free radicals can potentially aid in the prevention of cancer progression (Ghagane et al.,2017). The inhibitory effect of acetone and water extracts of *T. violacea* on the growth of human oral cancer cells was observed to be dose dependent (Fig. 5.7) with IC_{50} values of 0.2 mg/mL and 1 mg/mL, respectively. Cell death of KB suggests the potential of the crude extracts of *T. violacea* to inhibit the progression of cancer. Cell death can occur via apoptosis or necrosis.

To determine the cause of cell death morphological and biochemical markers were examined. Morphological features of cells undergoing apoptosis include cell shrinkage, rounding of the cells and the formation of membrane blebs. All these morphological changes were observed in the KB cells (Fig. 5.8) indicating that the KB cells did undergo apoptosis. Staining of the cells with the AOEB dual stain is a further way of indicating apoptosis or necrosis. Acridine orange is a fluorescent dye that stains nuclear DNA when the cell membrane is intact whereas ethidium bromide stains cells that have lost membrane integrity (Liu et al., 2004).

Treatment of KB cells with acetone and water extracts of *T. violacea* resulted in an increase in the number of apoptotic cells in a dose dependent manner (Fig. 5.9). The cells treated with acetone and water extracts at concentrations 0.4 mg/mL and 2 mg/mL, respectively, showed a higher number of cells that stained yellow and red indicating membrane disruption. Similar results were observed with the positive control, Melphalan. The untreated cells had an even distribution of bright green staining which suggests that they did not undergo apoptosis.

Multicaspase activity and cell cycle arrest were the molecular markers that were examined to further confirm the apoptotic effects of the acetone and water extracts on the KB cells. The activation of multicaspase activity in the KB cells treated with *T. violacea* acetone and water extracts was examined by flow cytometry. The human oral cells were stained with caspase reagent and 7-AAD which can enter the cells only when the plasma membrane is damaged. This allows early apoptosis cells (Caspase reagent +, 7 –AAD -) to be distinguished from late apoptosis (Caspase reagent +, 7-AAD +) and necrotic cells (Caspase Reagent -, 7-AAD +). The percentage of apoptotic cells following treatment with the acetone and water extracts at concentrations of 0.5 x IC_{50}, 1x IC_{50} and 2 x IC_{50} was 9.8%, 14.9%, 18.7% and 6.8 %, 22.6 %, 30.5 %, respectively, compared to 1.8% in the untreated control (Fig. 5.10). These results suggest that both the acetone and water extracts of *T. violacea* induced apoptosis in a dose dependent manner. However, it was observed that the acetone extract also induced necrosis as the concentration of the extract increased.

Cysteine-dependent aspartate-specific proteases (Caspases), are triggered during the early stages of apoptosis. The caspases are synthesized as inactive zymogens but, once turned on, can initiate a proteolytic deluge, resulting in the breakdown of key cellular components required for normal cellular function (Turan et al., 2017). Elevated caspase activity is regarded as a marker for apoptosis (Du et al., 2017) and the observed cell death in this study most likely occurred through apoptotic induction. Loss of DNA content is a common feature of apoptosis (Ravi et al., 2012). This is verified by staining cells with propidium iodide and enumerating the cells at different phases of the cell cycle. In this study, cell cycle analysis after treatment for 24 h and 48 h with both the acetone and water extracts of *T. violacea* showed an increase in the percentage of cells in the G_2/M phase and a reduction in the G_0/G_1 phase while no major changes were observed in the S phase (Fig. 5.11).

These results suggest that the extracts caused cell cycle arrest at the G_2/M phase in the Kb cells while the positive control, Melphalan, induced S phase arrest. Recent studies have indicated that cancer can be viewed as a disease of the cell cycle due to inefficient cell cycle checkpoint control (Turan et al., 2017;Teiten et al., 2013; Prelowska et al., 2017). Cell cycle arrest of cancer cells is thus considered as one of the target mechanisms in cancer treatment. The continuous dividing ability of cells can be interupted by blocking cells in G_1, S, G_2 or M phases of the cell cycle. Therefore the ability of *T. violacea* extracts to arrest the cell cycle at G_2/M phase in the human oral cancer cells is of significance. According to Teiten et al. (2013) allicin metabolites and diallyl polysulfides found in *Allium sativum* have been reported to be strong

inducers of early mitotic arrest, aberrant tubulin depolymerisation that prevents the synthesis of normal spindle microtubules and interruption of microtubular dynamics, thereby leading to G_2/M arrest. The genetic relationship between *A. sativum* and *Tulbaghia* species may suggest that similar mechanisms of cell disturbances may be present in the species under consideration in this study. The apoptotic effect of the *T. violacea* extracts observed in this study is in agreement with the findings of Saibu et al. (Saibu et al., 2015) who demonstrated induction of apoptosis in a panel of four cancer cell lines treated with *T. violacea* water extract. The anticancer activity of the extracts of *T. violacea* can be attributed to some secondary metabolites such as phenolic compounds which have been shown to inhibit the formation of tumours by interfering with the growth, proliferation, metastasis as well as pro-apoptotic effects by blocking proteasome activity and inhibiting angiogenesis (Mbele et al., 2017).

In conclusion, acetone and water extracts of *T. violacea* were shown to exhibit inhibitory effects against the human oral cancer cells. This research provides strong evidence to suggest that death of the human oral cancer cells occurred through the induction of apoptosis and alteration of the cell cycle. Most cells showed cell cycle arrest at G_2/M phase. This study suggests that acetone and water extracts of *T. violacea* may have potential anticancer activity against human oral cancer cells supporting the traditional use of *T. violacea* in cancer treatment. Future studies are required to isolate the specific compound (s) with anticancer activity from *T. violacea.*

CHAPTER 6 THE IMMUNOMODULATORY ACTIVITY OF ACETONE AND WATER CRUDE EXTRACTS FROM *T. VIOLACEA* ON LPS STIMULATED RAW264.7 CELLS.

ABSTRACT

Tulbaghia violacea, commonly used as a medicinal plant in South Africa, has been shown to possess various pharmacological properties. However, there is limited information on its immuno-modulatory activities. The aim of this study was to evaluate the immuno-modulatory activity of acetone and water extracts of *T. violacea* on LPS-stimulated RAW264.7 macrophages. The toxicity of the extracts on LPS-stimulated RAW264.7 cells was assessed using the MTT assay after a 24 h treatment. The Greiss and Luminex assays were used to determine the effect of the extracts on the production of nitric oxide (NO) and the secretion of the cytokines in the treated cells, respectively. The results showed that cell viability was above 80% when concentrations of 50 µg/mL or less of both acetone and water crude was used. Treatment with the acetone extract had no significant effect ($p>0.05$) on the LPS induced NO production in RAW264.7 cells except at 50 µg/mL where significant inhibition was observed. The water extract had no significant effect ($p>0.05$) on NO production at all the concentrations. Treatment of LPS–induced RAW264.7 cells with acetone extract stimulated the production of IL-1α, IL-6 and TNF-α but had no significant effect ($p > 0.05$) on IL-1β. On the other hand, treatment with the water extracts stimulated the production of IL-1α, IL-6 but had no significant effect ($p>0.05$) on TNF-α and IL-1β. Treatment of LPS-induced RAW264.7 cells with the acetone extract had very little stimulatory effect on IL-4, IL-5 and IL-13 and no significant effect on IL10 whereas for the water extract a significant stimulatory effect was only observed for IL-4 after 48 h of treatment. High concentrations (>10000 pg/mL) of MCP-1, MIP1-α, MIP1-β, MIP-2, GCSF, GM-CSF, RANTES and IP10 were also observed in acetone and water extract treated RAW264.7 cells. The results of this study indicated that the acetone and water extracts of *T. violacea* have strong immuno-stimulative properties. Medication prepared from *T. violacea* may be beneficial to individuals by stimulating the immune system.

Key words: Cytokines, Inflammation, Immunomodulation, Lipopolysaccharide, *Tulbaghia*

6.1 INTRODUCTION

Tulbaghia violacea has been shown to possess medicinal properties such as antioxidant, antimicrobial and anticancer activity (Aremu and Van Staden, 2013). Anecdotal evidence also suggests that *T. violacea* has been used as an immune booster by communities that rely on medicinal plants for their wellbeing (Aremu and Van Staden, 2013). However, scientific evidence of the effects of *T. violacea* on the immune system is limited.

The human immune system is controlled by a series of immune responses mediated through different immune cells (Sun and Li, 2017). Inflammation is a normal physiological and immune response to tissue damage or injury (Ghosh et al., 2014). An inflammatory immune response usually occurs when the human body attempts to counteract potentially harmful agents, such as invading bacteria, viruses and other pathogens (Islam et al., 2013). Inflammation is controlled by a host of extracellular mediators in conjunction with regulators, including cytokines and growth factors (Kim et al., 2010, Mueller et al., 2010, Moro et al., 2012, Choi et al., 2015). Cytokines are the key modulators of inflammation, participating in acute and chronic inflammation via a complex network of interactions (Ghosh et al., 2014). Among the different types of immune cells, macrophages play an important role during inflammation. Macrophages reside in various tissues throughout the body in anticipation of any foreign agent or signal from damaged cells. When any tissue is breached and/or foreign agents gain entry into the body, the resident macrophages migrate to the site of injury. The macrophages are then activated to produce cytokines as well as enzymes such as inducible nitric oxide synthase (iNOS) and cyclooxygenases (COX) that result in the recruitment of additional cells of the immune system to the site of injury (Kim et al., 2010, Islam et al., 2013, Ayed et al., 2016).

Activated macrophages express iNOS which catalyzes the oxidative deamination of L-arginine to produce nitric oxide (Gao et al., 2012; Ferreira et al., 2013; Ayed et al., 2016). Nitric oxide has been shown to play an important role in the regulation of key biological activities in the vascular, neural and immune systems. However, the excess production of NO has been associated with gross tissue damage that results from sustained acute or chronic inflammation (Ghosh et al., 2014). Cytokines are proteins that play an important role in cell signalling (Ye et al., 2016). Cytokines have been categorized as either pro- or anti-inflammatory depending on their biological effects. Pro-inflammatory cytokines such as interleukin (IL)-1, IL6 and Tumour Necrosis Factor alpha (TNF-α) promote systemic inflammation whereas anti-inflammatory cytokines such as IL-4, IL5, IL10 and IL-13 inhibit an inflammatory response

(de Cassia da Silveira et al., 2014). Anti-inflammatory cytokines often prevent an inflammatory response by inhibiting the synthesis of inflammatory cytokines particularly IL-1 and TNFα in macrophages (Ye et al., 2016).

Although inflammation is primarily a protective response, any dysregulation in this response may result in detrimental consequences such as the lack of an immune response or gross tissue damage (Mueller et al., 2010). Therefore, inflammatory dysregulation warrants the need to modulate the immune system through the use of chemotherapeutic agents. The existing synthetic molecules like nonsteroidal anti-inflammatory drugs (NSAIDs) and selective COX-2 inhibitors produce side-effects such as increasing the incidence of adverse cardiovascular thrombosis (Damte et al., 2011). Alternative therapies such as natural plant extracts that have minimal side effects should be investigated to replace some of the existing anti-inflammatory drugs. Immunomodulation using medicinal plants can provide an alternative to conventional chemotherapy in the management of chronic inflammatory responses (Amirghofran et al., 2009). Some of the plants with immunomodulatory activity include (1) garlic which affects cell mediated immunity, (2) milk thistle that increases humoral and cellular activity and (3) Ginseng which enhances the production of macrophages, B and T cells (Amirghofran et al., 2009). The immunomodulatory activity of these plants has been attributed to various bioactive components such as resveratrol, organosulfur and polysaccharides (Wang et al., 2013).

Therefore, this study examined the effects of acetone and water extracts on the NO production and the concentration of cytokines in LPS stimulated RAW264.7 cells. The objectives of this study was to use different concentrations of acetone and water extracts from *T. violacea* and (i) assess the viability of RAW264.7 cells, (ii) determine the level of NO production, and (iii) determine their effect on the production of cytokines in RAW264.7 cells.

6.2MATERIALS AND METHODS

6.2.1 Plant material

Samples of *T. violacea* were obtained from an indigenous plant nursery in Gauteng, South Africa and maintained in the greenhouse at Vaal University of Technology. The plant was authenticated by a botanist, Professor Stefan Seibert at North West University (Potchefstroom, South Africa) where a voucher specimen (ST0008) was deposited in the AP Goossens Herbarium.

6.2.2 Preparation of *T. violacea* acetone and water crude extracts

The preparation of acetone and water extracts of *T. violacea* was done following the protocol previously described in section 4.3.2 and 4.3.3.

6.2.3 Culture of RAW 264.7-C macrophage cell line

The Abelson Murine leukemia macrophage adherent cell line (RAW 264.7-C) was obtained from Cellonex (Cellonex, Johannesburg, South Africa). This cell line was cultured in complete culture media consisting of high glucose phenol free DMEM (GE Health Life Sciences, Logan, UT) supplemented with 10% FBS (Thermo-Fisher Scientific, Waltham, MA) and 1 X penicillin, streptomycin and neomycin at 37^{o}C in a humidified atmosphere at 5% CO_2 in an incubator (ESCO, Changi South Street, Singapore).

6.2.4 Viability of RAW-264.7 cells after treatment with plant extracts

The RAW264.7 cells were seeded at a concentration of 1 x 10^5 cells/mL in 96-well plates in phenol free DMEM culture media and incubated at 24h at 37^{0}C in a humidified 5% CO_2 incubator. After 24 h of incubation, the media was aspirated and the cells washed twice with PBS. The cells were then treated with acetone and water extracts from *T. violacea* prepared in DMEM culture media at concentrations of 10, 20, 30, 40 and 50 µg/mL. LPS was added to all the treatments to a final concentration of 1 µg/mL. The control consisted of cells treated with LPS (1 µg/mL) only. The cells were incubated for 24 h at 37^{0}C in a humidified 5% CO2 incubator. After 24 h the media was aspirated and the cells washed twice with PBS buffer. Thereafter, 100 µL of the cell culture media was added into each well followed by 10 µl of the MTT solution prepared in PBS at a concentration of 5 mg/mL. The plates were then incubated for 4 h after which 85 µl of the media was removed from each well and 100 µL of DMSO was added. The plates were then gently shaken to solubilize the formazan. The amount of formazan produced was then measured at 570 nm using a microplate reader (Epoch 2, BioTek, Winooski,

VT). The percentage viability of RAW264.7 cells was calculated using the following equation: Cell Viability (%) = [(absorbance of LPS-treated control – absorbance of treated Sample)/absorbance of LPS-treated control] × 100.

6.2.5 Production of Nitric Oxide (NO)

The RAW 264.7 cells (2.0×10^5 cells/well) were seeded in 24-well plates in phenol free DMEM culture media with LPS at a concentration of 1µg/mL. The 24-well plates were incubated for 24 h at 37°C in a humidified 5% CO_2 incubator. After 24h, the cell culture media was replaced with 0.2 mL of phenol free DMEM media containing either *T. violacea* acetone or water crude extracts at concentrations of 10, 30 and 50 µg/mL. LPS was subsequently added to each well to a final concentration of 1 µg/mL. The plates were incubated for 24 h at 37^0C in a 5% CO_2 incubator. Thereafter the media was transferred to 2 mL tubes and was centrifuged at 500 x g for 5 min. The supernatant was stored at -20 ºC in aliquots of 100 µL. Supernatants from cells which were not treated with LPS served as a negative control while those treated with LPS only served as a positive control. Exactly 50 µL of the supernatant was used for determination of NO. The nitrite that accumulated in the culture medium was measured as an indicator of NO production based on the Griess reaction. Briefly, 50 µL cell culture medium were mixed with an equal volume of the Griess reagent (equal volumes of 1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid and 0.1% (w/v) naphthylethylenediamide- HCl), incubated at room temperature for 15 min, and the absorbance was measured at 540 nm using an Epoch 2 microplate reader (BioTeK, Winooski, VT). The amount of nitrite present in the samples was calculated by means of a standard curve generated using serial dilutions of $NaNO_2$ in fresh phenol free DMEM medium.

6.2.6 Immunomodulatory activity

The effect of acetone and water extract on cytokine expression in LPS- stimulated RAW264.7 cells was examined using the Milliplex MAP kit. Samples were prepared as in section 2.5. The Milliplex MAP kit consisted of Mouse Cytokine/Chemokine Magnetic Bead Panel (MYCTMAG70PMX25BK, MERK). The concentration of the cytokines in 25 µl of each of the supernatants was determined by the Luminex Multiple Assay according to the manufacturer's instructions (EMD Millipore Corporation, Bellerica, MA). The samples were run on a Luminex 200^{TM} system.

6.2.7 Data analysis

Statistical analysis and graphs were drawn using the Graphpad prism version 7 for Windows (Graphpad Software, La Jolla, CA). Normalized data was used to create heat maps using the CIMminer programme package (Weinstein et al., 1994).

6.3RESULTS

6. 3.1 Effect of acetone and water extracts from *T. violacea* on viability of LPS-stimulated RAW264.7 cells

The effect of acetone and water extracts at concentrations of 10, 20, 30, 40 and 50 µg/ml on the viability of LPS stimulated RAW264.7 cells was examined using the MTT assay after 24 h of incubation. Although there was variation in cell viability for the different concentrations of plant extracts, no significant difference ($p > 0.05$) in the viability was found between the treated and untreated samples. The viability of RAW264.7 cells was found to be 80% and higher (Fig. 6.1). Similar results were observed for the 48 h treatments (data not shown).

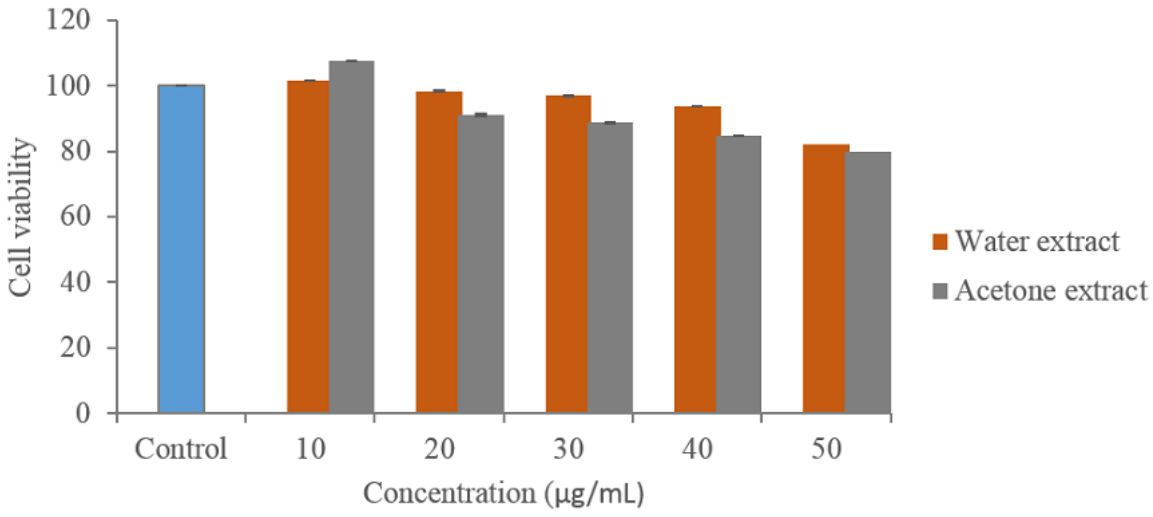


Figure 6.1 The effect of acetone and water extracts from *T. violacea* on the viability of RAW264.7 cells after 24 hr of incubation. Cell viability was expressed as percentage viability with respect to LPS treated cells which were taken to be 100% viable (Control). Data is expressed as mean ± SE of three replicates.

6.3.2 Effect of acetone and water extracts of *T. violacea* on NO production in LPS stimulated RAW264.7 cells

The amount of NO produced by the RAW264.7 cells decreased as the concentration of both the acetone and water crude extracts increased (Fig. 6.2). However, there was no significant ($p > 0.05$) difference in the mean NO production between the RAW264.7 cells that were treated with the crude water extracts and the control. Similar results were obtained for the treatments with the crude acetone extracts, with the exception of the cells treated with 50 µg/mL. A

significant (p< 0.05) decrease (46% vs LPS-treated only) in NO production occurred with the 50 µg/mL acetone extract (Fig.6. 2).

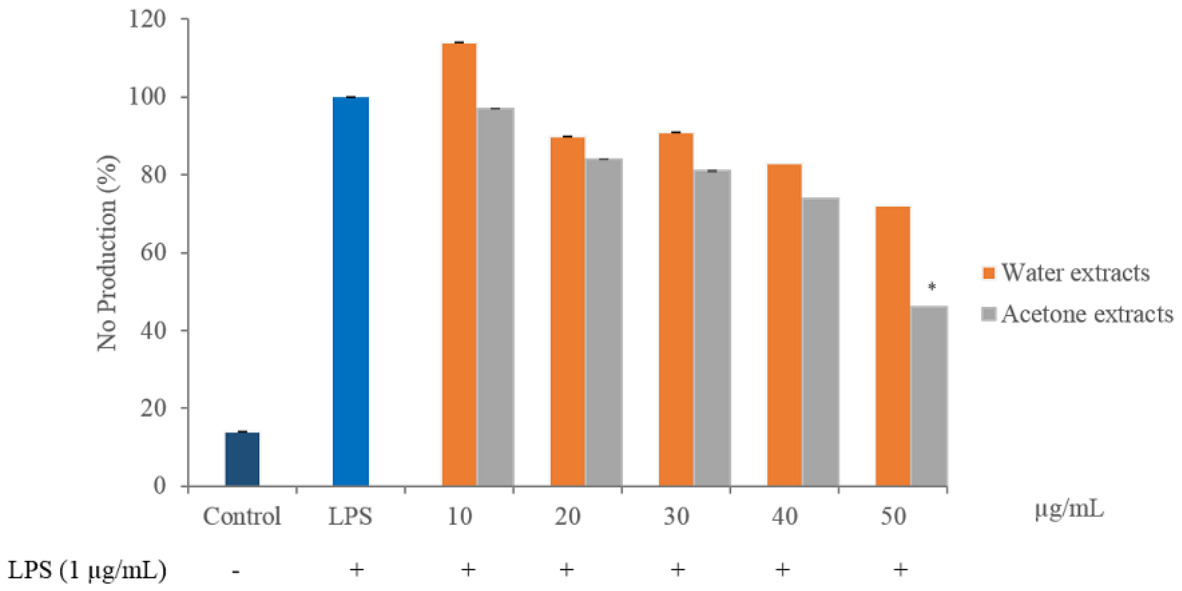


Figure 6.2 Effect of acetone and water extracts of *T. violacea* on NO production in LPS stimulated RAW264.7 cells. The cells were treated with different concentrations of *T. violacea* acetone and water extracts at 10 µg/mL, 20 µg/mL, 30 µg/ml, 40 µg/mL and 50 µg/mL. The media was collected after 24 h and assayed for nitrate production. Data is expressed as mean ± SE of three replicates. * Indicates a p< 0.05 compared to LPS – treated only.

6.3.3 Effect of acetone and water extracts from *T. violacea* on inflammatory cytokines

6.3.3.1 The effect of acetone and water extracts on pro-inflammatory cytokines

Treatment of the RAW264.7 cells with acetone extracts resulted in a significant (p<0.05) increase in the secretion of IL-1α after 24 h at all the tested concentrations of the extract (Fig. 6.3A) with the greatest amount obtained at a concentration of 30 µg/mL. However, after 48h a significant decrease (p<0.05) in IL-1α was observed at 10 µg/mL. No significant differences (p > 0.05) were observed between the LPS treated sample and the other treatments for IL-1β both after 24 h and 48 h (Fig. 6.3B). IL-6 was above the detection limit (15 000 pg/mL) in all the samples at the different concentrations of the acetone extracts after both the 24 and 48 h (Fig. 6.3C). The TNF-α production increased significantly (p < 0.05) after 24 h of stimulation with the crude acetone extract at a concentration of 30 µg/mL. There was a significant decrease

in the production of TNF-α when the crude acetone extract was used at concentrations of 10 and 50 µg/mL after 48 h (Fig.6. 3D).

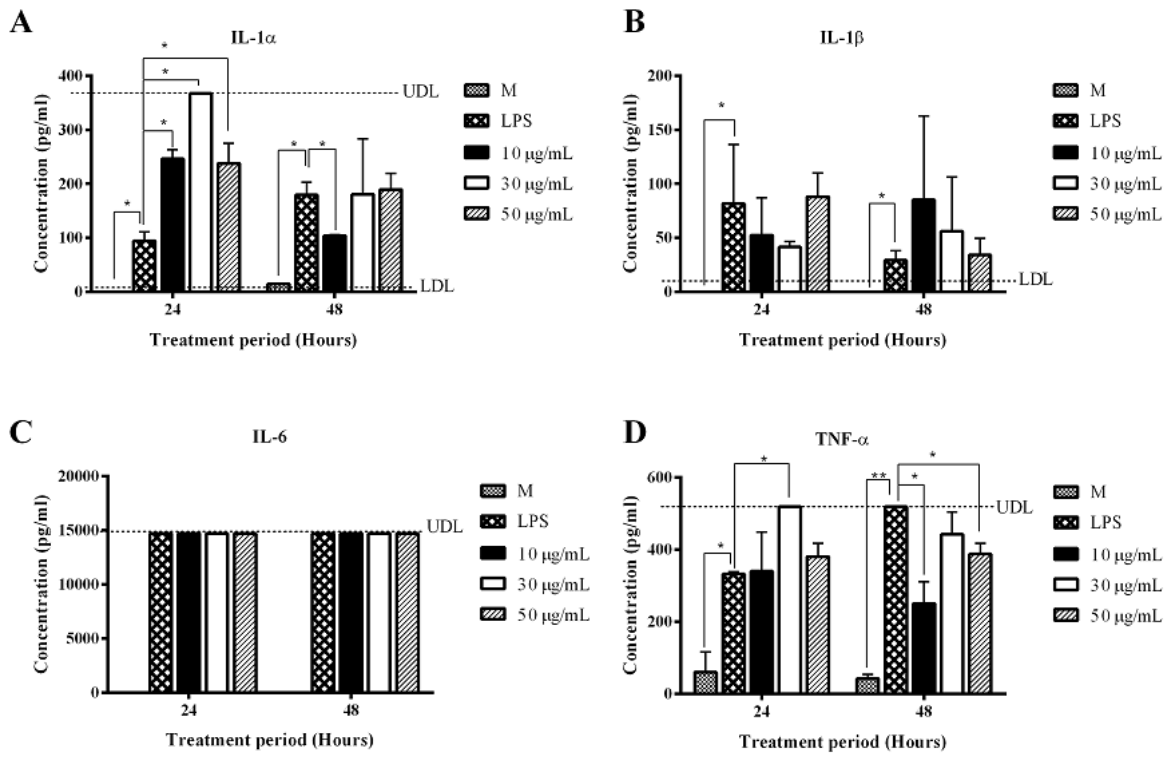


Figure 6.3 The effect of acetone extract on IL-1α, IL-1β, IL-6 and TNF-α production by LPS induced RAW264.7 cells. LPS (1µg/mL) stimulated RAW264.7 cells were treated with the crude acetone extracts at 10, 30 and 50 µg/mL for 24 and 48h. Error bars indicate SE of duplicate experiments, *p< 0.05, **p<0.01 treatment vs LPS alone. LDL: Lower detection limit, UDL: Upper detection limit.

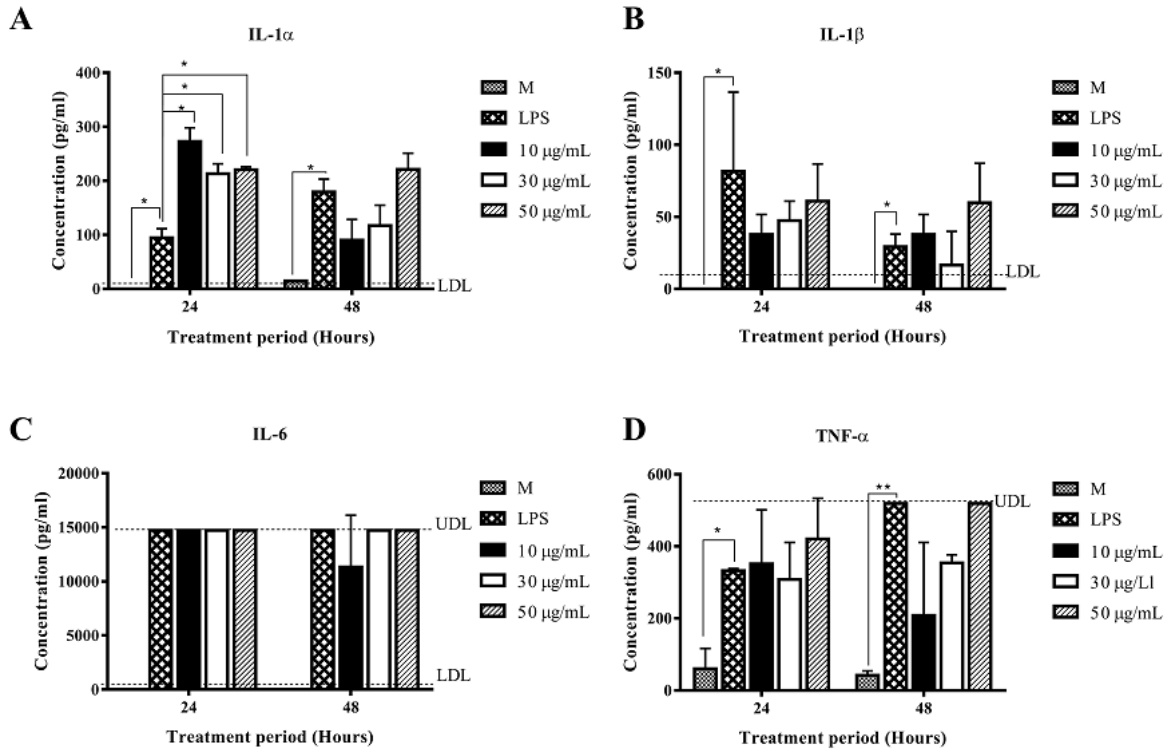


Figure 6. 4 The effect of crude water extract on IL-1α, IL-1β, IL-6 and TNF-α production by LPS induced RAW264.7 cells. RAW264.7 cells stimulated with LPS (1µg/mL) were treated with different concentration of 10, 30 and 50 µg/ml of water extracts for 24 and 48h. Data represent mean ± SD of duplicate experiments, *p< 0.05, **p<0.01 treatment vs LPS alone. LDL: Lower detection limit, UDL: Upper detection limit.

There was a significant increase (p<0.05) in IL-1α compared to the LPS control after 24 h for all three concentrations of the water extracts. However, after 48 h the water extract had no significant effect (p>0.05) on the IL-1α (Fig. 6.4A). No significant differences (p>0.05) in the mean production of IL-1β was observed in cells stimulated with LPS alone and those treated with all three concentrations of the crude water extract for both 24 h and 48 h (Fig. 6.4B). The production of IL-6 was above the detection limit (15 000 pg/mL) in all treatments involving the crude water extract at 24 and 48 hr. The only exception was the cells stimulated with 10 µg/mL of the crude water extract for 48 h (Fig. 6.4C) which showed a non-significant inhibition (p> 0.05). There was an increase in the production of TNF-α after 24 h of treatment with the crude water extract (Fig. 6.4D). However, after 48 h only the 50 µg/ml and LPS treated samples produced an increase in the production of TNF-α (Fig. 6.4D) which was on the upper detection limit.

6.3.3.2 Effect of acetone and water extracts from *T. violacea* on anti-inflammatory cytokines

Treatment with acetone extract at 30 µg/mL resulted in a significance ($p < 0.05$) increase in IL-4 after 24 h (Fig. 6.5A). However, it should be noted that the concentration of IL-4 was below 5 pg/mL. After 48 h a significant decrease in the secretion of IL-4 ($p < 0.05$) was observed at 50 µg/mL. For IL-5 a significant increase ($p<0.05$) was only observed for 30 µg/ml after 24 h while after 48 h no significant difference was observed between cells stimulated with LPS alone versus the treatments (Fig. 6.5B). The acetone extracts had no significant effect ($p > 0.05$) on the production of IL-10 at all concentrations although it increased as the concentration of the extract increased after the 24 h treatment (Fig. 6.5 C). After 48 h no significant difference ($p > 0.05$) was observed in IL-10. There was a significant ($p<0.05$) increase in IL-13 production at 30 and 50 µg/mL after a 24 h treatment. No difference was observed between LPS only and treated cells after 48 h for the IL-13 (Fig. 6.5D).

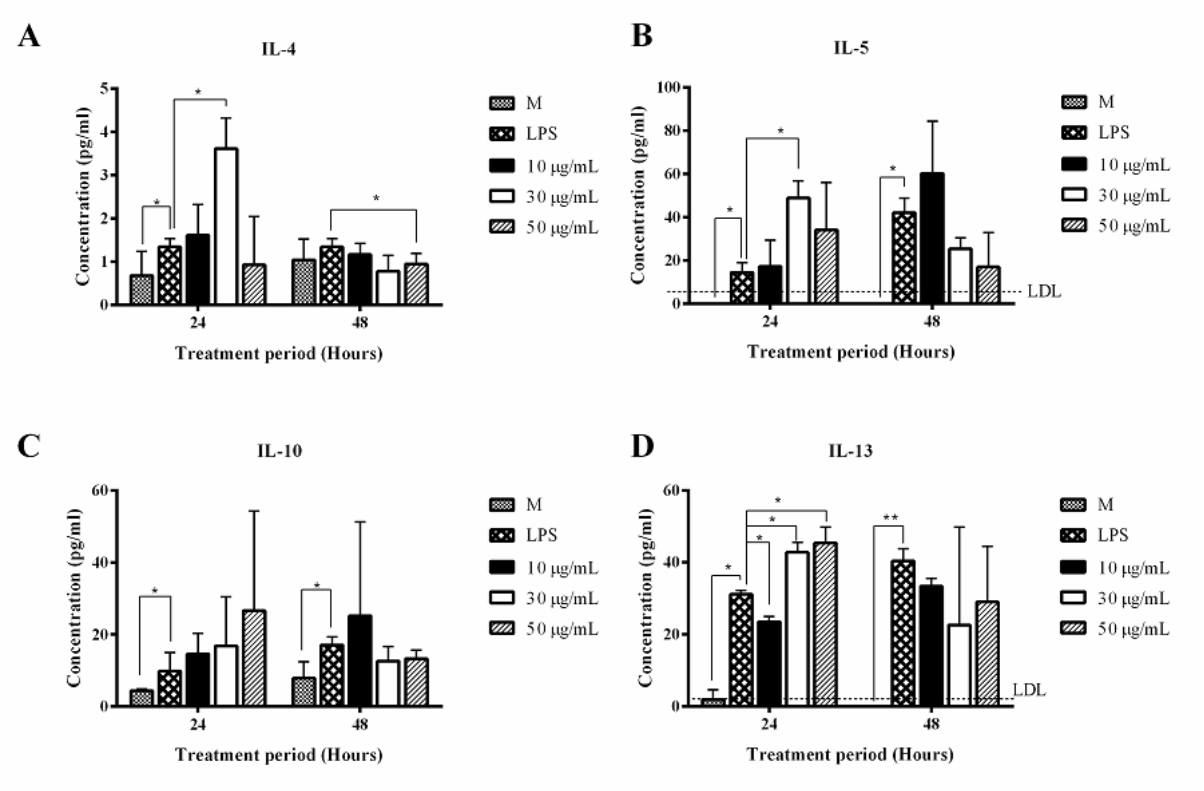


Figure 6. 5 The effect of acetone extract on IL-4, IL-5, IL-10 and IL-13 production by LPS induced RAW264.7. RAW264.7 cells stimulated with LPS (1µg/ml) were treated with different concentration of 10, 30 and 50 µg/ml of acetone extract for 24 and 48 h. Data represent mean ± SD, *$p< 0.05$, **$p<0.01$ treatment vs LPS alone. LDL: Lower detection limit, UDL: Upper detection limit.

For the water extract treatment no significant difference ($p > 0.05$) in IL-4 levels was observed between LPS alone versus the treatments after 24 h. A significant increase ($p<0.05$) in IL-4 was observed only for 50 µg/mL after 48 h (Fig. 6.6 A), although the concentration was below 4 pg/mL. For IL-5, IL-10, IL-13 there was no significant difference ($p>0.05$) between cells treated with LPS only vs the treatments for both 24 and 48 h (Fig.6. 6B, C, D).

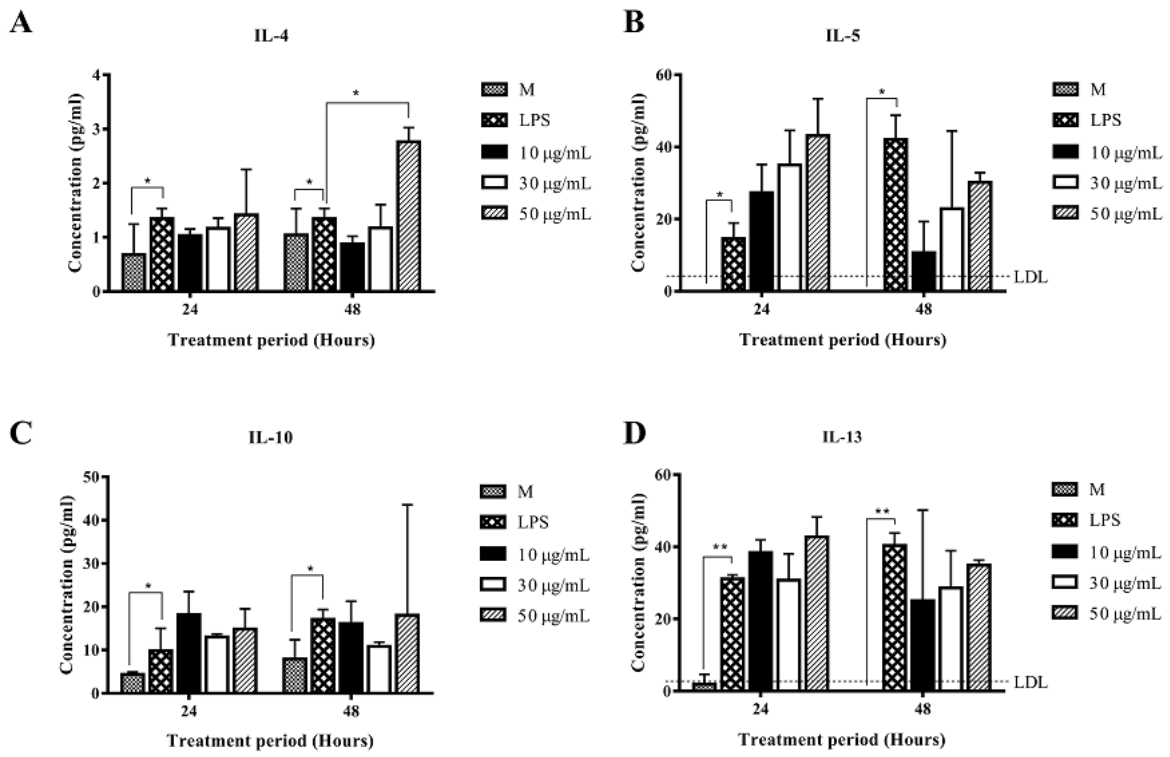


Figure 6. 6 The effect of water extract on IL-4, IL-5, IL-10 and IL-13 production by LPS induced RAW264.7. RAW264.7 cells stimulated with LPS (1µg/ml) were treated with different concentration of 10, 30 and 50 µg/ml of acetone extract for 24 and 48h. Data represent mean ± SD, *p< 0.05, **p<0.01 treatment vs LPS alone and untreated control.

6.3.3.3 Effect of acetone and water extract of *T. violacea* on MCP-1 and MIP-1α

The production of MCP-1 was above the detection limit (200 000 pg/mL) for all treatments of acetone extract at 24 and 48 h (Fig. 6.7A). Similar to acetone extract, treatment with water extract resulted in concentrations of MCP-1 above the detection limit for both 24 and 48 h (Fig. 6.7C). Treatment with acetone extract showed no significant difference ($p>0.05$) in the MIP-1α among the untreated control, LPS only and treatments for both 24 and 48 h (Fig. 6.7B). For MIP-1 α, treatment with water extract showed no significant difference ($p>0.05$) between

controls and treatments. However, elevated amounts of MIP-1α (10 000pg/mL) were observed after 48 h (Fig.6.7D).

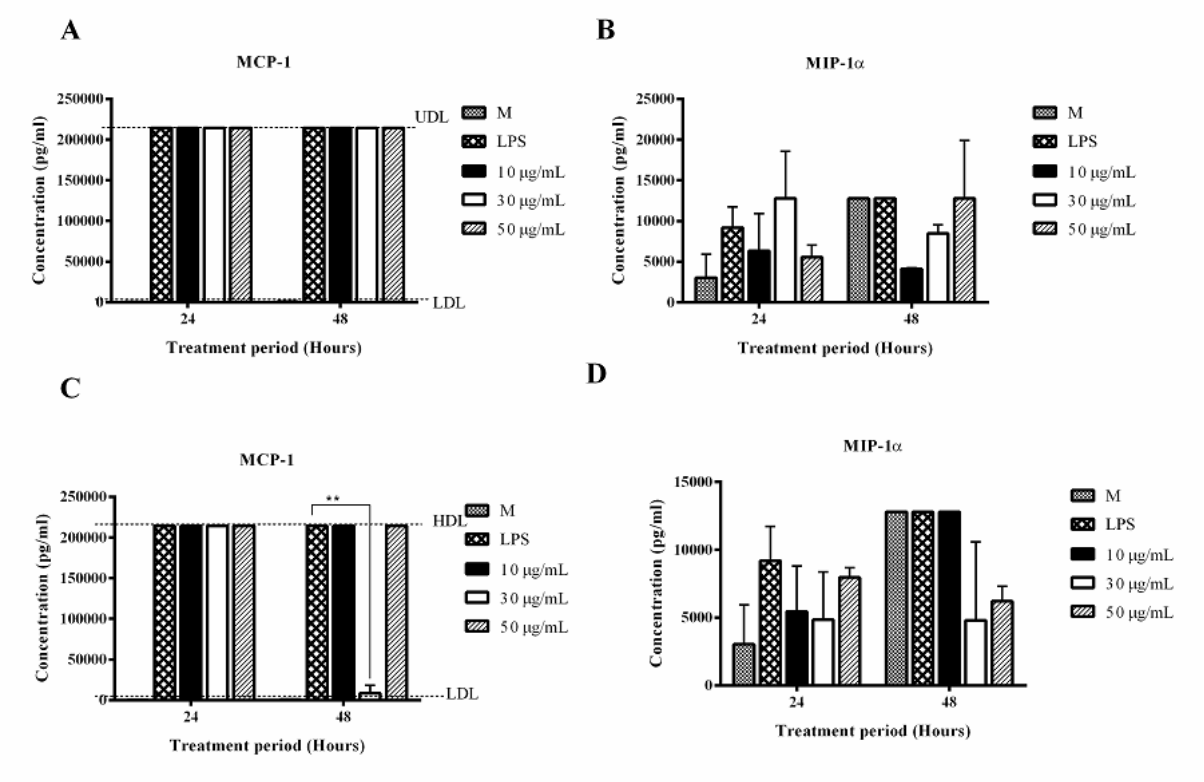


Figure 6. 7 The effect of acetone extract (A & B) and water extract(C & D) on MCP-1 and MIP-1α production by LPS induced RAW264.7. RAW264.7 cells stimulated with LPS (1µg/ml) were treated with different concentration of 10, 30 and 50 µg/ml of acetone extract for 24 and 48h. Data represent mean ± SD, *p< 0.05, **p<0.01 treatment vs LPS alone. LDL: Lower detection limit, UDL: Upper detection limit.

6.3.4 Heat map analysis of the 25- plex cytokine panel

Heat maps were constructed using the results from the entire 25-plex cytokine panel. After 24 and 48 h of stimulation both the acetone and water crude extracts induced the secretion of high levels of pro-inflammatory cytokines (Fig. 6.8 and 6.9). On the contrary, very little anti-inflammatory cytokines was produced after 24h and 48 h (Fig. 6.8 and 6.9). Two clusters of cytokines (cluster A and B) were observed following the 24h and 48h treatment with both the acetone or water crude extracts. After the 24h treatment cluster A was composed of IL-9,IL-15, IL-4, IL-β, IL-2, RANTES, IL-6, MCP-1, GM-CSF, IL-10, IL-5, IL-13, IL-α, TNFα. Cluster B consisted of IL-7,IFNG, IL-12P70, MIP-1β, IL-12P40, IP-10, KC, IL-17, MIP-1α, GCSF and MIP-2 (Fig 6.8). There was difference in the cytokine composition in both clusters after the 48h treatment (Fig 6.9). The anti-inflammatory cytokines IL-7, IFNG, IL-12P70, IL-12P40, IP-10, KC, IL-17 now grouped with cluster A while pro-inflammatory IL-9, MCP-1, IL-6, IL-13, RANTES, IL-α and TNF-α with cluster B (Fig. 6.9). After 48 h, cluster A was composed of pro-inflammatory cytokines with the exception of IL-13. The samples were also grouped in two clusters (Cluster C and D) following the 24 and 48h treatment. After 24 h, one of the samples (Mac- untreated macrophages) had its own node, while the acetone and water extracts were in the same cluster with samples treated with LPS only (Fig.6. 8). But after 48h the cluster that had Mac (Untreated macrophages) only now is clustered with W30 (water extract at 30 μg/mL).

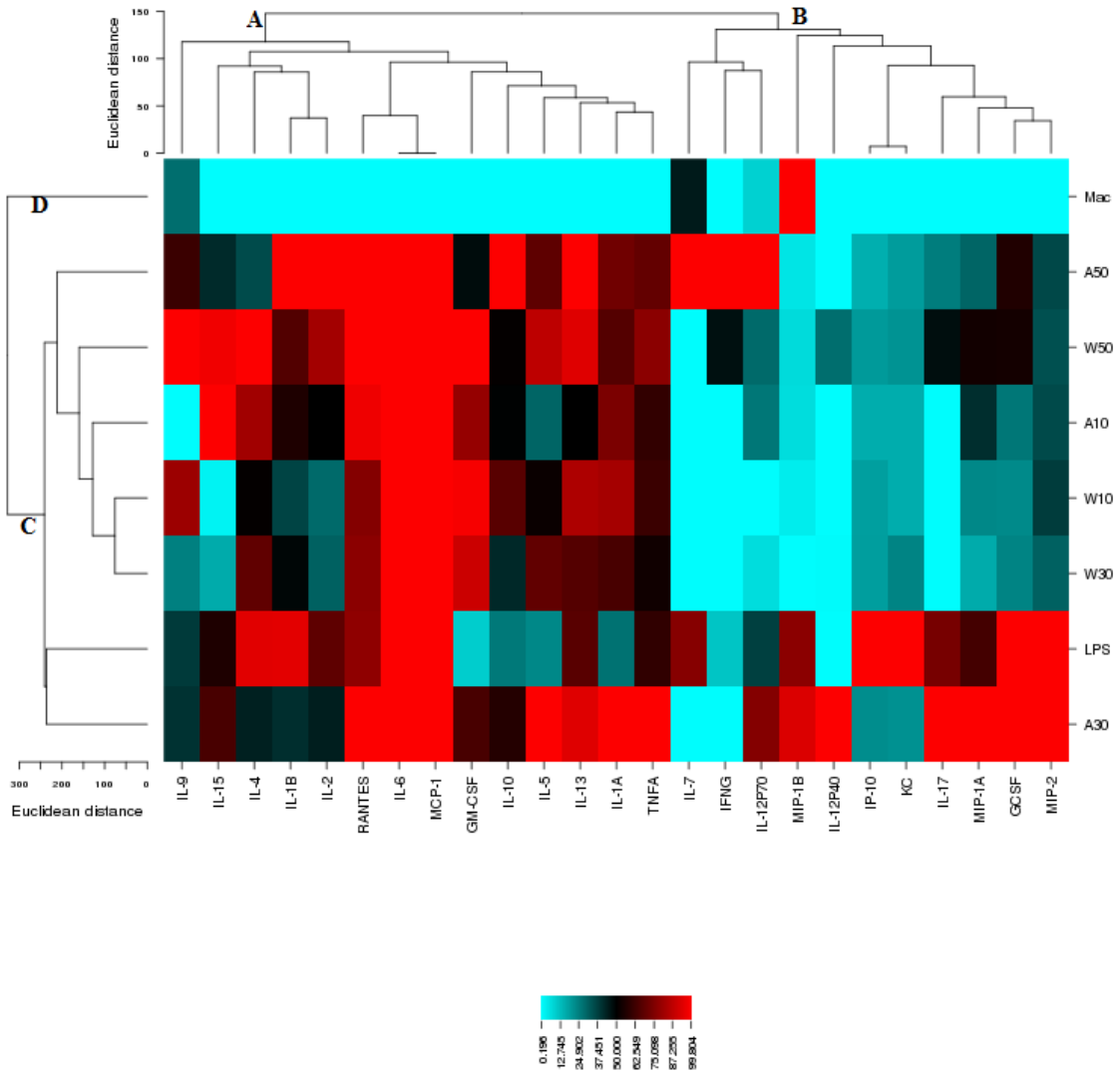


Figure 6. 8 Heat map reconstruction of 25-plex cytokine analyses of serum from RAW264.7 treated for 24 h with acetone and water extracts of T. violacea at concentrations of 10, 30 and 50 µg/mL. Mean cytokine concentration (pg/mL) are visually presented on a log scale with red, black and blue indicating, high, median and low, respectively (Indicated by color bar).

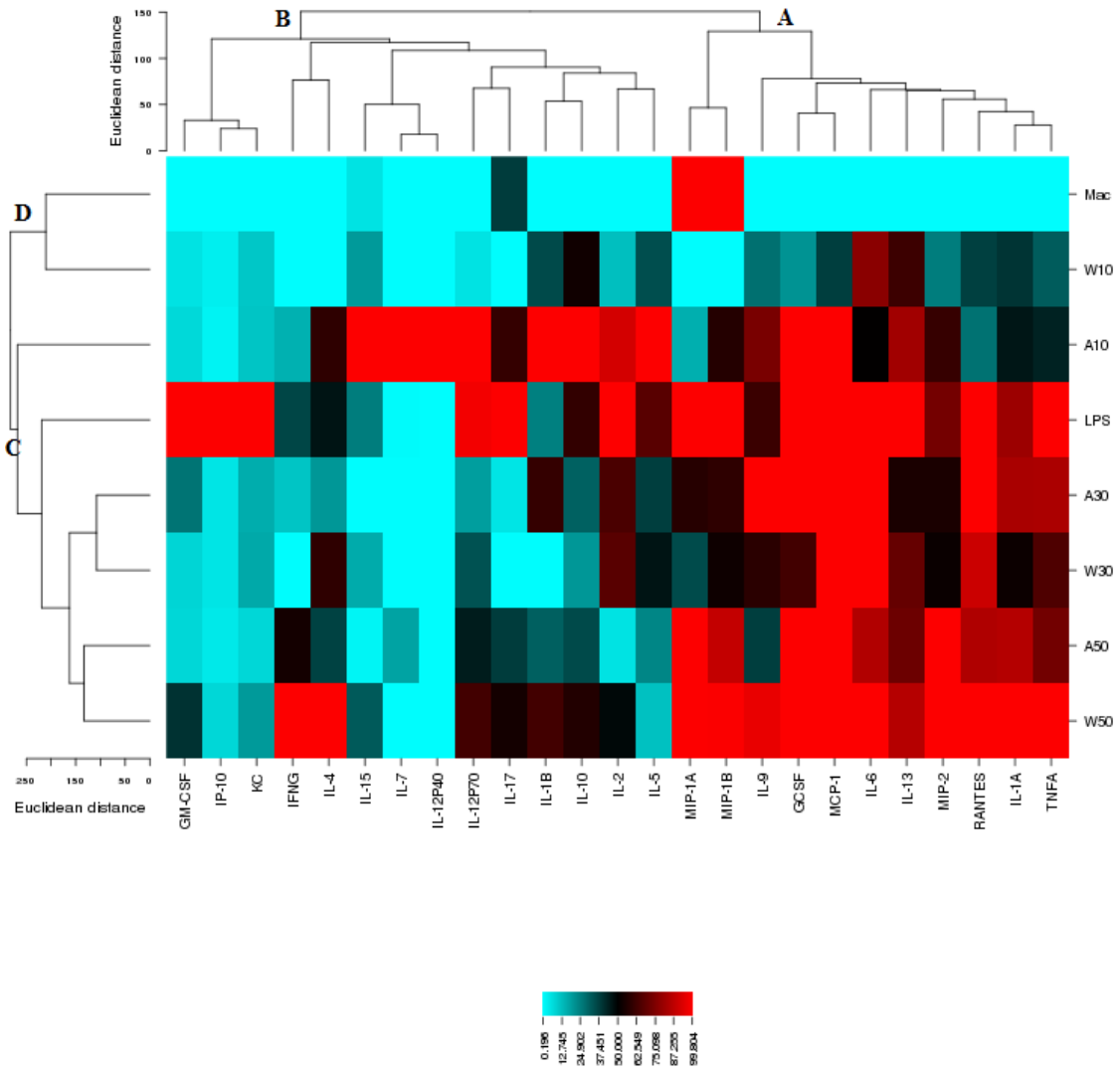


Figure 6. 9 Heat map reconstruction of 25-plex cytokine analyses of serum from RAW264.7 treated for 48 h with acetone and water extracts of *T. violacea* at concentrations of 10, 30 and 50 µg/mL. Mean cytokine concentration (pg/mL) are visually represented on a log scale with red, black and blue indicating, high, median and low, respectively (Indicated by color bar).

6.4 DISCUSSION AND CONCLUSION

Immunomodulators are compounds that are capable of interacting with the immune system to upregulate or downregulate specific aspects of the host response (Sunila and Kuttan, 2004). The activity of immunomodulators occurs through their effect on the different cell types involved in the innate (non-specific) and adaptive (specific) immune system, cytokine networks and signalling pathways (Moradali et al., 2007). Both immuno-stimulation and immune-suppression are needed to regulate the normal immunological functioning. The use of the immunomodulators is very important when the immune system does not function optimally especially in children, the elderly and those with a compromised immune system (Moraladi et al., 2007). Hence both immune-stimulating agents and immunosuppressing agents have their own standing and search for better agents exerting these activities from natural sources such as medicinal plants is of major interest (Makare et al., 2001).

The main goal of this study was to assess the immunomodulatory activities of acetone and water extracts of *T. violacea* by evaluating NO production and cytokine secretion in LPS-stimulated RAW264.7 macrophage cells. *Tulbaghia violacea* is known to induce cytotoxic effects *in vitro* and *in vivo* (Aremu and van Staden, 2013; Takaidza et al., 2018b). Therefore, it was important to first establish the concentrations at which the plant extract could be used with minimal cytotoxic effects. The viability of RAW264.7 cells treated with acetone and water crude extracts of *T. violacea* showed that concentrations of 50 µg/mL or less of both crude extracts maintained cell viability above 80% (Fig. 6.1). Therefore, concentrations within this range were used for treatments in further experiments and were considered to have no effect on the viability of the RAW264.7 cells.

The effect of acetone and water extracts on the production of NO in LPS stimulated macrophages was examined using the Griess reagent assay. The different concentrations of both extracts of *T. violacea* had no significant effect ($p>0.05$) on the production of NO. The only exception occurred when 50 µg/mL of acetone extract was used (Fig. 6.2). The results suggest that *T. violacea* acetone extract may have inhibitory effect on the inducible nitric oxide synthase (iNOS) which is responsible for the production of large amounts of NO (Damte et al., 2011; Ismal et al., 2013).

Pro-inflammatory cytokines such as TNF-α, IL-1 and IL-6 are involved in regulatory roles in the inflammatory process in order to restore tissue homeostasis (de Silveria e Sa et al., 2014). Macrophages can be stimulated by bacterial LPS to release TNF-α, which then stimulates the

release of IL-1β and IL-6. The induction of these cytokines is also dependent on NF-κB activation (Kim et al., 2008). In this study, the results indicate that the acetone extract of *T. violacea* had a stimulatory effect on the LPS-induced macrophages to produce TNF-α, IL-1α and IL-6 (Fig. 6. 3) but did not stimulate the production IL-1β. The water extract also showed a stimulatory effect on the production of IL-1α and IL- 6 but had no significant effect ($p > 0.05$) on TNF-α and IL-1β (Fig. 6.4). Macrophage inflammatory protein-1 (MIP-1) α, MIP-1β and monocyte chemoattractant protein-1 (MCP-1) are important chemokines (Low et al. 2001; Dai et al 2014). As indicated in Fig. 6.7 (A & C), high concentrations (>5000 pg/mL) of MCP-1 and MIP- 1α were observed in the samples treated with acetone and water extract of *T. violacea*. These results indicate that *T. violacea* acetone and water extract induced a pro-inflammatory activity. The high concentration of pro-inflammatory cytokines as illustrated in the heat maps (Fig. 6.8 and 6.9) suggests that the acetone and water extracts of *T. violacea* had strong a pro-inflammatory activity *in vitro*. The strong pro-inflammatory activity of *T. violacea* acetone and water extracts *in vitro* helps to explain some of the medicinal benefits attributed to the use of this plant in traditional medicine and could be used to provide a basis for isolation and identification of immune-regulating compounds from this plant.

The different functions of these cytokines mediate and regulate immunity. MIP-2 has been shown to be one of the major inducible chemokines with the ability to attract neutrophils to the site of inflammation (Martzer et al., 2001). TNF-α is a potent inflammatory mediator that is central to the inflammatory action of the innate immune system, including the induction of cytokine production, activation or expression of adhesion molecules, and growth stimulation. It stimulates the proliferation of normal cells, exerts cytolytic or cytostatic activity against tumour cells, and causes inflammatory, antiviral, and immunoregulatory effects. TNF-α has also been shown to perform a number of additional functions linked with lipid metabolism, coagulation, insulin resistance, and endothelial function. Indeed, it has been shown to be one of the most important and pleiotropic cytokines mediating inflammatory and immune responses (Turner et al., 2014).

IL-6 is expressed by an a number of cells, including mononuclear phagocytes, T cells, B cells, fibroblasts, endothelial cells, keratinocytes, hepatocytes, and bone marrow cells. It is involved in haematopoiesis, and is critical in the final maturation of B-cells into antibody-producing plasma cells (Turner et al., 2014). GM-CSF is a growth factor mainly produced by activated leukocytes and recognized through the GM-CSF receptor, by granulocytes, monocytes and macrophages. GM-CSF is known to stimulate chemotaxis, proliferation and differentiation,

and is also generally recognized as a pro-inflammatory cytokine (Grasse et al., 2018). Human granulocyte colony-stimulating factor (GCSF) is a hematopoietic growth factor, produced by a number of tissues that stimulate bone marrow to produce granulocytes and stem cells and release them into the blood. It also stimulates the survival, proliferation, differentiation, and neutrophil precursors and maturation functions. It plays a vital role in granulopoiesis, stimulate bone marrow to produce more WBC and enhance circulating neutrophils. GCSF has found its use for new therapies for cancer patients, for combating life threatening infections, healing and regeneration of tissues (Adusumilli et al., 2012).

RANTES, brings T cells, dendritic cells, eosinophils, NK cells, mast cells, and basophils to sites of inflammation and infection. It can also be involved in direct antimicrobial activity by inducing NO in macrophages However, RANTES can have detrimental effects via the recruitment of immune cells that enhance inflammatory processes such as arthritis, atopic dermatitis, nephritis, colitis, and other disorders (Levy 2009). C-X-C motif chemokine 10 (CXCL10) also known as interferon γ-induced protein 10 kDa (IP-10) or small-inducible cytokine B10 is a cytokine belonging to the CXC chemokine family. CXCL10 specifically activates CXCR3 receptor, a seven *trans*-membrane-spanning G protein-coupled receptor (GPCRs), which is predominantly expressed on activated T, B lymphocyte, natural killer (NK), dendritic and macrophage cells. CXCL10 induces chemotaxis, apoptosis, cell growth inhibition and angiostasis (Liu et al., 2011). The different functions of the pro-inflammatory cytokines suggest that the acetone and water extracts of *T. violacea* could act as an immune booster as suggested by Aremu and Van Staden (2013) and perhaps supports the use of this plant for that purpose.

Anti-inflammatory cytokines are a series of immuno-regulatory molecules that control the pro-inflammatory cytokine response. Anti-inflammatory cytokines include interleukin receptor antagonists such as IL-4, IL-5, IL-10 and IL-13 (Opal and DePalo, 2000). Treatment of LPS induced RAW264.7 cells with acetone extract of T. violacea stimulated the production of IL-4, IL-5 and IL-13 after 24 h but had no significant effect ($p > 0.05$) on the production of 1L-10 (Fig. 6.5). The water extract stimulated the production of only IL-4 (fig. 6.6) However, the concentration of anti-inflammatory cytokines in both the acetone and water treatments was below 100 pg/mL. This suggests that these extracts exhibited a poor anti-inflammatory potential. This is in agreement with a previous study by Adebayo et al. (2015) on the anti-inflammatory activity of *T. violacea* acetone extract assessed by using the anti-15 LOX model of inhibition.

The immunomodulatory activity of the acetone and water extracts of *T. violacea* may be attributed to the many bioactive compounds present in the extracts (Takaidza et al., 2018a). It is possible that either one or several active compounds in the plant plays an equally important role in modulating the cytokines (Ye et al., 2016).

The use of *T. violacea* in traditional medicine as an enhancer of the immune system is probably due to the activation of macrophages and the increased production of pro-inflammatory cytokines. Such activities have been observed for *Ganoderma lucidum* which is a widely used medicinal mushroom known to have immune enhancing activities such as the increased production of NO and cytokines in macrophages (Kozarski et al., 2011; Shi et al., 2013). Consequently, the pro-inflammatory activity of the acetone and water extracts of *T. violacea* may have the ability of stimulating macrophages that protects the host against microbial infections. Pro-inflammatory cytokines have also been shown to have anti-tumour properties due to their ability to inhibit tumour growth or cause cell death (Shen et al., 2002). TNF-α, IL-β and IL-6 can regress tumours and increase median survival time in a variety of cancer patients (Shen et al., 2002). We also found that *T. violacea* has anti-cancer properties (Takaidza et al., 2018b). This may be attributed to the pro-inflammatory properties of the extracts from this plant. Brief or acute inflammation is a self-limiting process and has a possible therapeutic outcome, whereas the incomplete resolution of inflammatory responses owing to dysregulation in immune response becomes detrimental to tissues (Mueller et al., 2010).

In conclusion, the acetone and water extracts of *T. violacea* exhibited immuno-stimulative properties and poor immuno-suppressing activity. According to Martel et al. (2017) and Wang et al. (2013) plants contain molecules that modulate immune cell activities and the solvent used to prepare a plant extract may determine whether the extract produces immunosuppressive or stimulant effect. Thus there is need to further examine the effect of solvent extraction on the immune cell activity. This will enable identification of new immunomodulatory compounds as well as facilitate the preparation of extracts producing specific immunological effects on humans.

CHAPTER 7 EVALUATING THE TOXICITY OF CRUDE EXTRACTS FROM SELECTED *TULBAGHIA* SPECIES

A. The effect of acetone and aqueous crude leaf extracts from *T. alliacea, T. simmleri* and *T. violacea species* on the viability of RAW264.7 macrophage cell line

ABSTRACT

The genus *Tulbaghia* has been used in traditional medicine to treat diseases such as tuberculosis, oesophageal cancer and stomach ailments. Plants used in traditional medicine are assumed to be safe due to their long usage in the treatment of diseases. However, recent scientific studies have shown that these plants may be potentially toxic. Currently, there is contradicting evidence with regards to the toxicity of the species in the genus *Tulbaghia*. Therefore the aim of this study was to evaluate the effect of acetone and aqueous crude leaf extracts from *T. alliacea, T. simmleri* and *T. violacea* on the viability of RAW 264.7 cells. The effect of the extracts on the metabolic activity and cell membrane integrity of treated RAW264.7 cells was assessed using the MTT and LDH assays, respectively. For the MTT assay, significant inhibitory effect ($p<0.05$) on the viability of RAW264.7 cells was observed after 48h treatment with *T. alliacea, T. simmleri and T. violacea* acetone extracts resulting in IC_{50} values of 0.48 mg/mL, 0.72 mg/mL and 0.03 mg/mL, respectively. Treatment with water extracts showed minimal inhibitory effect indicated by higher IC_{50} values of 0.95 mg/mL, 2.49 mg/mL and 0.3 mg/mL for *T. alliacea, T. simmleri* and *T. violacea*, respectively. The LDH release by macrophages after 24 h treatment with acetone extracts was observed to be concentration dependent while treatment with water extracts did not induce LDH release. In conclusion, *T. violacea* showed the most potent activity. The cytotoxic effect of the plant extracts on RAW264.7 cells was observed to be concentration and time dependent. Plant extracts can be utilised as sources of natural therapeutic agents at non-toxic concentrations over a short time period.

Key words: LDH, Macrophages, Medicinal plants, MTT, Toxicity, *Tulbaghia*.

7.1 INTRODUCTION

Medicinal plants are known to contain a variety of secondary metabolites which attribute to their therapeutic properties. Among these secondary metabolites are saponins, terpenoids, tannins, amino acids, glycosides, alkaloids and lectins (Mounanga et al., 2015) just to mention a few. However, if consumed in excess some plant secondary metabolites like lectins, may result in toxicity (Elgorashi et al., 2003). The amount of a potentially toxic compound in a plant is influenced by extrinsic and intrinsic factors (Makhafola et al., 2014). Extrinsic factors such as the climate and the soil may directly or indirectly influence the dose of toxic secondary metabolites in the different parts of the plant (Celik 2012). Despite the known bioactivities of medicinal plants, it is crucial to validate their safety as well as their efficacy when studying their traditional uses (Erena, 2014).

The genus *Tulbaghia* has been widely used in traditional medicine for the treatment of a various ailments. Three species from *Tulbaghia* genus that are dominantly used in Southern Africa are *T. alliacea*, *T.simmleri* and *T. violacea*. Their uses include the treatment of fits (*T. alliacea*) fevers, rheumatism and paralysis (*T. alliacea* and *T. violacea*), stomach ailments ,constipation/purgative (*T. violacea*), pulmonary tuberculosis and use as anthelmintics (*T. alliacea* and *T. violaceae*) (Lindsey and van Staden, 2004; Ncube et al., 2011; Jäger and Stafford, 2012; Ncube et al., 2012; Aremu and Van Staden, 2013; Moodley et al., 2014; Ranglová et al., 2015). *Tulbaghia simmleri* is commonly used as a substitute for both *T. violacea* and *T. alliacea* in an event where the latter are not available. *Tulbaghia* species have also found use in the treatment of oral fungal infections (Jäger and Stafford, 2012).

The consumption of medication prepared from *Tulbaghia violacea* has been implicated to cause a variety of symptoms including abdominal pain, gastroenteritis, stoppage of gastro intestinal peristalsis as well as the contraction of the pupils and sloughing of intestinal mucosa (Hutchings et al., 1996).

Medicinal plant extracts have potential in the pharmaceutical industry, however this industry is regulated by rules that require full knowledge regarding the toxicity of any given compound (Morobe et al., 2012; McGaw et al., 2014; Nemudzivhadi and Masoko, 2014). Cytotoxicity studies are a necessary initial step in determining the potential toxicity of plant extracts or their derivatives the biologically active compounds. Current safety and toxicology testing for preclinical studies comprise of *in vitro* and *in vivo* models. The levels of cytotoxicity as well

as the potential toxic effects of medicinal plants can be evaluated *in vitro* using model host cells.

When model cells are exposed to a potentially harmful substances they may undergo changes in their morphology, proliferation and metabolism (Mounanga et al., 2015). In this study, RAW264.7 macrophage cells were used. Macrophages are present in almost all tissues of the body and perform a variety of functions such as host defence, inflammatory regulation and wound healing (Martinez-Pmares, 2007; Mosser and Edwards, 2008; Acosta-Iborra 2009).

Lactate dehydrogenase (LDH) and (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) (MTT) assays have reliably been used to assess viability and cytotoxicity of plant compounds or extracts on cells. LDH and MTT assays are able to assess the integrity of the cell membrane and mitochondrial activity, respectively (Saravanan 2003; Boncler 2014). In this study, the effect of acetone and water crude leaf extracts from *T. alliacea, T. violacea,* and *T. simmleri* on the viability of macrophage cell line was investigated using the MTT and LDH assays.

7.2 MATERIALS AND METHODS

7.2.1 Plant material

Three plant samples were selected from the eight *Tulbaghia* species used in this study. *Tulbaghia violacea* was selected for toxicity testing as it showed the best bioactivities. *Tulbaghia simmleri* and *T. alliacea* were also selected as they are both commonly used in traditional medicine.

7.2.2 Preparation of the crude acetone and water extracts

The preparation of acetone and water extracts was done following the protocol previously described in section 4.2.2 and 4.2.3.

7.2.3 Cell culture conditions

The Abelson Murine leukemia macrophage adherent cell line was obtained from Cellonex (CRAW 264.7-C, Cellonex, Johannesburg, South Africa). This cell line was grown in complete culture medium consisting of high glucose DMEM (GE Health Life Sciences, lagon,UT) supplemented with 10% FBS (ThermoScientific, Cramlington, Northumberland) and 1 X penicillin, streptomycin and neomycin at 37ºC in a humidified atmosphere at 5% CO_2 (ESCO, Changi South Street, Singapore).

7.2.4 Treatment of the macrophages with crude aqueous and acetone plant extracts

The RAW264.7 cells were seeded at a concentration of 1 x 10^5 cells/mL in 96-well plates and incubated at 37ºC in a humidified atmosphere at 5% CO_2. After 24 h incubation, cells were washed with PBS and then treated with crude aqueous acetone and water extracts. The extracts from *T. violacea, T. simmleri* and *T. alliacea* were prepared in DMEM culture media at concentrations of 25, 50, 75, 100, 250, 500, 750 and 1000 µg/mL. Hydrogen peroxide at 2 µg/mL was used as a positive control. After 24 and 48 h of incubation the metabolic activity and cell membrane integrity was assessed using the MTT and LDH assays, respectively. Hydrogen peroxide and the untreated macrophages were used as controls.

7.2.5 Assessment of cell morphology

After 24 and 48 h treatment with the crude aqueous and acetone plant extracts from *T. violacea, T. simmleri* and *T. alliacea* the macrophages were observed at X20 magnification using an inverted microscope (Olympus IX 53, Tokyo, Japan).

7.2.6 MTT Cell viability assay

The Vybrant® MTT Cell Proliferation Assay Kit was used (ThermoFisher Scientific, OR) to assess the state of metabolic activity of the macrophages with slight modifications. In brief, after 24 h and 48 h treatment of RAW264.7 cells, the cell culture medium was replaced with 100 µL of fresh culture medium. Then 10 µL of the 12 mM MTT stock solution was added into each well followed by a 4 hour incubation at 37ºC in a humidified atmosphere at 5% CO_2. Afterwards, 85 µL of the medium was removed from each well and was replaced with 50 µL of DMSO. The 96 well plates were then incubated at 37°C in a humidified atmosphere at 5% CO_2 for 10 minutes. Absorbance was read at 540 nm using a Microplate reader (Promega, Sunnyvale, CA). Cell viability was calculated using the equation below:

$$\% \text{ Viability} = \frac{Abs\ of\ treated\ cells - Abs\ of\ blank}{Abs\ of\ untreated\ cells - Abs\ of\ blank} \times 100$$

The IC_{50} was calculated as the concentration of the test extract resulting in a 50% reduction of absorbance compared to untreated cells.

7.2.7 LDH cytotoxicity assay

The Pierce LDH cytotoxicity assay kit (Thermoscientific, Rockford, IL) was used to assess the integrity of the macrophage cell membrane according to the manufacturer's instructions. Treatments at concentrations of 250, 500, 750 and 1000 µg/ mL were used for the LDH assay. After 24 h treatment of RAW264.7 cells, 50 µL of each sample medium was transferred to a new 96 well plate and 50 µL of reaction mixture was added to each sample well. The plates were then incubated at room temperature in the dark for 30 minutes. Afterwards, 50 µL of stop solution was added to each well and the absorbance was read at 490nm and 680nm. To determine the LDH activity the absorbance values at 680 nm were subtracted from those of 490 nm. The cytotoxicity was calculated as follows:

$$\% \text{ Cytotoxicity} = \frac{Compound\text{-}treated\ LDH\ activity - Spontaneous\ LDH\ activity}{Maximum\ LDH\ activity\ - Spontaneous\ LDH\ activity} \times 100$$

7.2.8 Statistical analysis

All experiments consisted of at least three independent biological replicates and the data were expressed as means ± SE. IC_{50} values were calculated using the software program Graphpad Prism version 7.0 for Windows, Graphpad Software, Lajolla, CA. To define statistically significant differences, the data were analysed with one way analysis of variance ANOVA assuming equal variances at a $p<0.05$ level.

7.3 RESULTS

7.3.1 Morphology of treated RAW264.7 cells

Treatment with plant extracts at concentrations of 25 µg/mL had no effect on the morphology of the RAW264.7 cells. As the concentration increased from 50 µg/mL to 1000 µg/mL the morphology of the cells was altered from smooth round shape to irregular, rough, enlarged cells (fig. 7.1 and fig. 7.2).

7.3.2 The effect of treatment with plant extracts on the metabolic activity of RAW264.7 cells

At low concentrations (25 – 50 µg/mL) all plant extracts exhibited no significant (p>0.05) effect on the cell viability. Treatment with acetone crude extract from *T. violacea* showed the a significant reduction (p<0.05) in viability of RAW264.7 cells at concentration ≥ 100 µg/mL after 24 h and at ≥ 75 µg/mL after 48 h. *Tulbaghia alliacea* acetone crude extract showed significant reduction in viability at concentration ≥ 500 µg/mL after 24 h treatment and at ≥250 µg/mL after 48 h. For *T. simmleri* a significant reduction in viability was observed at ≥ 750 µg/mL after 24 h treatment and at ≥ 500 µg/mL after 48 h (Table 7.1).

Treatment with water extracts significantly reduced the viability of RAW264.7 cells at concentration ≥ 500 µg/mL for all three extracts after 24 h period. After 48 h *T. violacea* viability was significantly reduced at concentration ≥ 100 µg/mL while for both *T. alliacea* and *T. simmleri* it was observed at ≥ 250 µg/mL (Table 7.2). *Tulbaghia violacea* exhibited the most reduction in the viability of RAW264.7 cells compared to *T. alliacea* and *T. simmeri*. *Tulbaghia violacea* had the lowest IC_{50} values of 0.1 mg/mL and 0.3 mg/mL for acetone and water extracts, respectively after 48 h. *T. alliacea* had IC_{50} values of 0.48 mg/mL and 0.95 mg/mL while *T. simmleri* had 0.72 mg/mL and 2.49 mg/mL for acetone and water extracts, respectively after 48 h treatments (Table 7.3).

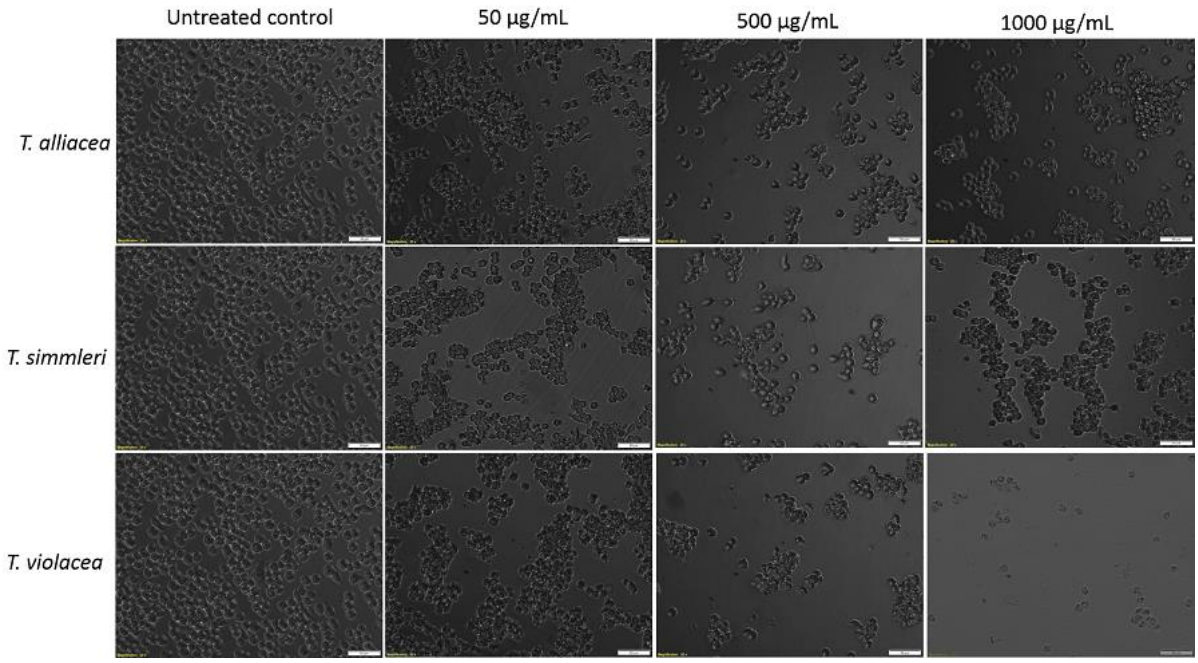


Figure 7. 1 Representative images of RAW264.7 after treatment with *T. violacea* acetone extract at different concentrations after 48h treatment.

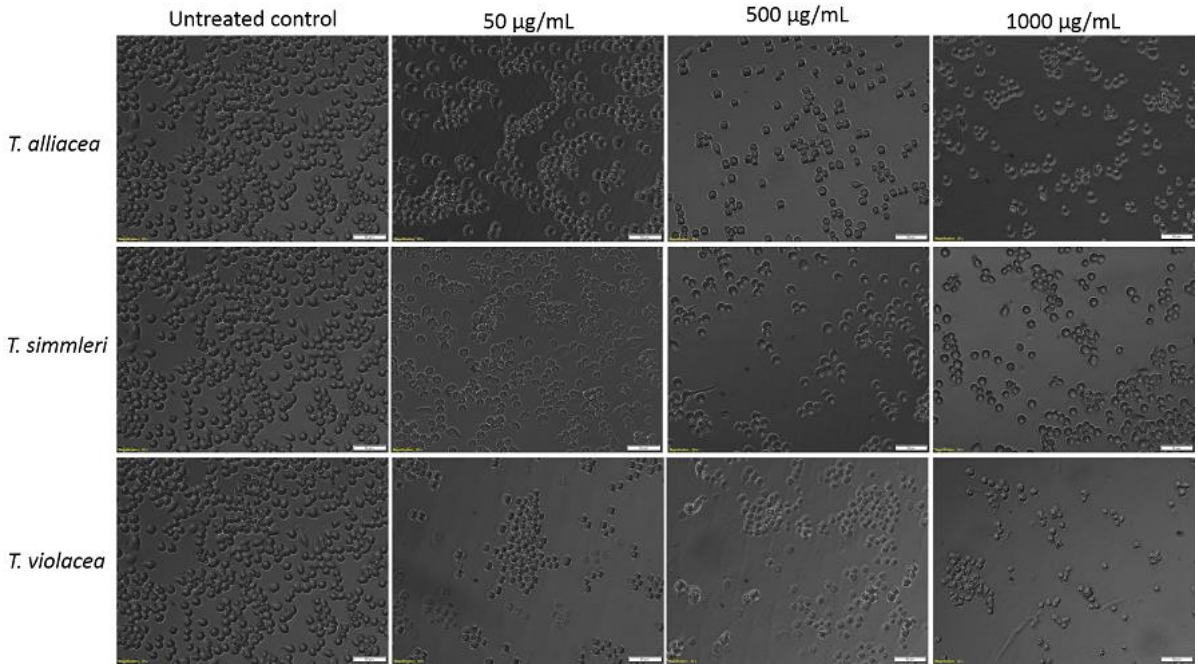


Figure 7. 2 Representative images of RAW264.7 after treatment with *T. violacea* water extract at different concentrations after 48h treatment.

Table 7.1 Percentage mean ± SE of the metabolic activity of macrophages stimulated with crude acetone plant extract

	24 hrs			**48hrs**		
	T. alliacea	*T. simmleri*	*T. violacea*	*T. alliacea*	*T. simmleri*	*T. violacea*
H_20_2	10± 1.13	10±1.13	10±1.13	12±1.58	12±1.58	12±1.58
0 µg/mL	100± 11	100±11.21	100±11.21	103±8.34	103±8.34	103±8.34
25 µg/mL	101±13	111±16.02	91±10.30	80±2.98	101±4.25	100
50 µg/mL	101±10.61	127±4.10	108±29.43	80±6.31	98±15.25	84
75 µg/mL	75±24.27	163±4.15	81±1.73	117±4.83	159±20.67	**75**
100 µg/mL	94± 16.95	139±3.98	**78± 101**	**91±1.14**	**85 ±4.85**	**60**
250 µg/mL	80±20.94	131±11.81	**58±44.53**	**64 ±0.47**	**80 ±3.03**	**13±12.6**
500 µg/mL	**68±0.37**	89±2.72	**6±14.84**	**58 ±1.39**	**72 ±0.72**	**0±2.05**
750 µg/mL	**13±.58**	**57±12.94**	**2±10.29**	**45±0.65**	**59 ±2.40**	**0±13.24**
1000 µg/mL	**7±3.00**	**30±27.23**	**0**	**0±2.18**	**0±0.64**	**0±0.91**

Table 7.2 Percentage mean ± SE of the metabolic activity of macrophages stimulated with the crude aqueous plant extract

	24 hrs			**48 hrs**		
	T. alliacea	*T. simmleri*	*T. violacea*	*T. alliacea*	*T. simmleri*	*T. violacea*
H_20_2	16±1.13	16±1.13	16±1.13	8±1.58	8±1.58	8±1.58
0 µg/mL	100±11.21	100±11.21	100±11.21	100±8.34	100±8.34	100±8.34
25 µg/mL	64±16.02	99±7.04	71±10.30	87±2.98	98 ±4.25	83
50 µg/mL	101±4.10	93±10.61	94±29.43	101 ±6.31	85 ±15.25	98
75 µg/mL	82±4.15	96±24.27	72±3.86	96±4.83	100 ±20.67	101
100 µg/mL	76±3.98	88±16.95	131±72.37	84±1.14	85 ±4.85	**74±9.34**
250 µg/mL	88±11.81	82±0.37	104±14.84	**62±0.47**	**67 ±3.03**	**63±0.96**
500 µg/mL	**67±2.72**	**66±2.82**	**66±10.29**	**41 ±1.39**	**48.1±0.72**	**36±2.05**
750 µg/mL	**62±12.94**	**63±1.58**	**49.6±4.53**	**39±0.65**	**44±2.40**	**23±13.24**
1000 µg/mL	**27±2.72**	**50±3.00**	**31±10.29**	**28±2.18**	**42±0.64**	**5±0.91**

The GraphPad prism 7 software was used to calculate the IC_{50} of the extracts. The lower the IC_{50} value the toxic the plant extract and the higher the IC_{50} value the less toxic.

Table 7. 3 IC_{50} values (mg/mL) of acetone and aqueous crude extract of *T. alliacea*, *T. simmleri* and *T.violacea* from MTT assay

Extract tested	IC_{50} @ 24hrs	IC_{50} @ 48hrs
T. alliacea (acet)	1.00(0.92-1.11)	**0.72**(0.66-0.76)
T. alliacea (aq)	1.02(0.97-1.24)	2.49(1.49-9.9)
T. simmleri (aq)	0.81(0.70-0.94	0.95(0.78-1.58)
T. simmleri (acet)	0.73(0.61-0.87)	**0.48**(0.29-0.68)
T. violacea (acet)	0.27(0.26-0.28)	**0.03**(0.02-0.06)
T. violacea (aq)	0.73 (0.67-0.80)	0.30(0.16-0.4)

7.3.3 The effect of treatment with plant extracts on the membrane integrity of RAW264.7 cells

After 24 h, a dose dependant increase in the percentage LDH or cytotoxicity of the RAW264.7 cells treated with acetone extract was observed. After 48 h there was no significant difference ($p>0.05$) in the amount of LDH in treated cells vs untreated (control) for acetone extracts (results not shown). For aqueous extracts no LDH was detected in the cell culture media even at high concentrations of the extracts for both 24 and 48 h treatments.

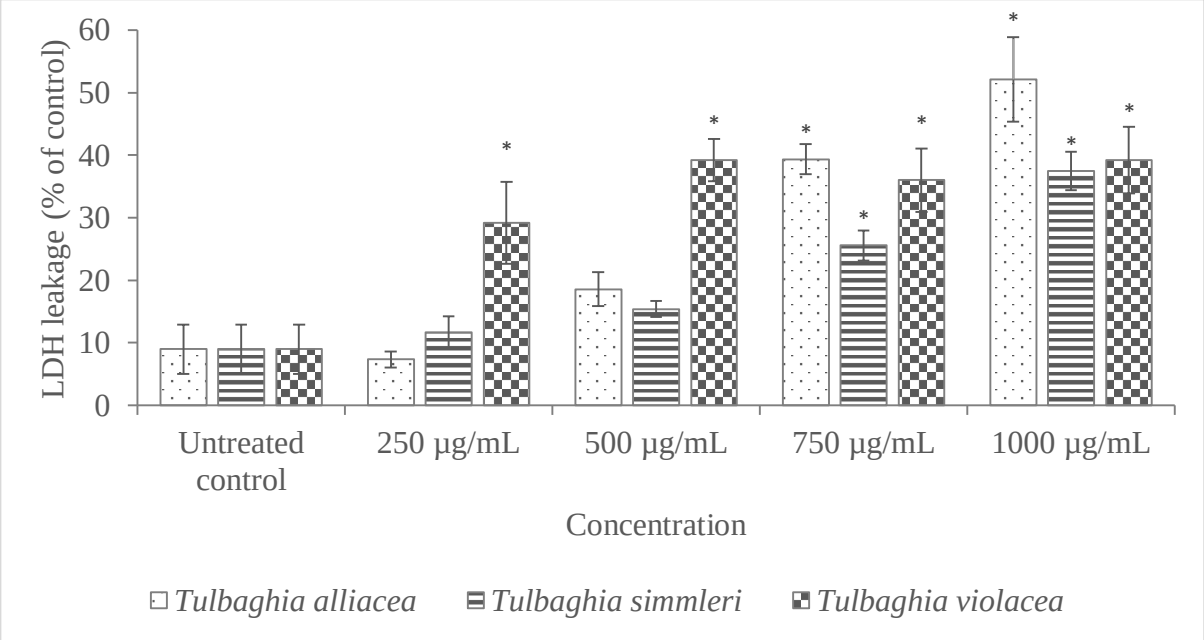


Figure 7. 3 The percentage leakage of LDH from RAW264.7 cells after treatment with *T. alliacea*, *T. simmleri* and *T. violacea* acetone extracts. Data is represented as mean values ± SE from three independent experiments done in triplicate. *$p < 0.05$ treatments vs untreated control.

7.4 DISCUSSION AND CONCLUSION

Tulbaghia has been used in traditional medicine to treat various ailments (Moodley et al 2014). The genus *Tulbaghia* is known to have sulphur containing compounds, which attribute to its garlic-like odour and medicinal properties (Lyantagaye 2011). Bioactive compounds such as kaempherol and marasmicin have also been reported to be present in *Tulbaghia* species (Kubec et al., 2013). A major concern about medicinal plant extracts is that some may contain compounds that may be toxic to humans and animals. Therefore, it is critical to evaluate safety of plant extracts that have a potential in pharmacology (Janakiraman and Johnson, 2016). In this study the effect of aqueous and acetone extracts of *T. alliacea*, *T. simmleri* and *T. violacea* on the viability of RAW 264.7 cell line was assessed using MTT and LDH assay.

In the MTT assay, a significant inhibition ($p< 0.05$) of RAW264.7 of cell growth was generally observed after 48h. Treatment with acetone extracts from *T. alliacea, T. simmleri and T. violacea* resulted in IC_{50} values of 0.72mg/mL, 0.48 mg/mL and 0.03 mg/mL, respectively. *T. violacea* was observed to be the most potent species. Treatment with aqueous extracts for 48h gave higher IC_{50} values of 2.49 mg/mL, 0.95 mg/mL and 0.3 mg/mL for *T. alliacea, T. simmleri* and *T. violacea*, respectively.

The results of MTT assay suggest that treatment with acetone and water extracts from *T. alliacea*, *T. simmleri* and *T. violacea* led to a certain degree of cytotoxicity of the RAW264.7 cells. The viability of treated RAW264.7 cells was reduced in a dose dependent manner. Thus a linear decrease in cell viability observed in RAW264.7 cells along with an increase in concentration of extracts indicates a significant alteration of the mitochondrial metabolism.

The MTT assay detects cells that are still metabolically active. It is used to assess how effective the mitochondrial dehydrogenases of viable cells metabolically reduce pale yellow MTT salt (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) to insoluble purple formazan product [1-(4, 5-dimethylthiazol-2-yl)-3, 5 diphenyl formazan]. Formazan crystals are impermeable to cell membranes, hence the resulting accumulation within healthy cells can be spectrophotometrically analyzed to estimate cell viability (Masson-Meyers et al., 2016).

The membrane integrity of treated RAW264.7 cells was measured using the LDH assay. The measurement depends on the leakage of lactacte dehydrogenase (LDH) from cells after different treatments (Lantto 2009). Treatment with water extracts did not have an effect on the membrane integrity as there was no significant ($p>0.05$) difference in the amount of percentage

LDH leakage or cytotoxicity between the treated and non-treated cells after 24 and 48 hr. For acetone extract a significant ($p < 0.05$) the percentage LDH leakage was detected at concentration of 500 µg/mL to 1000 µg/mL. As the concentration of the extracts increased the amount of LDH also increased.

The low cytotoxic effect of aqueous extracts even at high concentrations exhibited in both MTT and LDH assays is supported by numerous studies on medicinal plant pharmacological properties which show that the aqueous extracts seem overall to be less toxic than organic extracts (Nguta et al., 2012; Adamu et al., 2013; Mounanga et al., 2015). The phytochemical composition of both type of extracts is responsible of this observation. Acetone is a better extractant compared to water as it has the ability to extract compounds of wide polarity range (Adamu et al., 2013). The relative low toxicity of the aqueous extract could also be due to the fact they contain the total family of medicinal compounds just as they are found in their natural source and hence offer less risk of side effects (Celik 2012; Adamu et al., 2013; Monaunga et al., 2015).

In conclusion, the plant extracts used in this study showed a dose and time dependent effect on the treated RAW264.7 cells. The alteration of the cell viability of the treated cells suggests that there were substances in the extracts that interfered with the cell function. Therefore, plant extracts can be utilised as sources of natural therapeutic agents at non-toxic concentrations over a short time period.

B. Embryotoxicity and teratogenic effects of *Tulbaghia violacea* acetone and aqueous leaf crude extracts on *Danio rerio* (Zebrafish).

ABSTRACT

Tulbaghia violacea, a plant used in traditional medicine, is widely distributed in Southern Africa. Traditionally it has been used to treat various ailments such as tuberculosis, earache, heart problems and cancer of the oesophagus. Given its pharmacological potential there is a need to evaluate its toxicity. In this study, the zebrafish assay was used to evaluate the embryotoxicity and teratogenic effects of crude acetone and water extracts of *T. violacea* at 24 h intervals for 96 h post fertilisation (hpf). The percentage mortality, hatchability and heart rate were examined. The lethal dose (LD_{50}) for the acetone crude extract was 20 µg/mL whereas that for water was 85 µg/mL. The observed teratogenic effects included scoliosis, edema of the pericardial cavity, retarded yolk resorption, hook-like/bent tail and shorter body length. This study showed that the toxicity of *T. violacea* acetone and water extracts to zebrafish development is dependent on concentration and exposure time. Therefore, traditional medicine prepared from *T. violacea* should be taken with caution preferably in small doses over a short period of time.

Keywords: Embryotoxicity, teratogenic, *Tulbaghia violacea*, zebrafish

7.5 INTRODUCTION

Medicinal plants have been used traditionally to treat various ailments since time immemorial. Although medicinal plants have been assumed to be safe, many side effects have been reported (Mounanga et al., 2015). Some toxic substances from plants can affect the entire spectrum of vital human organs while others may affect key functional body systems. The most dominant toxins found in some medicinal plants are neurotoxins that interfere with the brain and central nervous system (CNS) by affecting the coordination and nerve functions, followed by cytotoxins and metabolic toxins that affect the functions of the kidneys, liver, heart and lungs. The severity of a toxic effect may depend on the route of administration, growth stage or part of the plant, the amount consumed, the species and susceptibility of the victim (Ndhlala et al., 2013). Considering this, medicinal plants should be used with caution. It is important that toxicological studies be conducted to increase the knowledge on the plant or plant preparations given to populations (Mounanga et al., 2015).

Tulbaghia violacea, a member of the family *Alliaceae*, has been widely used in traditional medicine for the treatment of numerous ailments. The bioactivities of *T. violacea* have been investigated in a wide range of biological functions including antimicrobial and antioxidant activity, anti-hypersensitivity/cardio vascular related diseases as well as anticancer properties (Aremu and van Staden, 2013).

The consumption of traditional medication prepared from *T. violacea* has been implicated to cause a variety of symptoms including abdominal pain, gastroenteritis, stoppage of gastro intestinal peristalsis, sloughing of the intestinal mucosa as well as the contraction of the pupils (Hutchings et al., 1996).

It is therefore important that medicinal plants with therapeutic potential such as *T. violacea* be assessed for their safety. To evaluate the toxicological effects of medicinal plants, appropriate methods such as the use of experimental animals should be utilised for selecting appropriate doses for human use (Ghorbani et al., 2016). In this study, the zebrafish assay was used to assess the embryotoxicity and teratogenic effects of acetone and aqueous leaf crude extracts of *T. violacea.* The Fish Embryo Acute Toxicity test (FET) was designed by the Organisation for Economic Co-operation and Development (OECD) as a guideline to evaluate the toxic effects of certain compounds on the early 96 h developmental stages of embryos (Selderslaghs et al., 2009). However, the zebrafish assay is not only limited to screening toxicity. Transgenic zebrafish have been developed through gene alterations and targeted mutations for studying

immunology, the digestive system, muscles and specific diseases such as cancer and diabetes (Crawford et al., 2011; Maes et al., 2012; Ismail et al., 2017; Meshalkina et al., 2017; Jia et al., 2017).

The advantages of using the zebrafish for drug screening include their high genetic and physiologic similarity with mammals (Gao et al., 2014; Aluru 2017; Kovacs et al., 2016; Racz et al., 2017; Lui et al., 2017; Hanigan et al., 2017). The small size, *ex-utero* development, optical transparency of the eggs offering clear visualization in all stages of organogenesis monitoring, low maintenance, rapid development and large numbers of embryos and larvae, make the zebrafish an ideal primary system for experimental analysis (Slederslaghs et al., 2009; Shi et al., 2017).

The small size of zebrafish embryos and larvae (1 to 5 mm) is also compatible with microtiter plates (ideally 24-well plates) for screening, requiring only microgram amounts of each extract, fraction, or compound to be tested. Due to the high fertility of zebrafish, large numbers of embryos and larvae can be produced and examined in a more cost-effective manner compared to other *in vivo* models involving organisms such as mice and/or rats. These features present zebrafish as an ideal *in vivo* model for the screening of bioactive natural products with therapeutic potential (Crawford et al., 2011; Zhang et al., 2017). This study examined the embryotoxicity and teratogenic effects of acetone and aqueous crude extracts from *T. violacea* on zebrafish embryo development.

7.6 MATERIALS AND METHODS

7.6.1 Plant material

Tulbaghia violacea plants were purchased from Our Nursery: Stepping Stone in Pretoria, South Africa and maintained in a greenhouse at the Vaal University of Technology. Identification of the plant was done by a botanist and the voucher specimen was deposited at AP Goossen Herbarium, North West University, Potchefstroom, South Africa.

7.6.2 Preparation of the leaf crude extracts

The preparation of acetone and water extracts was done following the protocol previously described in section 4.2.2 and 4.2.3.

7.6.3 Zebrafish egg production and the FET test

Embryos were obtained from spawning zebrafish adults with a sex ratio of 2 male: 1 female in breeding tanks at Sathyabama University, Chennai, India. Embryos were washed with embryo medium (E3) (OECD 2013) and only the fertilized embryos were selected for experimentation. Toxicity of the compounds was assessed with the OECD guideline, No. 236: Fish Embryo Acute Toxicity (FET) (Busquet et al., 2014). Ten fertilized embryos were transferred into each well of a 24-well plate containing a series of diluted extracts ranging from 6.25 µg/ml to 400 µg/ml. Exposure was done under semi-static conditions, namely, $\pm 28^0$C and 12 dark:12 light cycle periods. The semi-static method was used to avoid false non-toxicity that could be due to degradation of the test sample over time. Embryo development was monitored at 24 h intervals for 96 h. Morphological characteristics including coagulation of eggs, presence of the heartbeat and hatching rates were evaluated using an Inverted Microscope (Leica, Hesse, Germany). The LD_{50} values were extrapolated from the straight line graph that was plotted using different concentrations of the extracts vs the number of the surviving embryos at each concentration (data not shown). The experiments were conducted in triplicate.

7.7 RESULTS

7.7.1 Embryotoxicity

For the purposes of this experiment, mortality is defined as coagulation where dead embryos appear milky white instead of being transparent and there is no visual heartbeat in the embryos. The percentage survival of the embryos after 24, 48, 72 and 96 h exposure in varying concentrations of *T. violacea* water and acetone crude extracts are shown in Figs. 7.4 and 7.5, respectively.

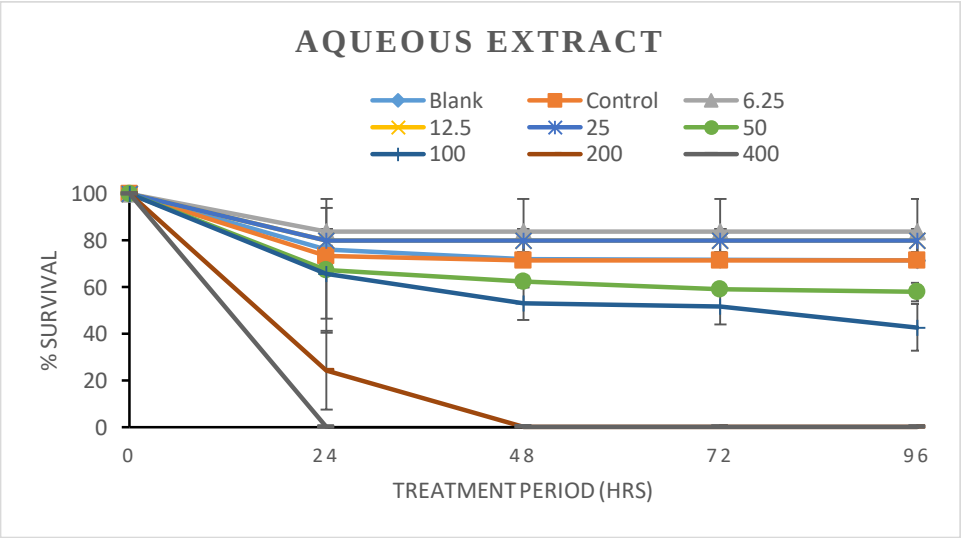


Figure 7.4 Survival rates of zebrafish embryos and larvae after exposure to various concentrations of aqueous extract of *T. violacea*.

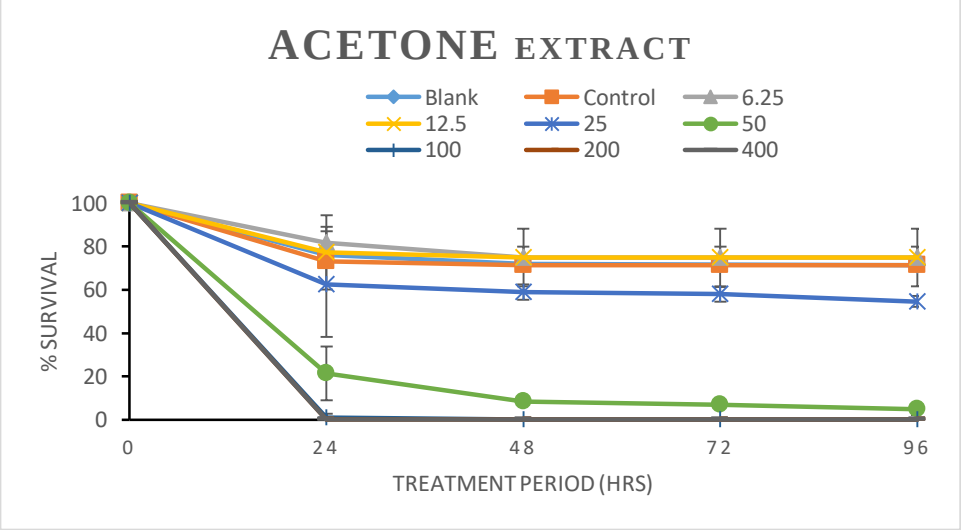


Figure 7.5 Survival rates of zebrafish embryos and larvae after exposure to various concentrations of acetone extracts of *T. violacea*.

A significant decrease in the survival and hatching rates was observed in both the water and acetone extracts at concentrations of 25 µg/mL and higher. There were no surviving embryos

after 24 hpf at ≥ 400 µg/mL and ≥ 100 µg/mL treatment for the water and acetone crude extracts, respectively (Table 7.4). The LD_{50} of the water extracts and acetone was 20 µg/ml and 85 µg/mL, respectively.

Table 7. 4 Heartbeat of Zebrafish embryos at 48 h exposure to varying concentrations of water and acetone extracts of *T. violacea*

Extract	Concentration (µg/mL)	Heart rate (/min) at 48 h
Water	6.25	156
	12.5	156
	25	152
	50	135
	100	133
	200	0
	400	0
Acetone	6.25	156
	12.5	154
	25	150
	50	130
	100	0
	200	0
	400	0
A. Control	1%	138
Blank	-	140

Values are expressed as mean of three replicates.

A. Control – Acetone control

Blank – E3 medium

The observed heart rate of the treated embryos was higher than that of the blank and the control and it decreased as the concentration of the extracts increased the heart rate decreased (Table 7.4).

7.7.2 Hatchability

The hatching rate is defined as the ratio of hatching embryos at specific exposure time to the total number of embryos at the beginning of the experiment. Zebrafish larvae usually hatch from the chorion at 48 to 72 hpf at 28^{0}C. In this study, the zebrafish embryos hatched between 48 h and 72 h (Figs. 7.6 and 7.7).

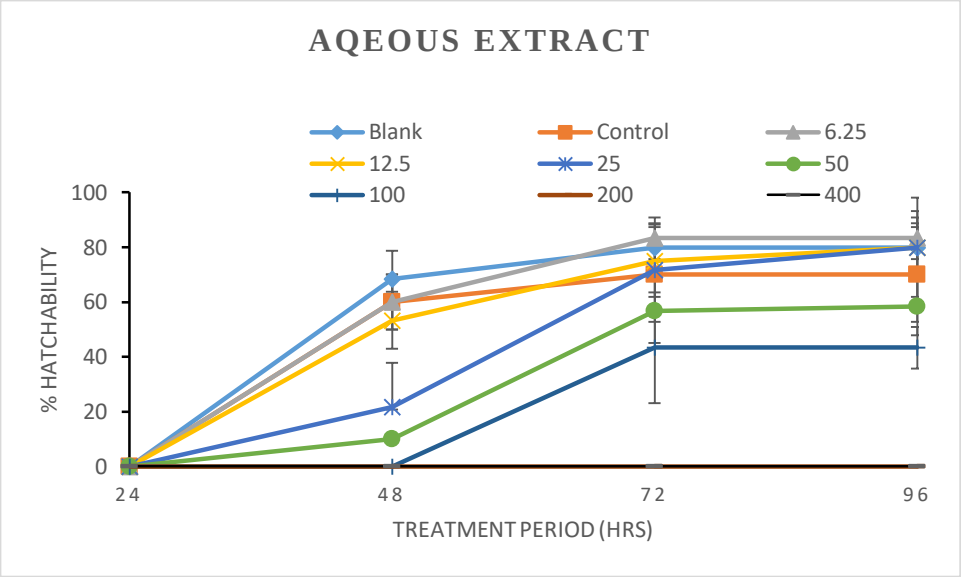


Figure 7.6. Hatchability of zebrafish embryos after exposure to various concentrations of aqueous extracts of *T. violacea.*

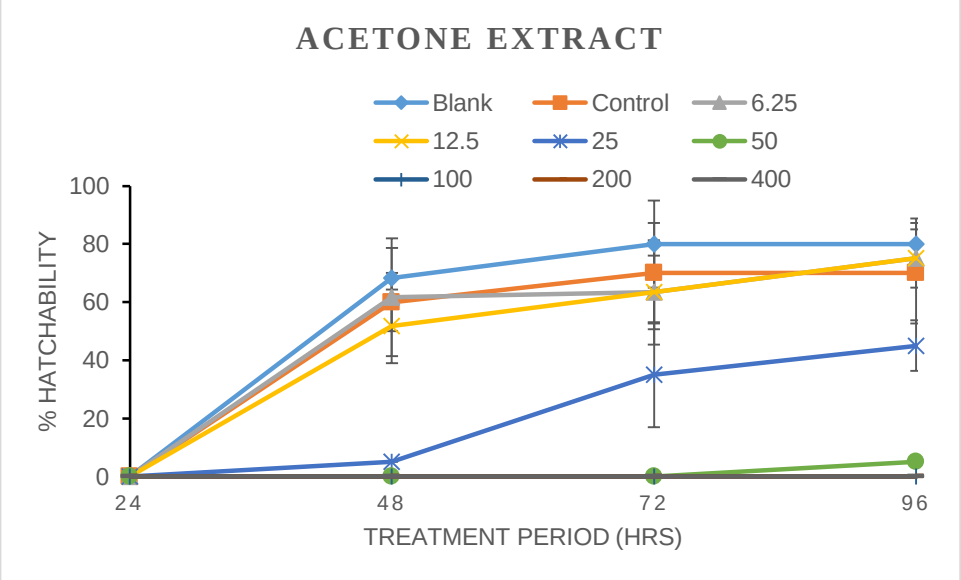


Figure 7.7 Hatchability of zebrafish embryos after exposure to various concentrations of acetone extracts of *T. violacea.*

Hatching retardation was observed in the embryos treated with ≥ 50 µg/mL for the water extract and ≥ 25 µg/mL for the acetone extract in a concentration dependent manner. Hatching did not occur when treated with concentrations of ≥ 400 µg/mL for the water extracts and ≥ 100 µg/mL for the acetone as all the embryos were dead after 24 hpf.

7.7.3 Morphological characteristics of zebrafish embryos

The morphological characteristics of the zebrafish from 24 h to 96 h for the water and acetone extracts treatments are shown in Figs. 7.8 and 7.9, respectively.

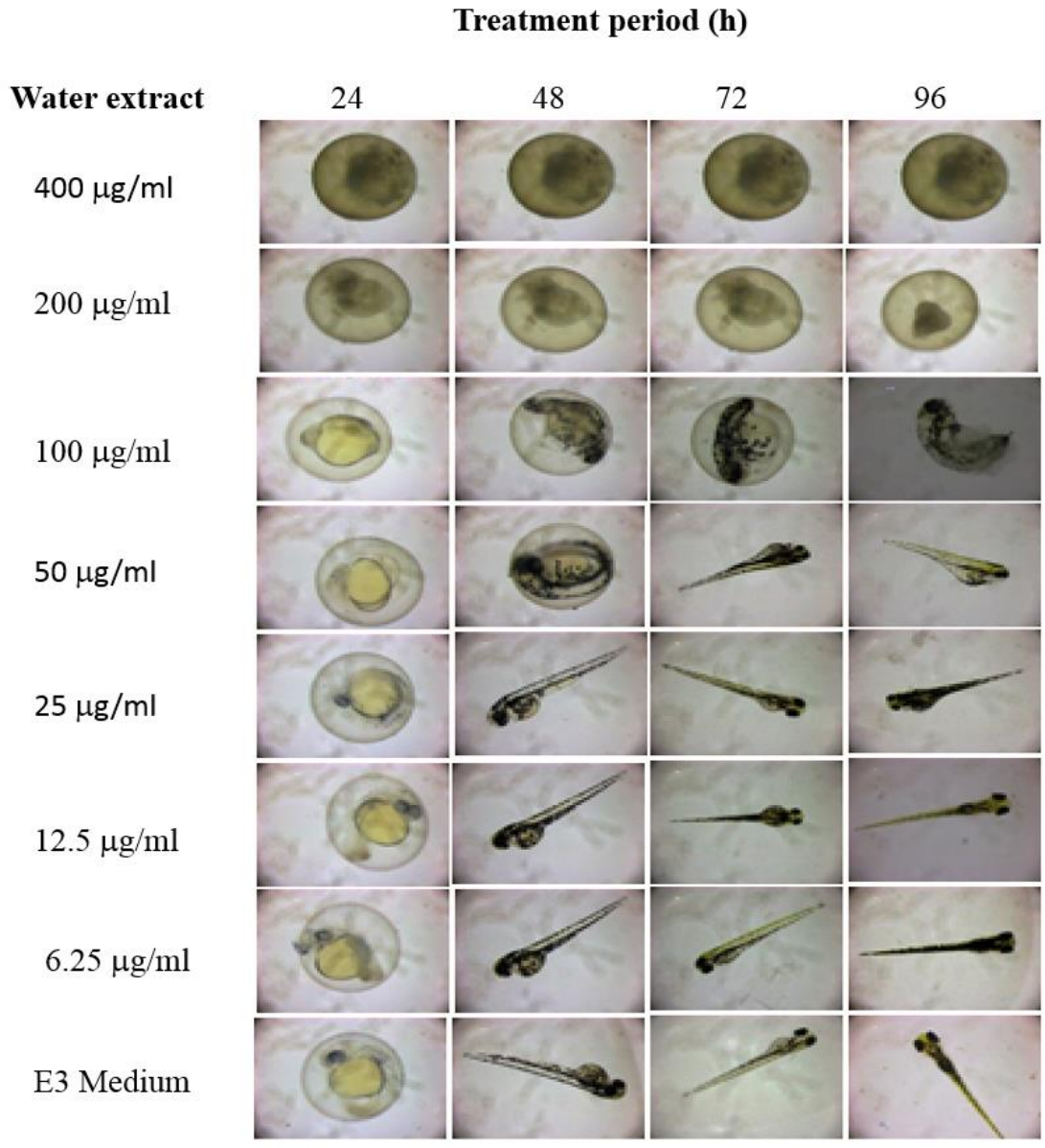


Figure 7.8 Effects of aqueous extracts of *T. violacea* on zebrafish embryos and larvae treated with different concentrations of the extract over a period of 96 h. E3 medium is the negative control.

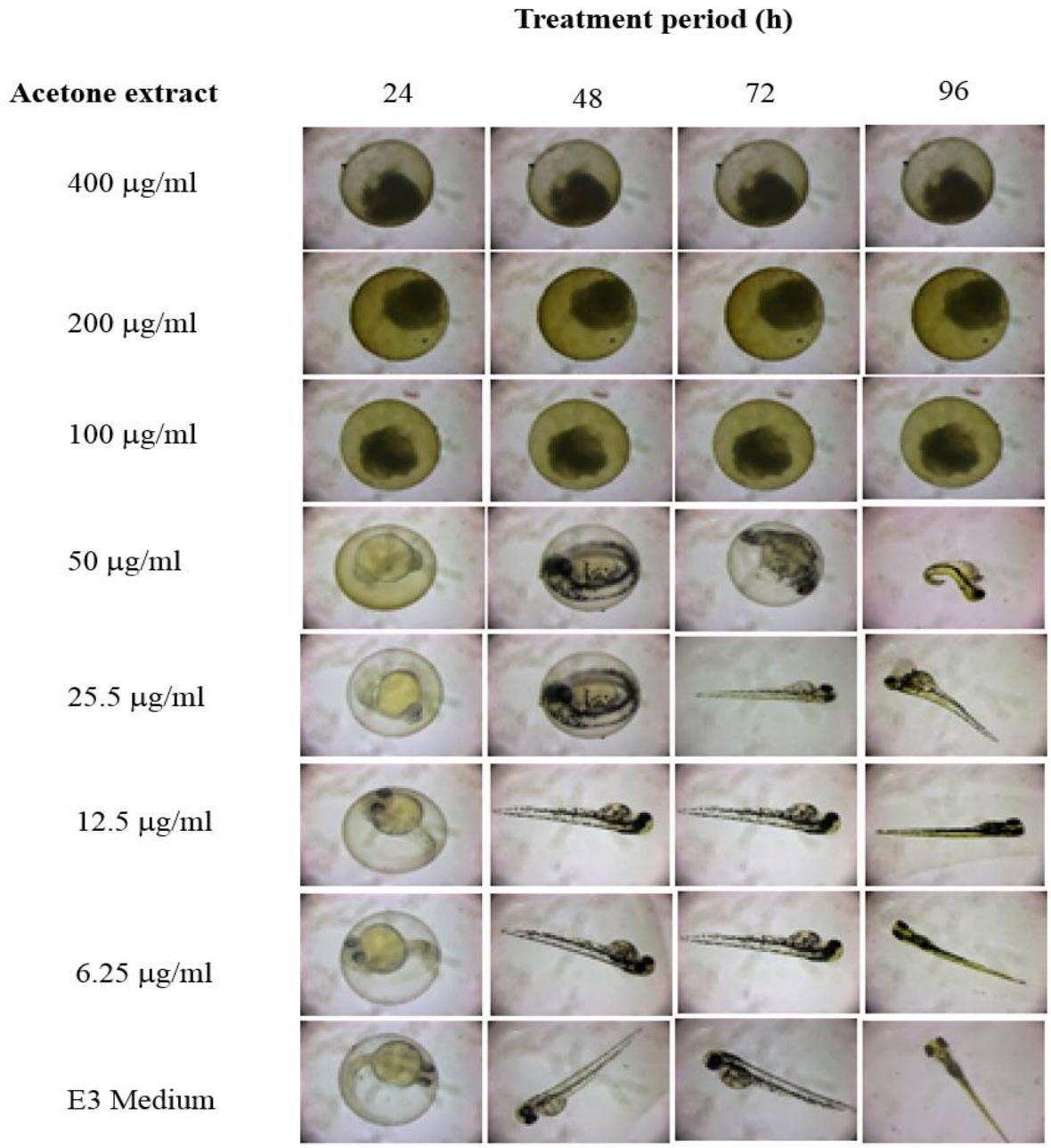


Figure 7.9 Effect of acetone extracts of *T. violacea* on zebrafish embryos and larvae treated with different concentrations of the extract over a period of 96 h.

The embryos developed normally when they are incubated in E3 medium (control) while various forms of growth retardation occurred at higher concentrations of both water and acetone extracts (Figs. 7.8 & 9). Coagulation of embryos was observed at concentrations ≥ 200 µg/mL and ≥ 100 µg/mL for the water and acetone extracts, respectively.

7.7.4 Teratogenic effect

The teratogenic effects of varying concentrations of both extracts on zebrafish embryos are shown in Fig. 7.10. Larvae that hatched from embryos treated with water extracts at concentration ≥ 50µg/mL and acetone extracts at concentrations ≥ 25µg/mL displayed several deformities including bent/hook-like tail, spinal column curving, short body length, edema of the pericardial sac and retarded yolk sac resorption (Fig. 7.10).

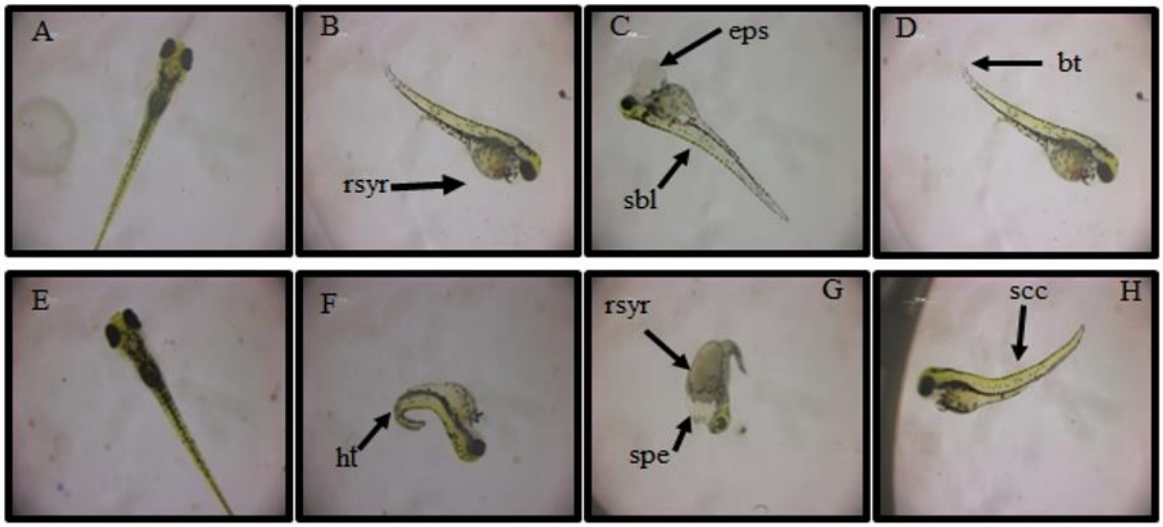


Figure 7.10 Teratogenic effects on development of zebrafish larvae after 96 h exposure (hpf). Phenotypes of normal larvae (A&E) and larvae hatching from embryos treated with *T. violacea* acetone and water crude extracts (B-D, F-H). Malformations are indicated by arrows: rsyr - retarded sac yolk resorption, bt - bent tail, ht - hook like tail, scc - spinal column curving (scoliosis), sbl - shorter body length, eps - edema of the pericardial sac, spe - severe pericardial edema.

7.8 DISCUSSION AND CONCLUSION

This is the first study that assessed the embryotoxicity and teratogenic effects of aqueous and acetone crude extracts of *T. violacea* on zebrafish. Medicinal plants with pharmacological potential such as *T. violacea* need to be tested for their efficacy and toxicity. As embryogenesis in zebrafish progresses through a standard embryological development a sequence of events controlled by numerous signalling pathways takes place. Any disturbance of these molecular pathways leads to distinct and predictable defects in the embryo (Gao et al., 2014). Based on the principle that destabilization at the anatomical, physiological and behavioural levels is possible following modification of a developmental event, the FET tests are able to determine the toxicity of substances by relating their effects on various developmental stages to levels of exposure (Hallare et al., 2014).

In this study the embryotoxicity and teratogenic effects of crude extracts of *T. violacea* were examined over the pharyngula and hatching stages of zebrafish embryo development. The pharyngula stage which is observed between 24 h and 48 h is a phylotypic stage. It is characterised by spontaneous movement, tail detachment from the yolk and early pigmentation within the 24 h period (Kimmel et al., 1995). The heartbeat is observed at 48 h. The hatching period (48 to 72 h) is characterised by tail pigmentation, strong circulation, single aortic arch pair, early motility, regular heartbeat, tapering of yolk, extension of dorsal and ventral stripes and development of some segmental blood vessels (Nagel 2002).

In this study, extract concentrations ≥ 400 µg/mL for the water extract and ≥ 100 µg/mL for the acetone extract (Figs. 7.4 and 7.5) were highly toxic to the zebrafish and resulted in the coagulation of the embryos and lack of a heartbeat within 24 h (Table 7.4). Coagulation usually results from the denaturation of yolk proteins and results in the disruption of yolk resorption. This involves the inability of nutrients to pass through the yolk syncytial layer leading to tissue damage and ultimately death of the embryo (Busquet et al., 2014). The heart is one of the first organs to form and function in zebrafish embryo development and is the most commonly affected organ in toxicity studies. The normal heart beat of zebrafish embryos ranges between 120 and 180 beats per min (Jose et al., 2016). A concentration dependant decrease in the heart beat was observed in this study (Table 7.4). There was no heart beat in embryos treated with 100 µg/mL and 200 µg/mL for acetone and water extracts respectively due to early arrested growth and development. The heart may also be affected by the abnormality of the pericardium resulting in an irregular heartbeat and blood flow rate (Sun and Lin, 2017). Cardiac glycosides which are compounds known to be present in *Tulbaghia* species (Aremu and van Staden, 2013)

are a specific type of toxic glycosides that affect the cardiac muscle sometimes causing fatal toxicities (Madike et al., 2017). The lethal dose (LD_{50}) was found to be 85 μg/mL and 20 μg/mL for the aqueous and acetone extracts, respectively. The percentage survival rate was observed to be dependent on the concentration of the extracts and also showed that the aqueous extract is less toxic than that of acetone. Most traditional remedies from plants are prepared as simple water extracts thus reducing the potential toxic effect. On the other hand the organic nature of acetone is known to extract a wider variety of polar compounds that can contribute to its toxicity (Adamu et al., 2013).

Hatching is a critical stage of zebrafish embryogenesis (Qin et al., 2014). It is therefore a key point in developmental toxicity. Hatching is a result of biochemical and physical mechanisms. Hatching failure of zebrafish embryos can occur due to various reasons such as the inhibition of the hatching enzymes e.g. chorionase and/or behavioural defects resulting in a weakened spontaneous muscular movement (Li et al., 2017). In this study, concentrations ≥ 50 μg/mL for the water extract and ≥ 25 μg/mL for that of acetone inhibited embryo hatching (Fig 7.6 and 7.7). The inhibitory effects of the extracts on embryo development showed dose-dependence. The malformations of the zebrafish embryos may be due to increased concentrations (Figs 7.8 and 7.9).

Some of the developmental anomalies observed in this study included scoliosis (spinal column curving), edema of pericardial cavity, retarded yolk resorption, hook-like tail, shorter body length and bent tail (Fig. 7.10). During the normal hatching process of the zebrafish embryos, the chorion is digested by the hatching enzyme which is a proteolytic enzyme secreted from hatching gland cells of the embryo (Bai et al., 2010). It is likely that the extracts of *T. violacea* inhibited the hatching gland or directly caused the downward spinal curvatures that may have prevented the embryos from hatching out of the chorion. The short body length further confirms the inhibitory effects of *T. violacea* extracts on the growth of zebrafish embryos.

The potential toxic effect of *T. violacea* has also been demonstrated by Olorunisola et al., (2011) in the brine shrimp lethality test. The toxic effects are assumed to be associated with the sulphur compounds and steroidal saponins known to be part of the phytochemical components of *T. violacea* (Lewis, 1999; Aremu and van Staden, 2013). In another study by Olorunissola et al. (2011) it was observed that a single oral administration of a 5g/kg dosage of extract from the rhizome of *T. violacea* did not produce mortality or significant behaviour changes during a 14-day observation on albino wistar rats. Species differences between the

zebrafish and the wistar rat as well as method of drug delivery and metabolism may account for the differences in mortality in the two experiments. Despite the differences between zebrafish and wistar rats (Gaoet al., 2014) showed that the log LC_{50} values in zebrafish correlated well with other mammalian models. Therefore the zebrafish is a valuable tool for assessing and predicting compounds/ drug toxicity (Haq et al., 2016).

Developmental deformities observed in this study have also been reported in other toxicity screenings of natural products (Wu et al., 2007; Kumar et al., 2013; Ismail et al., 2017). In conclusion, the study showed that the extracts of *T. violacea* reduced the survival rate, hatching rate and induced malformations on the zebrafish embryos in a dose and time dependent manner. Both aqueous and acetone extracts were toxic to the embryos at concentrations ≥ 400 µg/mL and ≥ 100µg/mL, respectively. Clearly, the acetone extract was more toxic than the water extract. The dominant malformations were bent/hook like tail, spinal curving, short body length and edema of the pericardial sac. This clearly indicates that caution must be exercised when consuming extracts from medicinal plants for curing different ailments.

CHAPTER 8 GENERAL CONCLUSION AND RECOMMENDATIONS

8.1 GENERAL CONCLUSION

In South Africa, like many other developing countries a high percentage of the population still depends on traditional medicine for primary health care (van Wyk and Prinsloo, 2018). However, there is limited scientific evidence on the phytochemical composition and biological activities of some of these medicinal plants. Many plant species and their constituents remain unexplored. In addition, a number of plant derived bioactive compounds are yet to be discovered. Medicinal plants, either as a source of pure compounds or as standardised plant extracts, provide opportunities for new drug leads due to their high chemical diversity ((Moussauoi and Alaoui, 2016; Elisha et al., 2017).

Species in the genus *Tulbaghia* have been widely used in Southern Africa as traditional medicine. These medicinal plants are used to treat various symptoms and diseases such as sore throat, earache, stomach ailments and oesophageal cancer (Aremu and van Staden, 2013). However, constituents of these plants and the nature of their biological activities still need to be comprehensively studied. Thus, this study was conducted in order to investigate the phytochemical components and biological activities of the crude extracts from selected *Tulbaghia* species.

The following are conclusions drawn from the experimental data:

8.1.1 Phytochemical analysis and antioxidant activity

Phytochemical analysis revealed the presence of flavonoids, glycosides, tannins, terpenoids, saponins and steroids in *T. alliacea, T. acutiloba, T. cernua, T. leucantha, T. ludwigiana, T. natalensis, T. simmleri* and *T. violacea*. The amount of total phenol and flavonoid content varied in different plant extracts ranging from 4.50 to 11.10 miligrams gallic acid equivalent per gram (mg GAE/g) of fresh material and 3.04 to 9.65 milligrams quercetin equivalent per gram (mg QE/g) of fresh material respectively. The bioactivities of the extracts observed in this study may be attributed to the presence of these compounds.

In this study, the antioxidant activity was observed to be concentration dependent. The IC_{50} values based on DPPH (0.06 and 0.08 mg/mL) and ABTS (0.06 and 0.03 mg/mL) for *T. alliacea* and *T. violacea* respectively, were generally lower showing potential antioxidant activities. The antioxidant activity was observed to be higher in ABTS assay than DPPH assay. *Tulbaghia violacea* showed the most potent antioxidant activity and the highest amounts of total phenolic and flavonoid content compared to the other species. However, all the eight

Tulbaghia plant extracts exhibited poor antioxidant activity in comparison to the control ascorbic acid. The poor antioxidant activity observed in the extracts used in this study does not imply low medicinal value of the plants. It is possible that the presence of different components in the crude extracts act in synergy giving rise to activities that are not exhibited by single compounds. Certain compounds are known to work best as single entities. The extracts are also likely to show a different reaction *in vivo.*

8.1.2 Antimicrobial activity

The aqueous extracts for all the species, except *T. violacea*, showed poor antimicrobial activity (MIC values greater than 10 mg/mL) in all the bacterial and fungal strains. The sensitivity of the microbial strains to the acetone extracts from the most to the least sensitive were as follows: *S. aureus* (1.90 mg/mL) > *C. neoformans* (2.41 mg/mL) > *C. albicans* (2.46 mg/mL) > *E. facaelis* (3.46 mg/mL) > *P. aeruginosa* (2.90 mg/mL) > *K. pneumonia* (4.91 mg/mL). Among the species of *Tulbaghia* used in this study the acetone and water extracts of *T. violacea* appear to have contained the most active compounds especially against the proliferation of the fungal species *C. albicans* and *C. neoformans*. The potent antifungal activity of the *Tulbaghia* species is of great significance as it has potential to provide an effective, easily accessible and cheaper treatment against problematic fungal infections. The strong antimicrobial activity exhibited by *T. violacea* supports its wide ethno-pharmacological use. *Tulbaghia violacea* may contain potential antimicrobial compounds that may be of great use for the development of pharmaceutical products which can be therapeutic agents against various diseases.

8.1.3 Anticancer activity

In this study, the crude acetone and water extracts of *T. violacea* were proven to have anticancer activity against esophageal and oral cancer cells *in vitro*. The inhibitory activity of the extracts on cell growth in HKesc-1 and KB cell lines was shown to be concentration dependent. The activation of multicaspase activity in HKesc-1 and KB cells treated with *T. violacea* extracts was dose-dependent leading to cell death by apoptosis and cell cycle arrest at the G_2/M phase.

8.1.4 Immunomodulatory activity

The crude acetone and water extracts of *T. violacea* increased the production of pro-inflammatory cytokine in the LPS stimulated macrophages. The crude acetone extract of *T. violacea* stimulated IL-1α, IL-6 and TNF-α while the water extract stimulated IL-1α and IL-6. High concentrations (>10 000 pg/mL) of the cytokines MCP-1, MIP1-α, MIP1-β, MIP-2,

GCSF, GM-CSF, RANTES and IP-10 were observed in both acetone and water crude extracts of *T. violacea*. This indicates that *T. violacea* extracts have immune modulating activities which may initiate innate immunity.

8.1.5 Toxicity studies

The acetone and water extracts from *T. alliacea, T. simmleri and T. violacea* showed a concentration and time dependent effect on the viability of treated RAW264.7 cells. The alteration of the cell viability of the treated cells suggests that there were substances in the extracts that interfered with the cell function.

This study on zebrafish embryos showed that the toxicity of *T. violacea* acetone and water extracts to zebrafish embryo development was dependent on concentration and exposure time. The potent cytotoxic activity of *T. violacea* supports its use to treat cancer in traditional medicine.

The toxicity of plant samples depend on their composition which may be influenced by a number of factors including geographical source of plants, seasonal and ecological variation, extraction process and plant part used (Akhalwayo et al., 2018). These factors couple with the variation in the assays used in toxicity studies could be the reason there are discrepancies between results in this study and other literature.

8.2 GENERAL RECOMMENDATIONS

8.2.1 Phytochemical analysis and antioxidant activity

Further investigations need to be undertaken to isolate and identify individual compounds from *T. violacea* for antioxidant activity as some compounds work best as single entities. Further analysis of the extracts from *T. violacea* must be conducted using different chromatographic techniques such as GC-MS to provide more elaboration in the phytochemical composition of the species studied.

8.2.2 Antimicrobial activity

The results from this study supports the use of extracts from *T. violacea* to manage microbial infections in traditional medicine. However given that the antimicrobial activity of the *Tulbaghia* species examined in this study was done on collection strains, there is still need to further investigate the effect of acetone and water extracts of *T. violacea* on clinical isolates of interest. Future research should also focus on bioassay guided isolation of compounds that may have antibacterial or antifungal activities.

8.2.3 Anticancer activity

Both acetone and water extracts from *T. violacea* exhibited anticancer activity against Hkesc-1 and KB cell lines. The use of water extracts in traditional medicine should be recommended since it is expected to have lower cytotoxicity on non-cancerous cells *in vivo* compared to acetone extract. Further assays using the animal models to confirm anticancer activity of *T. violacea* extracts should be done. Future studies should also focus on bioassay guided fractionation, isolation and identification of the bioactive compound(s) responsible for the anti-cancer activity which may lead to new oral or esophageal cancer chemotherapeutic agents with novel structures and mechanism of action.

8.2.4 Immunomodulatory activity

Medication prepared from *T. violacea* may be recommended to individuals with weak immune system. The mechanisms by which the extracts effect the immune modulation needs to be examined. Further studies are required to isolate and identify compounds with specific immunomodulatory effect that is either pro-inflammatory or anti-inflammatory activity.

8.2.5 Toxicity studies

This study clearly indicates that caution must be exercised when consuming extracts from medicinal plants for curing different ailments. Traditional medicine prepared from *Tulbaghia* species should be taken preferably in small doses over a short period of time. Given the presence of toxic compound(s) within a plant extract in its crude form, further investigations regarding compounds of therapeutic value should be done.

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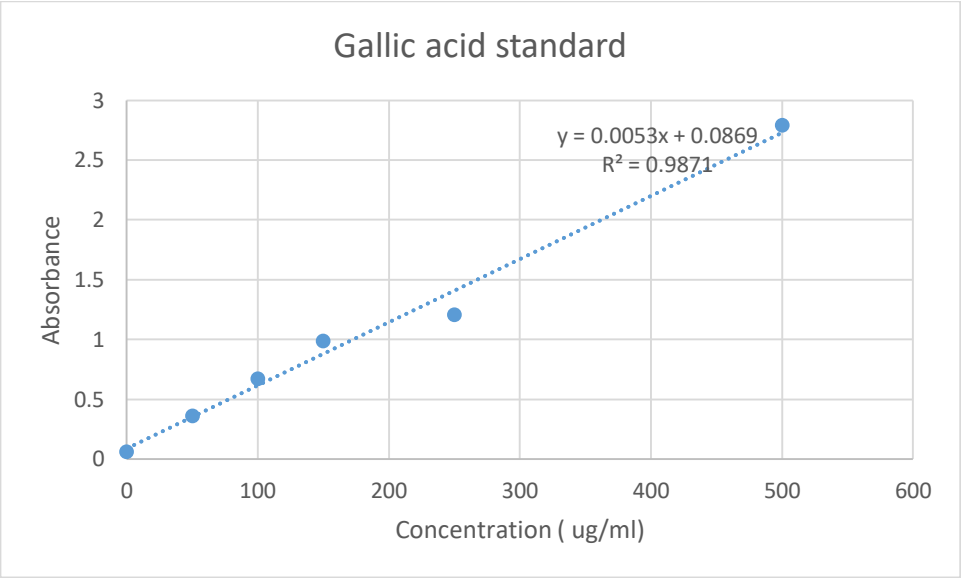
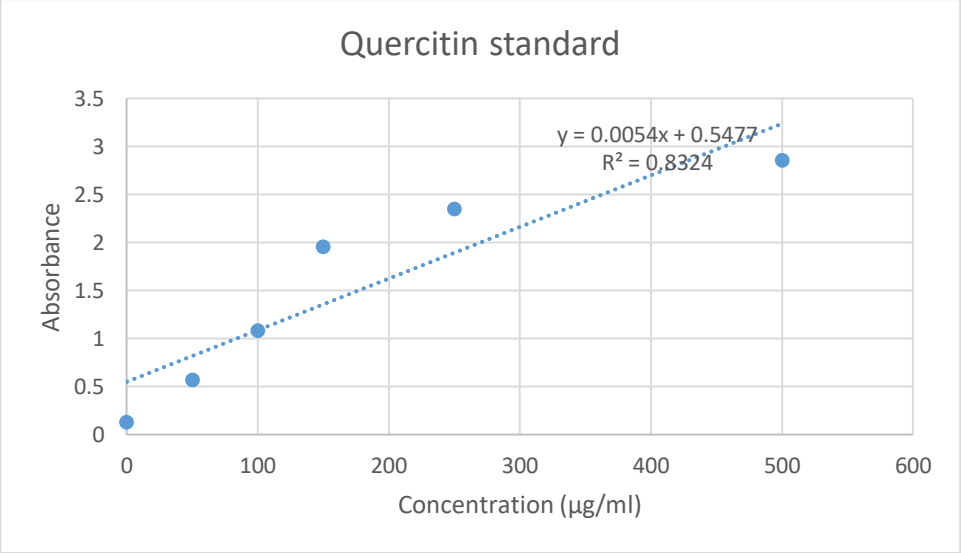
APPENDIX A: GALLIC AND QUERCITIN STANDARD CURVES





T= C x V/M

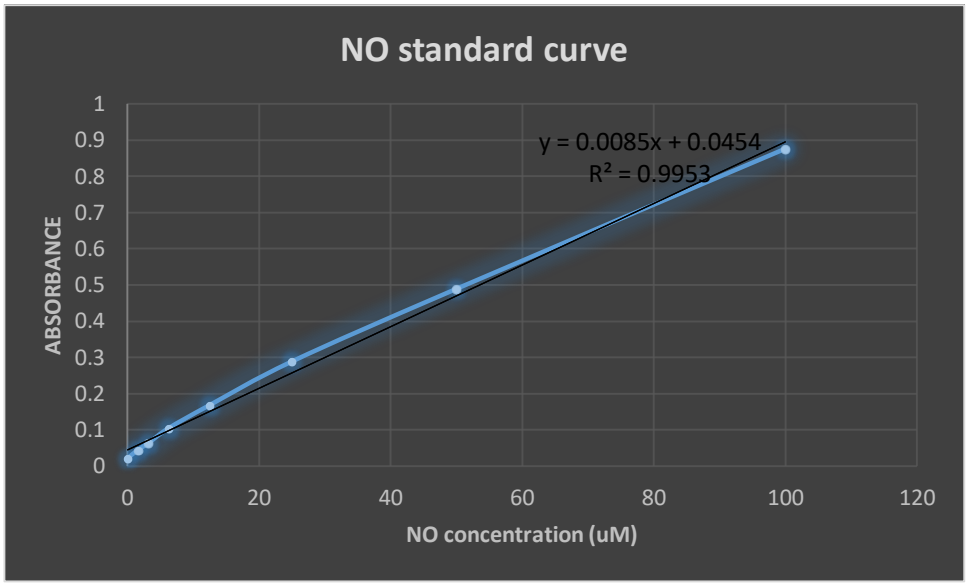
C= the concentration of gallic acid obtained from calibration curve.

V= volume of extract in mL.

M=mass of extract in grams

T= total content of phenolic compounds in mg/g plant extract in GAE.

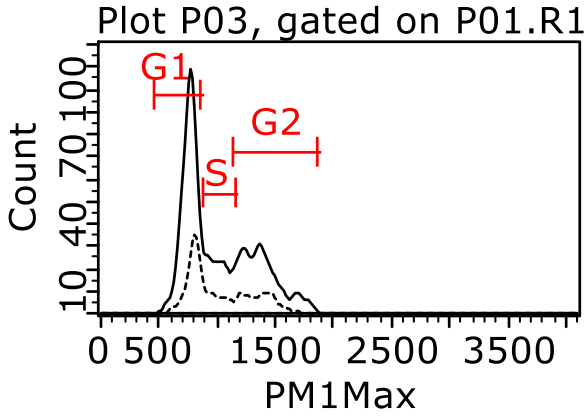
APPENDIX B: NO STANDARD CURVE



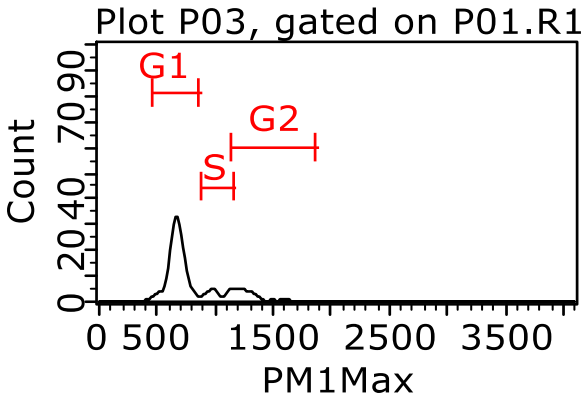
APPENDIX C: CELL CYCLE OVERLAY IMAGES OF HKESC

1. Cell cycle overlay images for HKESC cell line treated with *T. violacea* acetone extract

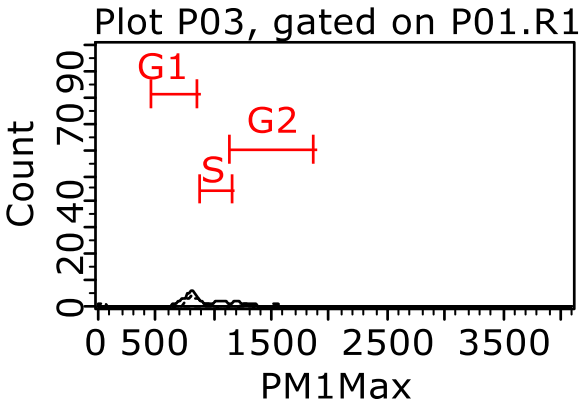
Untreated control



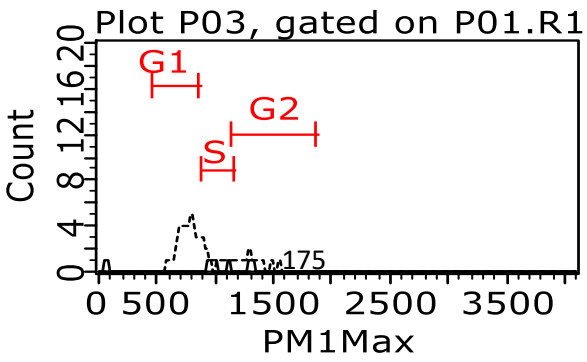
0.2 mg/mL



0.4 mg/mL

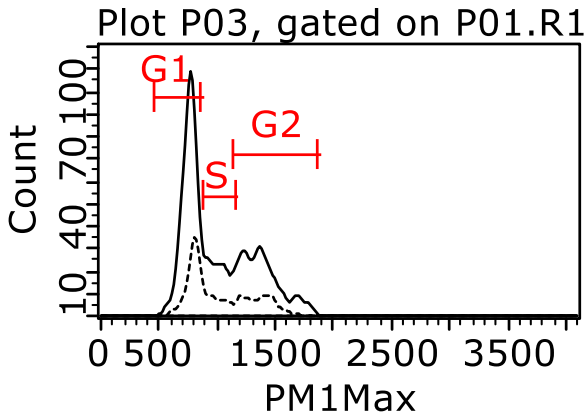


0.8 mg/mL

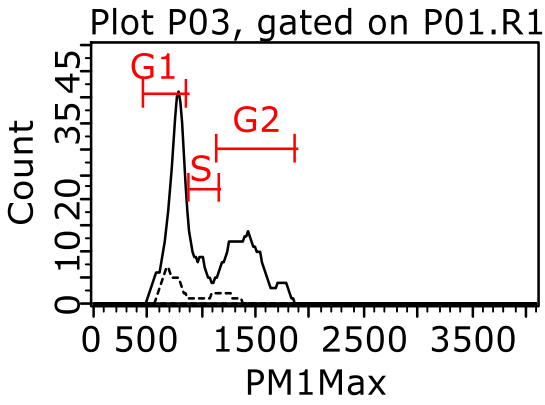


2. Cell cycle overlay images for HKESC cell line treated with *T. violacea* water extract

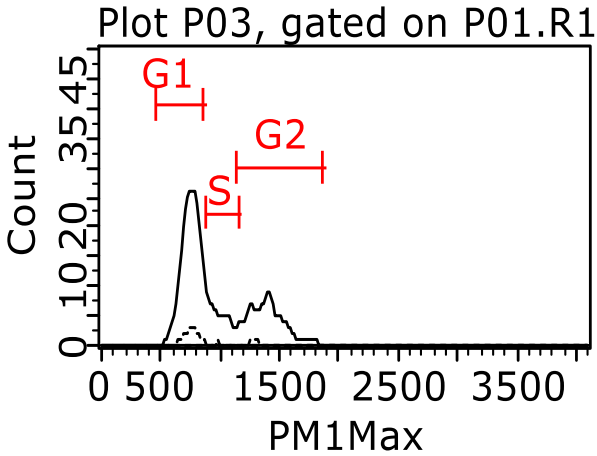
Untreated control



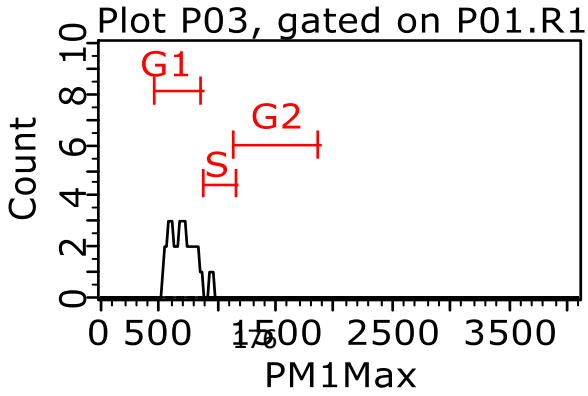
0.625 mg/mL



1.25 mg/mL



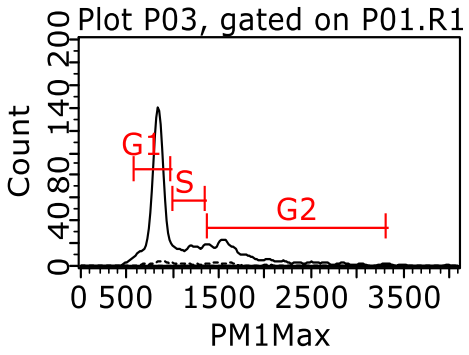
2.5 mg/mL



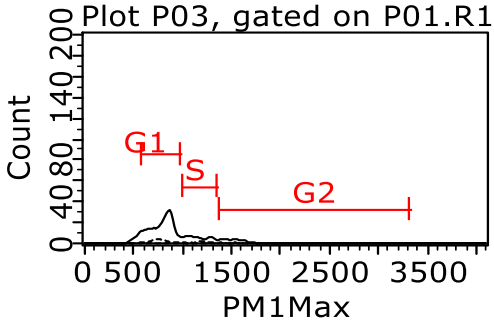
APPENDIX D: CELL CYCLE OVERLAY IMAGES OF KB CELL LINE

1. Cell cycle overlay images of KB cell line treated with *T. violacea* acetone extract

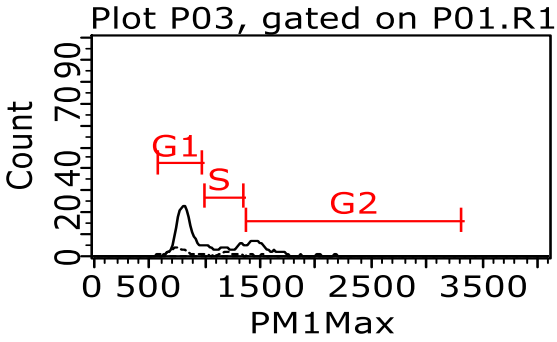
0.1 mg/mL



0.2 mg/mL

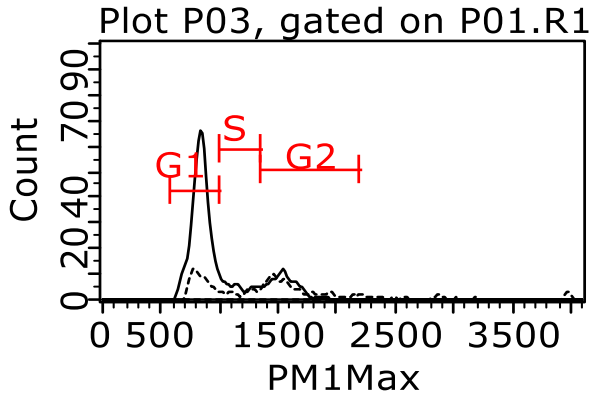


0.4 mg/mL

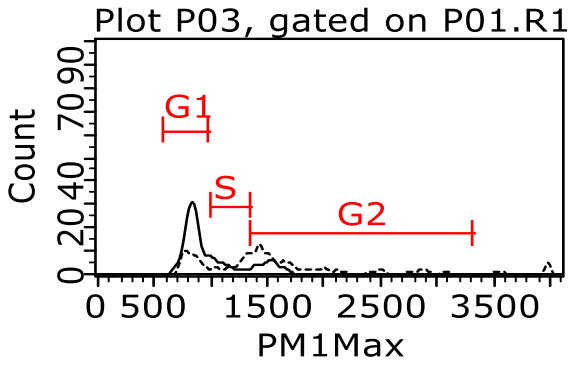


2. Cell cycle overlay images of KB cell line treated with T. violacea water extract

0.5 mg/mL



1 mg/mL



2 mg/mL