An investigation of the phytochemistry and biological activity of *Tulbaghia violacea*

by

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DECLARATION

I, **DIJENG EUGINIAH RAMPANA**, hereby certify that the dissertation submitted by me for the degree MASTER OF HEALTH SCIENCES IN BIOMEDICAL TECHNOLOGY (M HBIO), is my own independent work and complies with the Code of Academic Integrity, as well as other relevant policies, procedures, rules and regulations of the Central University of Technology (Free State). I hereby declare that this research project has not previously been submitted to any university or faculty for the attainment of any qualification. I further waive copyright of the dissertation in favour of the Central University of Technology (Free State).

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DIJENG E. RAMPANA   **DATE**

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LIST OF ABBREVIATIONS AND ACRONYMS

%	Percentage
CNS	Central nervous system
CSIR	Council for Scientific and Industrial Research
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
et al.	*Et alia* (and others)
HELA	Cervix cancer cell
IC50	Concentration required to attain 50% radical scavenging effect
K10	Renal cancer cell
MH	Mueller Hinton
MIC	Minimum inhibitory concentration
NCI	National Cancer Institute
PC-3	Prostate cancer cell
PSA	Prostate-specific antigen
RCC	Renal cell carcinoma
ROS	Reactive oxygen species
WHO	World Health Organisation

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ABSTRACT

Medicinal plants are the most important source of life saving drugs for most of the world's population. The aim of this study was to assess *Tulbaghia violacea* as an anticancer agent, to determine the antimicrobial activity, anticancer activity, antioxidant and total polyphenol activity, cytotoxicity and genotoxicity effects to be used in the development of a novel anticancer remedy. *Tulbaghia violacea* bulb, root and leaf extracts were collected, dried and finely ground with aqueous and methanol/dichloromethane solvents. The extracts were tested for their growth inhibitory effects using the sulforhodamine B assay; genotoxicity activity was determined using NucRed Live 647; antimicrobial activity was determined using p-Iodonitrotetrazolium chloride assay; antioxidant activity was determined using 2,2-diphenyl-1-picrylhydrazyl assay; and the gallic acid method was used to determine the concentration of phenols. The extracts from *T. violacea* were classified as weak or inactive against the human cell lines TK-10 (renal), PC3 (prostate) and HeLa (cervix) cancer. Aqueous and methanol/dichloromethane extracts of *T. violacea* were both non-toxic when tested against the WI-38 cell line. The result of the test for genotoxicity determination showed that leaf water extract induced the formation of micronuclei and could be considered genotoxic. The extract of *T. violacea* exhibited high antioxidant activity compared to the standard. Total phenolic determination showed that water leaf extract had the highest concentration of phenolics and bulb methanol/dichloromethane extract the lowest. The antibacterial activity showed interesting results with certain plant extracts, indicating Gram specificity. Water leaf extracts and water bulb extract from *T. violacea* both showed activity against *Klebsiella pneumoniae* (Gram-negative) but not against *Staphylococcus aureus* (Gram-positive). Root water extract showed a similar result at the lower concentrations. The high concentrations of root methanol/dichloromethane extract showed a more drastic increase in the percentage cell death of *S. aureus* than that of *K. pneumoniae*. Methanol/dichloromethane bulb extract also seemed to be effective against *S. aureus.* The extracts of *T. violacea* have the potential to be developed as an antimicrobial agent, but the standard has higher antimicrobial activity. These results indicated the safety and importance of *T. violacea* as a potential for the development of a novel drug.

CHAPTER 1

Literature Review

1.1 Introduction

Plants are known to be significant sources of chemotherapeutic agents (Mohammad, 2006). Medicinal plants play a major role in primary health care in rural areas (Parekh, *et al.,* 2006). It has been reported that 65% to 80% of the world's population rely mainly on traditional medicinal plants for the treatment of different diseases and infections (WHO, 2002; Tullayakorn *et al.,* 2012). Throughout history, natural products have proven to be rich repositories of medicines with different chemical structures and bioactive compounds that are effective against many diseases (Tullayakorn *et al.,* 2012). The use of medicinal plants as treatment has increased rapidly in the past three decades (Valli *et al.,* 2002). Rural people in various parts of the world have a universal tendency to depend on medicinal plants growing in areas close to them (Tullayakorn *et al.,* 2012). Treating infectious diseases with natural products rather than conventional drugs is more advantageous and affordable to many patients in the Southern African situation, since these products are readily available in the area in which they live, and some are cultivated to ensure product quality and safety (Muleya *et al.,* 2014).

Skills have been developed and learned over many years, affecting different and independent systems of drug use (Engel, 1977). The availability of medical plants and the cultural use of plants make this an area of significance for the discovery of therapeutic species and ethno-medicinal research (Cox *et al.,* 1994). It is important to note that some plant-based remedies are mostly used by traditional people to cure easily treated diseases and protect them from many complex diseases (Gurib-Fakim, 2006).

There is an ongoing need for development of novel drugs that are highly effective in the treatment of various diseases, such as cancer (Elham *et al.,* 2014). The pharmacological evaluation of plants is a significant means of finding new, safe and effective drugs (Farnsworth *et al.,* 1985). Natural products, such as medicinal plants, whether used as a pure compound or as a standardised extract, provide unlimited possibilities for new drug development because of the unmatched accessibility of chemical variety (Sasidharan *et al.,* 2011).

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A growing number of people are turning to plant-derived medicines and remedies for various reasons, which include their low cost compared to Western pharmaceuticals, and are looking for natural alternatives, which are widely believed to have fewer side effects (Kumar *et al.*, 2003). The traditional medicine industry is a multi-million-rand industry in South Africa and is continually growing (Ndhlala *et al.*, 2010). Although the Medicines and Related Substances Control Act 101 of 1965 was amended in 2002, the regulation of commercially labelled medicines, herbal formulations and nutritional supplements is still complex (Ndhlala *et al.*, 2011). Medicine regulation is in the public interest and includes three integral aspects: quality, safety and efficacy (Sasidharan *et al.*, 2011).

There are some concerns about the quality of the hygiene and potential contamination of traditional products used in herbal medicines, which are sold on pavements and in markets where the materials are often exposed to sputum, urine and faeces, contrasting with the pharmaceutical manufacturing standards that are required for the production and packaging of other medicines (Kunle *et al.*, 2012). In terms of efficacy and safety, it is essential to determine the relative risk from traditional African herbs and remedies (Ndhlala *et al.*, 2010).

1.2   Drug development

Innovations in the health sciences have resulted in dramatic changes in the ability to treat disease and improve the quality of life (Kazis *et al.*, 1989). The cost of pharmaceuticals has grown faster than other key components of the health care system since the late 1990s (Grabowski *et al.*, 2002). Therefore, discussions on rising health care costs and the development of new therapeutic technologies have focussed increased attention on the pharmaceutical industry, which is both a main contributor to the health care industry and a main source of advances in health care technologies (DiMasi *et al.*, 2003). Furthermore, great ecological awareness has led to increased curiosity about natural plant formulations worldwide (Lindemann-Mathies, 2005). The effectiveness of many plant preparations has been established by valid phytopharmaceutical techniques and the number of herbal-based medicines or health

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foods has increased progressively to meet the increasing demand (Atta-ur-Rahman *et al.*, 2001).

1.2.1 Anticancer activity

In the 1960s, camptothecin (CPT) was discovered by Wall and Wani as an anticancer treatment with an exclusive mode of action, that is, inhibition of deoxyribonucleic acid (DNA) topoisomerase I (Wall *et al.*, 1996). This naturally occurring alkaloid was first extracted from the stem wood of the Chinese ornamental tree *Camptotheca acuminata,* also known as the 'tree of joy' and 'tree of love', through the screening of thousands of plants in an examination for steroids (Srivastava *et al.*, 2005). Initial research discovered significant anticancer activity in a standard *in vitro* assessment system, also in mouse leukaemia cells (Hertel *et al.*, 1990). These astounding discoveries stimulated significant interest in this herbal product as a possible anticancer agent. Currently, the first-generation analogues of CPT, hycamtin (topotecan) and camptosar (irinotecan, CPT-11), marketed by Glaxosmithkline and Pfizer, are used for the treatment of ovarian cancer, lung cancer and colon cancer (Diwaker *et al.*, 2012).

The alkaloids, vinblastine and vincristine, from the Madagascar periwinkle, *Catharanthus roseus* (L.) (*Apocynaceae*), were clinically used as anti-tumour agents, among others to treat leukaemia, lymphoma, advanced testicular cancer, breast and lung cancer and Kaposi's sarcoma (Shah *et al.*, 2013). The discovery of paclitaxel from the bark of the Pacific yew, *Taxus brevifolia* (Taxaceae), is another example of herbal product treatment discovery (Itokawa *et al.*, 2008). Paclitaxel was reported to be important in the treatment of ovarian cancer, advanced breast cancer and lung cancer (Shah *et al.*, 2013). In South Africa the root and bark of *Combretum caffrum* have been found to have anticancer properties and it is likely that the combretastatin molecule is the most successful cytotoxic phytomolecule identified to date (Pettit *et al.*, 1982; Mohammad 2006; Kharb *et al.*, 2012).

1.2.2 Antimicrobial activity

Since ancient times, human beings have used herbs to treat common infectious ailments and some plant-based therapy is still included as part of the usual treatment of numerous diseases (Ncube *et al.*, 2008). *Arctostaphylos uva-ursi* and *Vaccinium macrocarpon* have been used to treat urinary tract infections (Bag *et al.*, 2008). The essential oils of thyme, origanum, mint, cinnamon, salvia and clove also have antimicrobial properties (Rios *et al.*, 2005).

The roots of *Crytolepis sanguinolenta* have antimicrobial properties and are also used in the treatment of urinary infections (Elujoba *et al.*, 2005). In the Centre for Research on Pharmacopoeia and Traditional Medicine in Rwanda, *Datura stramonium, Eucalyptus globes, Capsicum frutescens* and *Plantago lanceolate* are readily available in the dispensary of plant-based remedies (Elujoba *et al.*, 2005). These are administered for their antispasmodic, pulmonary disinfectant, counter-irritant and anti-tussive activities (Elujoba *et al.*, 2005). The root and stem-bark extracts of the two *Zanthoxylum* species have high antimicrobial activity (Rios *et al.*, 2005). The essential oils of the eugenol purified from *Ocimum gratissimun* also have antimicrobial properties to treat pneumonia, diarrhoea and conjunctivitis (Khan *et al.*, 2009).

1.2.3 Antioxidant activity

Free radicals are formed as part of normal metabolic processes, and are unstable and very reactive (Valko *et al.*, 2007). Antioxidant enzymatic systems and chemical substances such as endogenous enzymes or naturally occurring dietary antioxidants and some hormones control the cellular reduction oxidation state (Lobo *et al.*, 2010). The increased formation of free radicals overpowers the antioxidant defence and enforces oxidative stress on the physiological system (Chong *et al.*, 2017). Too much oxidative stress can harm cellular lipids, proteins or DNA by suppressing their normal role (Marnett, 2000). The human body naturally continually activates a battery of detoxifying enzymes or inhibits the generation of reactive oxygen species (ROS) and reactive nitrogen species through several mechanisms, such as antioxidant enzymes and molecules (Mates 2009). Numerous synthetic substances, such as vitamins,

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butylated hydroxy anisole, butylated hydroxytoluene, propyl gallate and tert-butylhydroquinone are used as antioxidants but are suspected of being toxic to humans and experimental animals (Shahidi *et al.*, 2005). Hence, the development of new drugs and the use of more effective antioxidants of natural origin are very significant. Furthermore, antioxidant evaluation of medicinal plants might yield understanding of the mechanism of their pharmacological activities (Olorunnisola *et al.*, 2011).

1.3   *Tulbaghia violacea*

Tulbaghia violacea Har*v.* (Figure 1.1) of the family Alliacea is a small perennial bulbous herb with corm-like rhizomes and narrowly linear, evergreen aromatic leaves (Lyantagaye 2011). The flowers are tubular, mauve or pale purple, occurring in groups of about 10 at the tip of the slender stalk (Raji *et al.*, 2012). The plant prefers partial shade or full sun and dry to moist soils (Meerow *et al.*, 1993). The developed height ranges from 30 cm to 120 cm, depending on environmental circumstances (Lyantagaye, 2011). The plant can be grown successfully in a tub and transferred to a greenhouse or a frost-free place to overwinter (Saibu *et al.*, 2015). The herb has a robust odour of onion or garlic when bruised, hence its common names, wild garlic or society garlic (Kubec *et al.*, 2002). Despite its garlic-like flavour, the consumption of *T. violacea* is not accompanied by the development of bad breath (Lyantagaye, 2011). This plant is commonly called different names by diverse groups in South Africa, such as wild garlic (English), wildeknoffel (Afrikaans), Isihaqa (Zulu) and Moelela (Sotho) (Lyantagaye, 2011).

This plant is used in traditional medicine in the Eastern Cape and KwaZulu-Natal for treating diseases and clinical symptoms such as fever, colds, asthma, tuberculosis, stomach-ache and cancer of the oesophagus (Olorunnisola *et al.*, 2012). The bulbs of *T. violacea* are used as a remedy for pulmonary tuberculosis and to destroy intestinal worms (Lyantagaye, 2011; Raji *et al.*, 2012). The Zulu-speaking traditional people use the bulb to make an aphrodisiac medicine (Lyantagaye, 2011). Some Rastafarians eat copious amounts of this plant and chili during winter, allegedly to keep the blood warm

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and stop aches and pains (Lyantagaye, 2011). In the Eastern Cape it is used for colic, wind, restlessness, headache and fever, largely for young children (Lyantagaye, 2011). The plant is also used to keep snakes away (Bungu *et al.*, 2008; Adeyemi *et al.*, 2013).

Figure 1.1 *Tulbaghia violacea* plant (https://www.amazon.com/Society-Garlic-Tulbaghia-Violacea-Gallon/dp/B0789MB71C)

1.4   Aim of the study

The current study was conducted with the aim of investigating the biological activity and phytochemical analysis of aqueous and dichloromethane extracts of *T. violacea*.

1.5 Objectives of the study

The objectives of the study were to:

- conduct *in vitro* anticancer screening of *T. violacea* extracts;
- assess antimicrobial activity of both aqueous and dichloromethane extracts;
- determine total polyphenol composition of the plant extract; and
- evaluate the genotoxicity and establish the safety of the plant extracts through cytotoxicity assays of both the aqueous and dichloromethane extracts.

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1.6   Chapter outline

This study is presented in five chapters. Chapter two presents the anticancer activities of the aqueous and dichloromethane extracts from *T. violacea* using sulforhodamine B (SRB) assay. The safety of the plant extract is dealt with in chapter three through cytotoxicity assays. The antioxidant activity and the total polyphenol analysis of the plant extract are covered in chapter four. The fifth chapter is an assessment of the antimicrobial activity of the plant extract against *Staphylococcus aureus* (ATCC 25923) and *Klebsiella pneumoniae* (ATCC700603) and the sixth chapter presents the genotoxicity analysis of the extract.

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CHAPTER 2

Anticancer screening of *Tulbaghia violacea*

2.1 Introduction

Cancer is a disease of complex aetiology, defined as uncontrolled growth of cells (Ruffa *et al.*, 2002). Cancer is a major cause of death registered worldwide by public health institutions, exceeded only by cardiovascular diseases (Yaacob *et al.*, 2010). Cancer starts with the deformation of a natural cell, triggered by genetic mutations in DNA (Ruffa *et al.*, 2002). The World Health Organisation (WHO) has projected that if instant action is not taken, the incidence of cancer will be increased by 80% by 2030, with most cases occurring in low- and middle-income countries (Khazir *et al.*, 2014). External factors (such as tobacco, infectious organisms, chemicals and radiation) and internal factors (inherited mutations, hormones, immune conditions and mutations that occur from metabolism) are mostly responsible for cancer (Khazir *et al.*, 2014). These causal factors may act together or in sequence to initiate or promote the growth of cancer (Khazir *et al.*, 2014). The transformation of a normal to a cancerous cell involves three distinct phases, namely initiation, promotion and progression (Foulds, 1954). Cancer is a significant health problem in developing and developed countries (Kooti *et al.*, 2017). According to the WHO, 14 million people suffer from cancer and 8 million die from cancer worldwide annually (Khazir *et al.*, 2014).

Various methods exist for the treatment of cancer, namely chemotherapy, radiotherapy and surgery. Despite being highly efficient, surgery is not always practical under all circumstances (Asadi-Samani *et al.*, 2016). For radiotherapy, systemic radiotherapy is conducted in various ways, including external radiation therapy, brachytherapy and internal radiation therapy (Pijls-Johannesma *et al.*, 2010). The effect of therapy leads to changes in skin health, such as alopecia and destruction of epithelial cells (scaling) and epithelial moisture (dermis revealing and skin secretion of serous fluid) and potentially to mouth ulcers and complications, bone marrow suppression and development of anaemia, leukopenia and thrombocytopenia (Asadi-Samani *et al.*, 2016). Chemotherapy treatments, including antimetabolites (such as methotrexate), inactive material of DNA (such as cisplatin and doxorubicin), antitubulin agents (such as taxis), and hormones that are most often used cause unwanted effects, including hair loss, bone marrow suppression, drug resistance, gastric ulcers, neurological dysfunction and cardiac toxicity (Majid *et al.*, 2016). Most cancer

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chemotherapies severely affect the host's normal cells (Maeda *et al.*, 2009). The most significant problem in cancer treatment is destroying tumour cells among natural cells, without harming the natural cells (Kooti *et al.*, 2017). These available cancer treatments are expensive and inaccessible to people living in resource-limited and poor communities (Takaidza *et al.*, 2018).

The use of medicinal plants is considered as of outstanding value in cancer treatment (Mans *et al.*, 2000). Cancer is one of the most prominent ailments in humans and at present there is significant scientific and commercial interest in the discovery of new anticancer agents from natural products. Natural products such as flavonoids, terpenes, alkaloids, etc. have received significant attention in recent years owing to their various pharmacological properties, including cytotoxicity and anticancer features (Shaktikumar *et al.*, 2011). Only a few of them have been scientifically proven to have potent abilities to treat cancer (Couzin-Frankel, 2013). The probability of using natural products as anticancer agents was recognised in the 1950s by the US National Cancer Institute, which has since made major contributions to the discovery of new naturally occurring anticancer agents (Fouché *et al.*, 2008). South Africa's rich plant biodiversity, with over 20 000 different species, is an excellent source of interest to the scientific community (Turpie, 2003). Despite the approach of using natural products to develop anticancer drugs, reports on plants used for the treatment of cancer are rare in South Africa (Fouché *et al.*, 2008). Natural products are known to serve as a helpful source of cancer chemo-preventive agents (Pezzuto, 1997). There is a possibility that regular use of medicinal plants could reduce the incidence of cancer by inhibiting the development of carcinogenesis (Ruffa *et al.*, 2002). However, many drugs of natural origin still need to be investigated as new anticancer agents, to increase the range obtainable and to discover less toxic and more effective drugs. Samples with pharmacological usage should be considered when selecting plants to treat cancer, as some ailments reproduce disease states of significance to cancer or cancer-like symptoms (Bellamy *et al.*, 1990).

Prostate cancer is a leading cause of cancer-related death in males (Jemal *et al.*, 2005). Prostate cancer has surpassed lung cancer as the most common cancer in men (Mohler *et al.*, 2010). A relatively low death rate suggests that increased public

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awareness, with earlier detection and treatment, has started to affect mortality from this common cancer (Mohler *et al.*, 2010). However, early detection and treatment of prostate cancers that do not threaten life expectancy result in unnecessary side effects (Mohler *et al.*, 2010). Prostate tumours are particularly heterogeneous, i.e. composed of cells with different phenotypic characteristics and different proliferative and malignant potentials (Collins *et al.,* 2005). In leukaemia and some solid cancers, only a small subset of cells is clonogenic in culture and *in vivo* (Collins *et al.,* 2005). Androgen deprivation therapy plays an essential role in the management of locally advanced, recurrent and metastatic prostate cancer (Collins *et al.*, 2005).

However, most patients go on to develop androgen-independent disease that remains incurable by current treatment strategies (Collins *et al.*, 2006). Prostate-specific antigen (PSA) in combination with digital rectal analysis forms the basis for current prostate cancer screening programmes (Mohler *et al.*, 2010). However, PSA examination has recently proven to reduce prostate cancer-specific mortality. Its limitations include the risk of unnecessary prostate biopsy and the diagnosis and treatment of cancers that might never have been symptomatic (Mohler *et al.*, 2010). The exact diagnosis of clinically significant prostate cancer remains a challenge (Kelloff *et al.*, 2009). Investigation of a correlation between the PSA level and biopsy results in the placebo group of a recent trial indicates that there is no normal PSA level (Mohler *et al.*, 2010). Acknowledging that the PSA level is a continuum rather than a dichotomous marker makes accurate diagnosis of clinically significant prostate cancer even more challenging (Mohler *et al.*, 2010).

Renal cell carcinoma (RCC) includes a diverse group of solid cancers originating from renal parenchyma. RCC, with a worldwide rising occurrence, is the seventh most common tumour in men and the ninth most common in women and constitutes about 2%-3% of adults' malignancies (Hirbod-Morabakeh *et al.*, 2017). RCC caused more than 140 000 deaths globally in 2012 (Hirbod-Morabakeh *et al.*, 2017). RCC arises from the renal epithelium and comprises approximately 80% of all kidney cancers (Drucker, 2005). The original treatment for localised RCC is surgery, but 20%-40% of patients characteristically relapse, with a median time to relapse of one to two years (Hudes *et al.*, 2011). In addition, about one third of RCC patients present with metastatic disease (Elson *et al.*, 1988). Until recently, patients with RCC whose

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disease had become metastatic had few treatment options (Albiges *et al.*, 2012). Therefore, patients treated with targeted therapies need close monitoring with specific management algorithms established to improve patient benefit (Hudes *et al.*, 2011). Patients with RCC may show local or systemic symptoms, although most symptoms are related owing to the widespread use of abdominal imaging (Escudier *et al.*, 2010). Cervical cancer is the third most diagnosed cancer and the fourth leading cause of cancer death in women globally; approximately 85% of these deaths happen in developing nations (Nour, 2009; Khaled, 2014; Sawadogo *et al.*, 2014). Cervical cancer has a great societal impact because it mainly affects females from their 30s to their 50s, who are frequently raising or supporting families (Sawadogo *et al.*, 2014). Thus far, cervical cancer has remained a common source of tumour death in women in developing countries lacking access to screening (Pap test) or vaccines against human papilloma virus (Vizza *et al.*, 2018). Persistent human papillomavirus infection is frequently associated with the development of cervical intraepithelial neoplasia grade 3 or carcinoma *in situ* "precancer", which can lead to cervical cancer (Sawadogo *et al.*, 2014).

Because of complications with the therapies currently available for treatment of cancer, the high cost of conventional therapies and the growing incidence of cancer in both developed and developing countries (Collins *et al.*, 2005), it seems necessary to develop more novel approaches with higher efficiency to decrease the disease intensity (Collins *et al.*, 2005). In this regard, there is considerable scientific and commercial interest in developing new anticancer agents from natural sources (Collins *et al.*, 2005). The development of new anticancer drugs has been turned into a significant research area. Researchers have attempted to find naturally derived combinations to synthesise new drugs to treat the disease (Collins *et al.*, 2005; Patel *et al.*, 2018). Plants have a long history of use in the treatment of cancer (Cragg *et al.*, 2005). It is important that over 60% of currently used anticancer agents are derived in one way or another from plants (Fouché *et al.*, 2006).

2.2 Methodology

2.2.1 Plant collection, preparation and extraction

The plant material of *T. violacea* was authenticated by scientists at the National Botanical Garden in Bloemfontein, South Africa. The collected material was washed to remove soil and then separated into leaves, bulbs and roots. The plant parts were oven-dried at 30-60ºC for five days. The dried plant parts were ground to a coarse powder using a hammer mill and stored at ambient temperature prior to extraction.

The dried and ground plant materials were blended in part of the extraction solvent (water and methanol/dichloromethane 1:1) using the Waring blender. After blending the rest of the solvent was added and solutions were allowed to steep for 24 h. After 24 h the solids were removed from each solution by filtration with a Millipore funnel with medium filter paper (Bright sign no 102) connected to a Millipore vacuum pump. Organic extracts were concentrated by rotary vacuum at 50-60ºC, then further dried *in vacuo* at ambient temperature. The aqueous extracts were concentrated to dryness using a freeze-dryer. The six plant extracts (Table 2.1) were stored at -20 ºC until further use.

Table 2.1: Extraction of *Tulbaghia violacea* with methanol/dichloromethane and water solvents

Sample No.	Part	Extraction solvent	Yield (g)
1	Leaves	Methanol/dichloromethane (1:1)	6.2716
2	Leaves	Water	7.1516
3	Bulbs	Methanol/dichloromethane (1:1)	4.8616
4	Bulbs	Water	6.8882
5	Roots	Methanol/dichloromethane (1:1)	6.2868
6	Roots	Water	3.4951

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2.2.2 Sulforhodamine B assay

The SRB assay was used to measure the growth inhibitory effects of the investigated plant extracts. The SRB is based on the ability of the protein dye SRB (Acid Red 52) to bind electrostatically in a pH-dependent manner to protein-basic amino acid residues of trichloroacetic acid-fixed cells. Under mild acidic conditions it binds to the fixed cellular protein, while under mild basic conditions it can be extracted from cells and solubilised for measurement. The SRB assay was performed at the Council for Scientific and Industrial Research (CSIR) in accordance with the protocol of the Drug Evaluation Branch, the National Cancer Institute (NCI).

The human cell lines TK10 and PC-3 were obtained from the NCI in the framework of a collaborative research programme between the CSIR and the NCI. The growth inhibitory effects of the plant extracts were tested in the three cell lines panel consisting of TK10 (renal), PC-3 (prostate) and HeLa (cervix) cancer cells by SRB assay. Cell lines were routinely maintained as a monolayer cell culture at 37ºC, 5% CO_2, 95% air and 100% relative humidity in Roswell Park Memorial Institute containing 5% foetal bovine serum, 2 mM L-glutamine and 50 µg/ml gentamicin. For the screening experiment, the cells (3-19 passages) were inoculated in 96-well microtiter plates at plating densities of 7-10 000 cells/well and were incubated for 24 h. After 24 h the cells were treated with the experimental plant extracts, which had previously been dissolved in dimethyl sulfoxide (DMSO) and diluted in medium to produce five concentrations. Cells to which no drug had been added served as control. The blank contained complete medium without cells. Parthenolide was used as a standard. The plates were incubated for 48 h after addition of the plant extracts. Viable cells were fixed to the bottom of each well with cold 50% trichloroacetic acid, washed, dried and dyed by SRB. Unbound dye was removed, and protein-bound dye was extracted with 10 mM Tris base for optical density determination at a wavelength 540 nm using a multi-well spectrophotometer.

2.2.2.1 Statistical assay

Data analysis was performed using GraphPad Prism software. The cell growth activities of the plant extracts were presented as IC_{50} (concentration required to attain 50% radical-scavenging effect) values and were divided into four categories (Table 2.2). Fifty percent of cell growth inhibition (IC_{50}) was determined by non-linear regression.

Table 2.2: CSIR standard criteria for anticancer activity

IC_{50}, μM	Status	IC_{50}, μg/ml	Status
> 100	Inactive	> 100	Inactive
< 100 > 50	Weak	< 100 >15	Weak
< 50 >10	Moderate	< 15 > 6.25	Moderate
< 10	Potent	< 6.25	Potent

2.3 Results and discussion

The anticancer activity of the crude extracts from *T. violacea* against cancer cell lines and the normal human foetal lung fibroblast cell lines were determined and presented in Tables 2.3-2.9. The CSIR anticancer activity criteria (Table 2.2) was used to interpret and categorise the activities observed. Therefore, the aqueous and methanol/dichloromethane leaf, root and bulb extracts of *T. violacea* were tested against TK10, PC3 and HeLa cancer cell lines. An extract was considered inactive if the IC_{50} value for two or three cell lines was more than 100 μg/ml. An extract with an IC_{50} value greater than 15 μg/ml but less than 100 μg/ml when tested against two or more cell lines was considered weak (Table 2.2).

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(I)   Determination of 50% inhibition concentration

Table 2.3: Parthenolide standard

Conc. (µg/ml)	Log Conc.	%Viability TK-10	SD	%Viability PC-3	SD	%Viability HeLa	SD	Parthenolide graph
100	2.0	3.89	0.38	0.40	0.13	10.42	0.65	
10	1.0	3.35	1.54	12.31	0.01	35.80	0.33	
1	0.0	92.27	2.28	93.04	1.15	101.27	0.45	
0.1	-1.0	105.03	0.25	100.87	0.80	99.64	2.71	
0.01	-2.0	102.81	1.78	101.95	0.74	100.86	1.45	
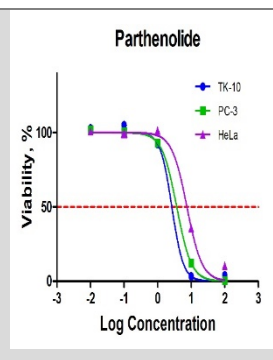
	TK-10	PC-3	HeLa
IC50, µg/ml	2.658	3.710	7.483

The parthenolide (standard) was tested against three cell lines, namely TK-10, PC-3 and HeLa. The standard drug (Parthenolide) (Table 2.3) had potent anticancer activity with an IC50 value of less than 6.25 µg/ml for all three cell lines. The control results obtained correlated with olive leaf extract, which showed a very strong reduction of cell viability in cervical cancer cells when treated with Parthenolide (Vizza *et al.*, 2019).

Therefore, the growth inhibitory effects of the plant extracts were then tested on the three cell lines panel consisting of TK10 (renal), PC-3 (prostate) and HeLa (cervix) cancer cells by SRB assay and compared to the control Parthenolide known activity.

Table 2.4: Anticancer activity of methanol/dichloromethane (1:1) leaf extract from *Tulbaghia violacea* on three human cell lines: TK10, PC3 and HELA.

Z' factor: 0.98

Conc. (µg/ml)	Log Conc.	% Viability TK-10	SD	% Viability PC-3	SD	% Viability HeLa	SD	
100	2.0	76.19	0.50	73.27	3.89	78.38	1.69	
10	1.0	85.10	0.37	91.86	2.45	95.35	3.68	
1	0.0	92.87	0.83	96.24	3.58	99.99	0.70	
0.1	-1.0	95.99	0.05	97.63	1.36	99.26	4.52	
0.01	-2.0	99.29	1.02	100.57	0.49	100.01	2.96	

CUT-I-17054A
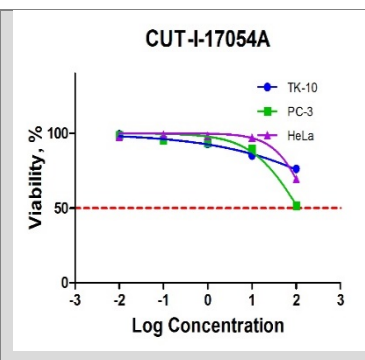
	TK-10	PC-3	HeLa
IC50, µg/ml	>100	>100	>100

The methanol/dichloromethane leaf extract was tested against three cell lines, TK-10, PC-3 and HeLa. The extracts' activity was generally weak; however, there was noticeable anti-cancer activity against PC-3 compared to TK-10 and PC-3. The results in Table 2.4 show that methanol/dichloromethane (1:1) leaf extract was inactive for all cell lines. In order to achieve 50% inhibition of the viable cells there was a need of > 100 ug/ml. This simply indicated that the performance of the extract was weak compared to the control used, as shown in table 2.3. The results obtained from the methanol/dichloromethane leaf extract differed from the published results of the study on the methanol leaf extract. It showed growth inhibition in HeLa and the three different cell lines, namely HT-29(colon cancer), MCF-7 (breast cancer and WHCO3 (oesophageal cancer), though a different solvent was used (Bungu *et al.*, 2006).

Table 2.5: Anticancer activity of water leaf extract from *Tulbaghia violacea* on three human cell lines: TK10, PC3 and HELA

Conc. (µg/ml)	Log Conc.	% Viability TK-10	SD	% Viability PC-3	SD	% Viability HeLa	SD	
100	2.0	40.17	0.17	32.52	1.81	38.80	1.27	
10	1.0	83.00	0.64	83.93	0.05	88.17	0.22	
1	0.0	89.68	0.26	96.84	0.02	95.03	0.15	
0.1	-1.0	98.99	0.08	97.57	0.04	98.15	0.44	
0.01	-2.0	101.24	0.11	97.44	0.23	100.63	0.89	

CUT-I-17054B

	TK-10	PC-3	HeLa
IC50, µg/ml	62.37	49.14	64.8

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The water leaf extract from *T. violacea* was tested against three human cell lines, namely TK10, PC-3 and HeLa. The results in Table 2.5 show that water leaf extract was weak for all cell lines; to obtain 50% inhibition of the viable cells there was a need of 62.37 ug/ml for TK-10, 49.14 ug/m l for PC-3 and 64.8 ug/ml for HeLa. The water leaf extract showed more activity when tested against TK-10 compared to PC-3 and HeLa human cell lines. The water leaf extract proved to have a better inhibitory effect on the PC-3 cell line compared to the other two cell lines. The results obtained from the water leaf extract did not correlate with the results published from the study, which showed water leaf extract of *Tulbaghia violacea* to have better inhibition with high significance between different concentrations on the human oral cell line (Takaidza *et al.*, 2018).

Table 2.6: Anticancer activity of methanol/dichloromethane (1:1) bulb extract from *Tulbaghia violacea* on three human cell lines: TK10, PC3 and HELA

Conc. (µg/ml)	Log Conc.	% Viability TK-10	SD	% Viability PC-3	SD	% Viability HeLa	SD	
100	2.0	82.55	0.10	69.00	1.39	77.92	0.79	
10	1.0	93.42	0.29	93.29	0.35	92.27	0.95	
1	0.0	97.48	0.05	91.78	0.20	98.05	0.99	
0.1	-1.0	97.52	0.01	95.65	1.78	98.36	0.21	
0.01	-2.0	101.24	0.61	97.24	2.38	98.72	0.50	

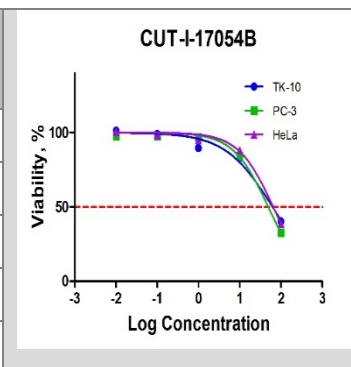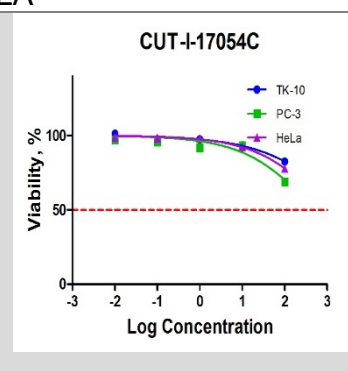|	TK-10	PC-3	HeLa
IC50, µg/ml	>100	>100	>100

The methanol/dichloromethane bulb extract was tested against three cell lines, TK-10, PC-3 and HeLa. All the bulb extracts' activity was weak, though anticancer activity PC-3 was better compared to TK-10 and PC-3. It is suggested that the concentration of extracts be increased to stimulate better activity (Table 2.6).

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Table 2. 7: Anticancer activity of water bulb extract from *Tulbaghia violacea* on three human cell lines: TK10, PC3 and HELA

Conc. (µg/ml)	Log Conc.	% Viability TK-10	SD	% Viability PC-3	SD	% Viability HeLa	SD	
100	2.0	53.02	0.41	57.63	0.03	57.46	1.49	
10	1.0	87.61	0.96	77.65	0.06	95.72	0.08	
1	0.0	94.18	0.77	95.96	0.00	98.67	0.07	
0.1	-1.0	98.18	0.28	98.36	0.16	103.52	0.80	
0.01	-2.0	98.10	0.09	102.06	0.16	104.09	0.14	
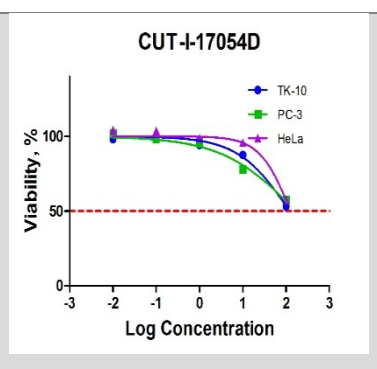
	TK-10	PC-3	HeLa
IC50, µg/ml	>100	>100	>100

The water bulb extract from *T. violacea* was tested against three human cell lines, namely TK10, PC-3 and HeLa, and the water bulb extracts were active. The water bulb extract showed more activity when tested against PC-3 compared to TK-10 and HeLa human cell lines (Table 2.7). The water bulb extract showed the least activity when tested against TK-10 cells.

Table 2.8: Anticancer activity of methanol/dichloromethane (1:1) root extract from *Tulbaghia violacea* on three human cell lines: TK10, PC3 and HELA

Conc. (µg/ml)	Log Conc.	% Viability TK-10	SD	% Viability PC-3	SD	% Viability HeLa	SD	
100	2.0	84.44	0.49	76.57	0.17	85.00	0.68	
10	1.0	100.63	0.12	92.50	0.70	96.24	0.00	
1	0.0	103.76	2.80	99.27	0.27	97.06	0.01	
0.1	-1.0	101.36	0.72	100.22	0.15	99.56	0.04	
0.01	-2.0	102.56	3.19	100.10	0.68	100.29	0.41	
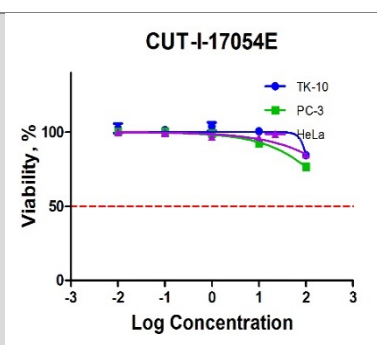
	TK-10	PC-3	HeLa
IC50, µg/ml	>100	>100	>100

The methanol/dichloromethane root extract was tested against three cell lines, TK-10, PC-3 and HeLa. All the root extracts showed weak activity, though PC-3 reacted better compared to TK-10 and PC-3. The root extracts showed weak activity when tested against the TK-10 cell line. It is suggested that the concentration of extract be increased to stimulate better results (Table 2.8).

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Table 2.9: Anticancer activity of water root extract from *Tulbaghia violacea* on three human cell lines: TK10, PC3 and HELA.

| Conc. (µg/ml)	Log Conc.	% Viability TK-10	SD	% Viability PC-3	SD	% Viability HeLa	SD
100	2.0	60.19	0.49	52.52	0.01	54.54	0.74
10	1.0	87.76	0.19	86.66	1.11	86.54	0.21
1	0.0	89.90	0.42	88.02	0.30	90.58	0.61
0.1	-1.0	106.13	0.68	91.72	0.66	98.95	3.08
0.01	-2.0	103.68	4.59	97.99	0.01	99.55	2.22
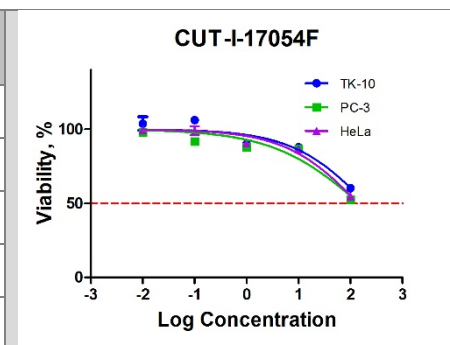
	TK-10	PC-3	HeLa
IC50, µg/ml	>100	>100	>100

The water root extract from *T. violacea* was tested against three human cell lines, namely TK10, PC-3 and HeLa and was active in all cases. The root extracts were more active when tested against PC-3 compared to TK-10 and HeLa human cell lines. The root extracts showed the least activity when tested against the TK-10 cell line.

Table 2.10: Summary of anticancer activity of the extracts from *T. violacea*

No.	Sample	Solvent	IC50 for TK-10, µg/ml	IC50 for PC-3, µg/ml	IC50 for HeLa, µg/ml
1	Leaves	Methanol/dichloromethane (1:1)	>100	>100	>100
2	Leaves	Water	62.37	49.14	64.8
3	Bulbs	Methanol/dichloromethane (1:1)	>100	>100	>100
4	Bulbs	Water	>100	>100	>100
5	Roots	Methanol/dichloromethane (1:1)	>100	>100	>100
6	Roots	Water	>100	>100	>100
	Parthenolide	Standard	2.658	3.710	7.483

The aqueous and methanol/dichloromethane *leaf, bulb and root extracts* from *T. violacea* at equal ratios were tested against three cell lines, TK-10, PC-3 and HeLa, as shown in Table 2.10. The methanol/dichloromethane leaf extracts from *T. violacea* were weak when tested against TK-10, PC-3 and HeLa cancer cell lines. The aqueous leaf extracts showed inhibition activity when tested against TK-10, PC-3 and HeLa cancer cell lines. The methanol/dichloromethane bulb extracts were weak when tested against TK-10, PC-3 and HeLa cancer cell lines. The aqueous bulb extracts were also weak when tested against TK-10, PC-3 and HeLa cancer cell lines. The

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methanol/dichloromethane root extracts were weak when tested against TK-10, PC-3 and HeLa cancer cell lines. The aqueous root extracts also proved to be weak when tested against TK-10, PC-3 and HeLa cancer cell lines. The aqueous leaf extract from *T. violacea* showed better inhibition activity against HeLa, followed by TK-10, then PC-3 cancer cell lines. The leaf extracts showed the best activity when compared to root extracts and bulb extracts. These anticancer activity results indicate that the leaf is the best part of the plant to use to conduct further testing of TK-10, PC-3 and HeLa. The water solvent is still the best solvent to use to test the three cell lines, with increased concentration of the plant extracts. The water extracts from *T. violacea* showed better inhibition activity; this correlates with the fact that traditional healers are using water to perform extraction.

2.4 Conclusion

The extracts from *T. violacea* were generally weak or inactive against the human cell lines TK-10 (renal), PC3 and HeLa, as shown in Table 2.2-Table 2.9. A higher concentration is suggested to test these three human cell lines against aqueous and methanol/dichloromethane extracts. The aqueous solvent proved to be the solvent of choice to perform extraction on the *T. violacea* plant. Also, the leaf extract would be the best part of the plant to test against TK-10, PC-3 and HeLa human cell lines. The values of IC50 for two to three cell lines show inactivity (>100 µg/ml) of *T. violacea* plant extracts. Acokanthera and Gomphocarus are causes of cardiac glycosides and are widely used throughout Africa as a traditional source of extremely toxic arrow poisons. Kalanchoe paniculate, Kalanchoe thyrstflora and Coryledon cuneate (0.4% hit rate), of the family Crassulaceae, are described as containing bufadienolides and these are toxic to livestock and a source of the serious poisoning syndrome known as nenta or krimpiekte (Fouche *et al.*, 2008). The aqueous extract of leaves supports the claim made by traditional healers for their use of the *T. violacea* plant for the treatment of cancer and other diseases. Further testing using other cell lines is required, also to identify the active compounds of *T. violacea* plant extracts.

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CHAPTER 3

Cytotoxicity and genotoxicity evaluation of *Tulbaghia violacea plant extracts*

3.1 Introduction

Many South Africans depend on plant-based medicine for their primary health care needs (Srivastava *et al.*, 1996). Unsuitable methods of collection, processing and storage, including unwanted contaminants in the products, have contributed to the negative view of African natural plant products competing on worldwide markets (Mulaudzi *et al.*, 2017). The dependence of such a great portion of people on herbal remedies can be attributed to several factors, such as moderately good availability of the herbs, affordability and wide indigenous knowledge and knowledge among the local people (Street *et al.*, 2008). Secondary metabolites obtained from herbs are not benign molecules (Sahoo *et al.*, 2010). In various nations treated, packaged and labelled medicinal plants are offered commercially, though in South Africa this is a novel trend that has thrived owing to variations in the socio-economic structure. Several medicinal plants have consequently been registered commercially (Madikizela *et al.*, 2017). Herbs have demonstrated chemical defence to prevent disease, or destroy threatening species (Dossey *et al.,* 2001).

Toxic substances from herbs can disturb the entire spectrum of vital human organs. Some may disturb important functional body systems such as the central nervous system (CNS) by interfering with the management of the nerve functions of the body (Gurib-Fakim, 2006). The most dominant toxins are neurotoxins that disturb the brain and CNS, followed by cytotoxins and metabolic toxins that disturb organs such as the kidneys, liver, heart and lungs (Li *et al.*, 2011). The harshness of a toxic effect will depend on the route of administration, growth phase or part of the herb, the quantity used, the species and the vulnerability of the target (Miltruprabu *et al.*, 2017). Further factors that may have an impact on the harshness of toxins include the solubility of the toxin in body fluids, the occurrence of intoxication and the age of the target (Ndhlala *et al.*, 2013).

Genotoxicity refers to the destructive effect that a compound or extract has on the nuclear material of the cell, causing mutations in the cell (Asharani *et al.,* 2009). To test whether a compound is genotoxic, the end-point assays used detect DNA damage (Moller *et al.*, 2000). A feature of a genotoxic compound is the resulting formation of

micronuclei after treatment of cells with the genotoxin (Van Goethem e*t al*., 1997). Micronuclei are extra-nuclear bodies that contain damaged chromosome fragments and/or whole chromosomes that have not been incorporated into the nucleus after cell division (Terradas *et al*., 2010). The formation of micronuclei can lead to cell death, genotoxic instability and cancer development (Luzhna *et al.,* 2013). While treated commercial medicinal plants sold in South Africa are prepared according to recipes and packaged like Western medicine, it is of concern that they are still not subject to enough safety and quality controls (Fennell *et al*., 2004). According to Madike *et al.* (2020) the water leaf extracts of *T. violacea* established variable selective toxicity towards HepG2, MCF7, H157, HT29 and KMST6, which are not cancer cell lines. It would consequently be imprudent to assume that plant extracts are invariably safe (Davey *et al*., 2002). The guidelines for plant-based medicine incorporate three important features: quality, safety and efficacy (Street *et al*., 2008).

3.2 Aim of the study

The current study was conducted with the aim of investigating the cytotoxicity activity and genotoxicity activity of *T. violacea* aqueous and dichloromethane extracts.

3.3 Methodology

3.3.1 Plant material

T. violacea plant material was authenticated by scientists at the National Botanical Garden in Bloemfontein, South Africa. The collected material was washed to remove soil and then separated into leaves, bulbs and roots. The plant parts were oven-dried at 30-60ºC for five days. The dried plant parts were ground to a coarse powder using a hammer mill and stored at ambient temperature prior to extraction.

3.3.2 Extraction

The dried and ground plant materials were blended in part of the extraction solvent (water and methanol/dichloromethane 1:1) using the Waring blender. After blending the rest of the solvent was added and solutions allowed to steep for 24 h. After 24 h

the solids were removed from each solution by filtration with a Millipore funnel with medium filter paper (Bright sign no 102) connected to a Millipore vacuum pump. Organic extracts were concentrated by rotary vacuum at 50-60ºC, then further dried *in vacuo* at ambient temperature. The aqueous extracts were concentrated to dryness using a freeze-dryer. The plant extracts generated were stored in a cold room at -20ºC until further use.

3.3.3 Cytotoxicity assay

The cytotoxic effects of the compounds were tested by SRB assay on the WI38 cell line. The SRB assay was used to measure drug-induced cytotoxicity. Its principle is based on the ability of the protein dye SRB (Acid Red 52) to bind electrostatically in a pH-dependent manner to protein basic amino acid residues of trichloroacetic acid-fixed cells. Under mild acidic conditions it binds to the fixed cellular protein, while under mild basic conditions it can be extracted from cells and solubilised for measurement. The SRB assay was performed at the CSIR in accordance with the protocol of the Drug Evaluation Branch, NC.

The WI-38 cell line - normal human foetal lung fibroblast from the European Collection of Authenticated Cell Cultures - was routinely maintained as a monolayer cell culture at 37ºC, 5% CO_2, 95% air and 100% relative humidity in Eagle's Minimal Essential Medium containing 10% foetal bovine serum, 2 mM L-glutamine and 50 µg/ml gentamicin. For the screening experiment, the cells (21-50 passages) were inoculated in 96-well microtiter plates at plating densities of 10 000 cells/well and were incubated for 24 h. After 24 h, the cells were treated with the experimental drugs, which had previously been dissolved in DMSO and diluted in medium to produce five concentrations: 100,10, 1, 0.1 and 0.01 µg/ml. Cells to which no drug had been added served as control. The blank contained complete medium without cells. Emetine was used as standard at different concentrations: 100,10, 1, 0.1 and 0.01 µg/ml. The plates were incubated for 48 h after addition of the compounds. Viable cells were fixed to the bottom of each well with cold 50% trichloroacetic acid, washed, dried and dyed by SRB. Unbound dye was removed, and protein-bound dye was extracted with 10 mM Tris base for optical density determination at a wavelength of 540 nm using a multi-well spectrophotometer.

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3.3.4 Genotoxicity assay

The NucRed Live 647 Probe (ThermoFischer Scientific) is a far red, cell-permeant nuclear stain for live and dead cells. It was used to determine the formation of micronuclei. Vero cells (African green monkey kidney cells) were seeded at 4 000 cells/well and left overnight to attach. The cells were treated with different concentrations of each extract: 200, 100, 50, 25 and 12.5 μg/ml. Cells were treated for 48 h (incubated at 37˚C). Medium and treated cells were aspirated and stained with NucRed by preparing NucRed working solution. Two drops of NucRed per ml PBS (+Ca +Mg) were added. In both the aspirate medium and treatments, 100 μl NucRed working solution was added to each well and incubated for 15 – 30 minutes at 37°C. The plates were examined using the ImageXpress XLS microscope.

3.3.5 Statistical analysis

Data analysis was performed using GraphPad Prism software. The cell growth activities of the plant extract were reported as IC_{50} values and were divided into four categories. Fifty percent (50%) of cell growth inhibition (IC_{50}) was determined by non-linear regression.

3.4   Results and discussion of cytotoxicity

The analysis of the different extracts was performed according to the CSIR criteria (Table 3.1). An extract with an IC_{50} value of less than 100 μg/ml but greater than 30 μg/ml when tested against a cell line was considered weakly cytotoxic (Table 3.1). An extract with an IC_{50} value of less than 30 μg/ml but greater than 5 μg/ml was considered moderately cytotoxic (Table 3.1). An extract with an IC_{50} of less than 5 μg/ml was considered highly hazardous.
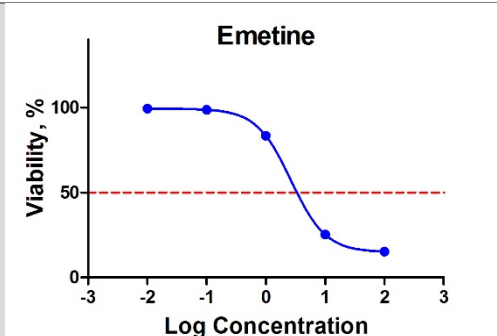
Table 3.1: Standard cytotoxicity criteria, according to IC50

IC50 µg/ml	Status
> 100	Low Hazard
< 100 > 30	Weak Hazard
< 30 >5	Moderate Hazard
<5	High Hazard

Determination of 50% inhibition concentration (IC)

Table 3.2 Emetine standard
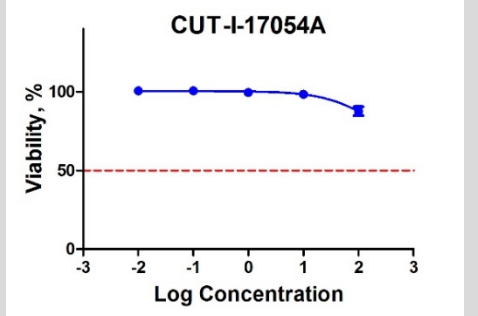
Concentration (µg/ml)	Log Conc.	% Viability	SD	
100	2.000	15.24	0.38	
10	1.000	25.25	0.09	
1	0.000	83.31	0.42	
0.1	-1.000	98.70	0.70	
0.01	-2.000	99.29	1.49	

Emetine was used as a control to determine 50% inhibition concentration against WI-38 cell lines at different concentration. The control was considered moderately toxic at a concentration of 100 µg/ml and 10 µg/ml for WI-38 cell lines, whereas the control at the lower concentrations was considered non-toxic against WI-38 cell lines with an IC_{50} value of greater than 5 µg/ml for WI-38 cell lines (Table 3.2). This suggests that it will be best to consider using lower concentrations of the Emetine standard when determining 50% inhibition against WI-cell lines.

Table 3.3 Cytotoxicity assay of methanol/dichloromethane (1:1) leaf extract from *Tulbaghia violacea* on WI-38 cell line

Concentration (μg/ml)	Log Conc.	% Viability	SD	
100	2.0	87.67	2.85	
10	1.0	98.43	2.24	
1	0.0	99.62	1.61	
0.1	-1.0	100.58	2.25	
0.01	-2.0	100.65	0.81	

The methanol/dichloromethane leaf extract from *T. violacea* was tested against the WI-38 cell line from a higher concentration to a lower concentration. The results obtained were >5 μg/ml from the higher concentration to the lower concentration, as shown in Table 3.3. These results indicated that the methanol/dichloromethane leaf extracts from *T. violacea* were non-toxic when tested against the WI-38 cell line (Table 3.3). The results obtained do not correlate with another study done in South Africa, where it was found that ethanol leaf extract was toxic to the murine macrophage cell line (Madike *et al*., 2020). The results support the hypothesis that the leaf extracts' cytotoxicity is purely dependent on the concentrations, type of extract and the cell lines used (Madike *et al*., 2020). The *T. violacea* ethanolic extract of the leaves in a study done in South Africa was toxic to murine macrophage cells even at the lowest concentration of 10 mg/ml; this simply indicated no correlation, although these results correlated with the control results (Madike *et al*., 2020).

Table 3.4 Cytotoxicity assay of water leaf extract from *Tulbaghia violacea* on WI-38 cell line
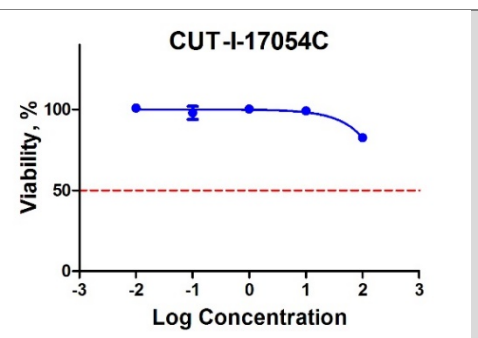
Concentration (μg/ml)	Log Conc.	% Viability	SD	
100	2.0	74.28	1.66	
10	1.0	98.84	0.96	
1	0.0	100.59	1.51	
0.1	-1.0	100.97	2.31	
0.01	-2.0	100.32	2.23	

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The water leaf extracts from *T. violacea* were tested against the WI-38 cell line at different concentrations. In order to achieve 50% inhibition, a concentration higher than 5 µg/ml was necessary, as shown in Table 3.4. These results indicated that the water leaf extracts from *T. violacea* were non-toxic when tested against the WI-38 cell line (Table 3.4). This further supports the study done in South Africa that showed that *T. violacea* leaf extracts' cytotoxic inhibition effect is purely dependent on the type of solvent and the cell line used (Madike *et al*., 2020). This was also seen even though the same plant and solvent were used; because of the different cell lines, namely murine macrophage and WI-38 used for testing, the results did not correlate at all (Madike *et al*., 2020).

Table 3.5 Cytotoxicity assay of methanol/dichloromethane (1:1) bulb extract from *Tulbaghia violacea* on WI-38 cell line

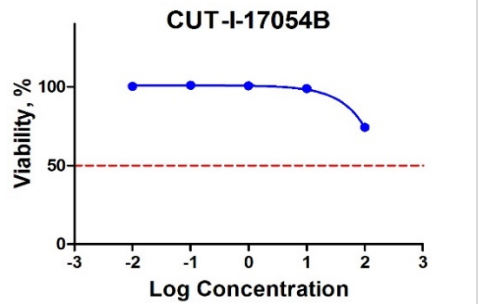| Concentration (µg/ml)	Log Conc.	% Viability	SD	
100	2.0	82.52	1.06	
10	1.0	99.15	2.23	
1	0.0	100.26	1.91	
0.1	-1.0	97.89	4.05	
0.01	-2.0	100.83	2.42	

The methanol/dichloromethane bulb extracts from *T. violacea* were tested on the WI-38 cell line from a higher concentration to a lower concentration. The results obtained were >5 µg/ml from the higher concentration to the lower concentration, as shown in Table 3.5. These results indicated that the methanol/dichloromethane bulb extracts from *T. violacea* were non-toxic when tested against the WI-38 cell line (Table 3.5).

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Table 3.6 Cytotoxicity assay of water bulb extract from *Tulbaghia violacea* on WI-38 cell line

Concentration (µg/ml)	Log Conc.	% Viability	SD	
100	2.0	75.05	0.17	
10	1.0	92.47	0.73	
1	0.0	99.62	0.16	
0.1	-1.0	97.00	0.09	
0.01	-2.0	100.62	0.40	

The water bulb extracts from *T. violacea* were tested on the WI-38 cell line from a higher concentration to a lower concentration. The results obtained were >5 µg/mL from the higher concentration to the lower concentration, as shown in Table 3.6. These results indicated that the water bulb extracts from *T. violacea* were non-toxic when tested against the WI-38 cell line (Table 3.6).
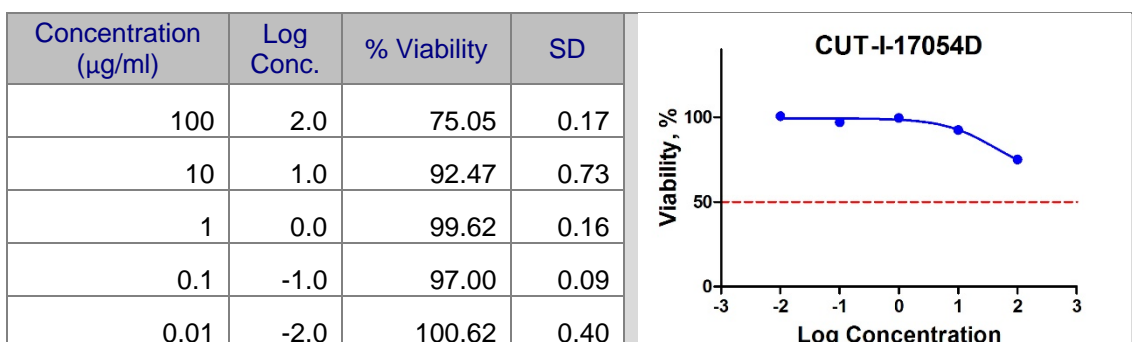
Table 3.7 Cytotoxicity assay of methanol/dichloromethane (1:1) root extract from *Tulbaghia violacea* on WI-38 cell line

Concentration (µg/ml)	Log Conc.	% Viability	SD	
100	2.0	81.83	0.48	
10	1.0	93.32	1.11	
1	0.0	96.75	3.12	
0.1	-1.0	94.48	2.59	
0.01	-2.0	100.54	3.03	

The methanol/dichloromethane root extracts from *T. violacea* were tested on the WI-38 cell line from a higher concentration to a lower concentration. The results obtained were >5 µg/mL from the higher concentration to the lower concentration, as shown in Table 3.7. These results indicate that the methanol/dichloromethane root extracts from *T. violacea* were non-toxic when tested against the WI-38 cell line (Table 3.7)

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Table 3.8 Cytotoxicity assay of water root extract from *Tulbaghia violacea* on WI-38 cell line

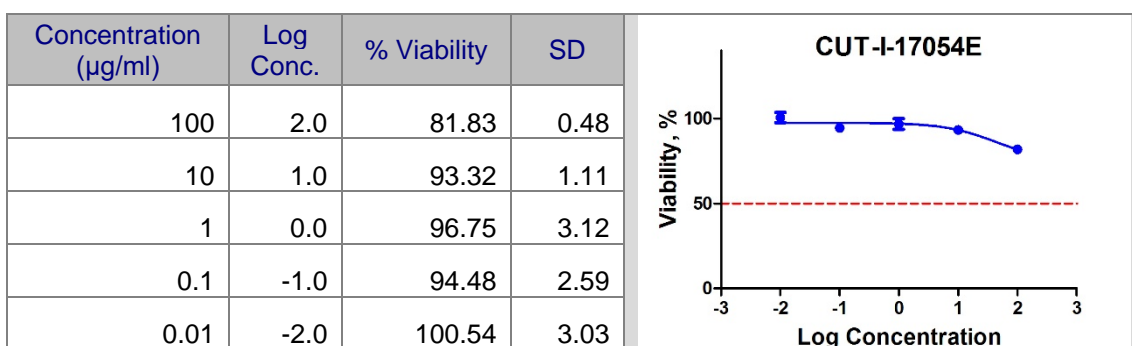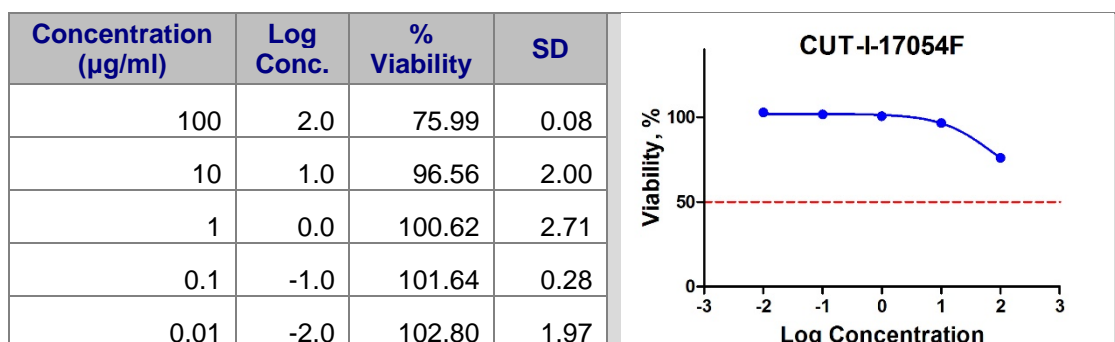| Concentration (µg/ml)	Log Conc.	% Viability	SD
100	2.0	75.99	0.08
10	1.0	96.56	2.00
1	0.0	100.62	2.71
0.1	-1.0	101.64	0.28
0.01	-2.0	102.80	1.97

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The water root extracts from *T. violacea* were tested on the WI-38 cell line from a higher concentration to a lower concentration. The results obtained were >5 µg/mL from the higher concentration to the lower concentration, as shown in Table 3.8. These results displayed that the water root extracts from *T. violacea* were non-toxic when tested against the WI-38 cell line (Table 3.8). Madike *et al*. (2020) argued that the crude extracts of the roots from *T. violacea* also showed that cell viability was reliant on both concentration and type of extract used. The number of viable cells decreased as the concentration of the water extracts increased.

Table3.9 List of plant extracts used in the cytotoxicity assay against WI-38 cell line

No.	Solvent	Name of sample	IC50 (µg/ml)
1	Methanol/dichloromethane	Leaf extract	>100
2	Water	Leaf extract	>100
3	Methanol/dichloromethane	Bulb extract	>100
4	Water	Bulb extract	>100
5	Methanol/dichloromethane	Root extract	>100
6	Water	Root extract	>100
7	**Standard**	**Emetine**	**2.66**

The aqueous and methanol/dichloromethane leaf, bulb and root extracts from *T. violacea*, at equal ratios, were tested against the WI-38 cell line, the normal human foetal lung fibroblast, as shown in Table 3.9. All extracts *of T. violacea* were non-toxic when tested against the WI-38 cell line. Madike *et al*. (2020) observed that the number of viable cells decreased as the concentration of the ethanolic extract increased on a different cell line. These cytotoxicity activity results indicated that the leaf, bulb and root extracts of the *T. violacea* were safe for cellular exposure compared to the

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standard drug (Emetine), which was considered highly toxic, with an IC_{50} value of less than 5 μg/ml for WI-38 cell lines.

3.5 Results of genotoxicity

The formation of micronuclei was determined using NucRed nuclear stain and the ImageXpress microscope, as shown in Figure 3.1. It is clear from these results that extracts (Table 3.10) did not induce the formation of micronuclei except for methanol/dichloromethane (extract no 5), which could possibly be considered genotoxic.

Table 3.10 List of plant extracts used in the genotoxicity assay against Vero cell

Sample No.	Part	Extraction solvent
1	Bulb	Methanol/dichloromethane
3	Root	Methanol/dichloromethane
5	Leaf	Methanol/dichloromethane
9	Root	Water
10	Leaf	Water
12	Bulb	Water

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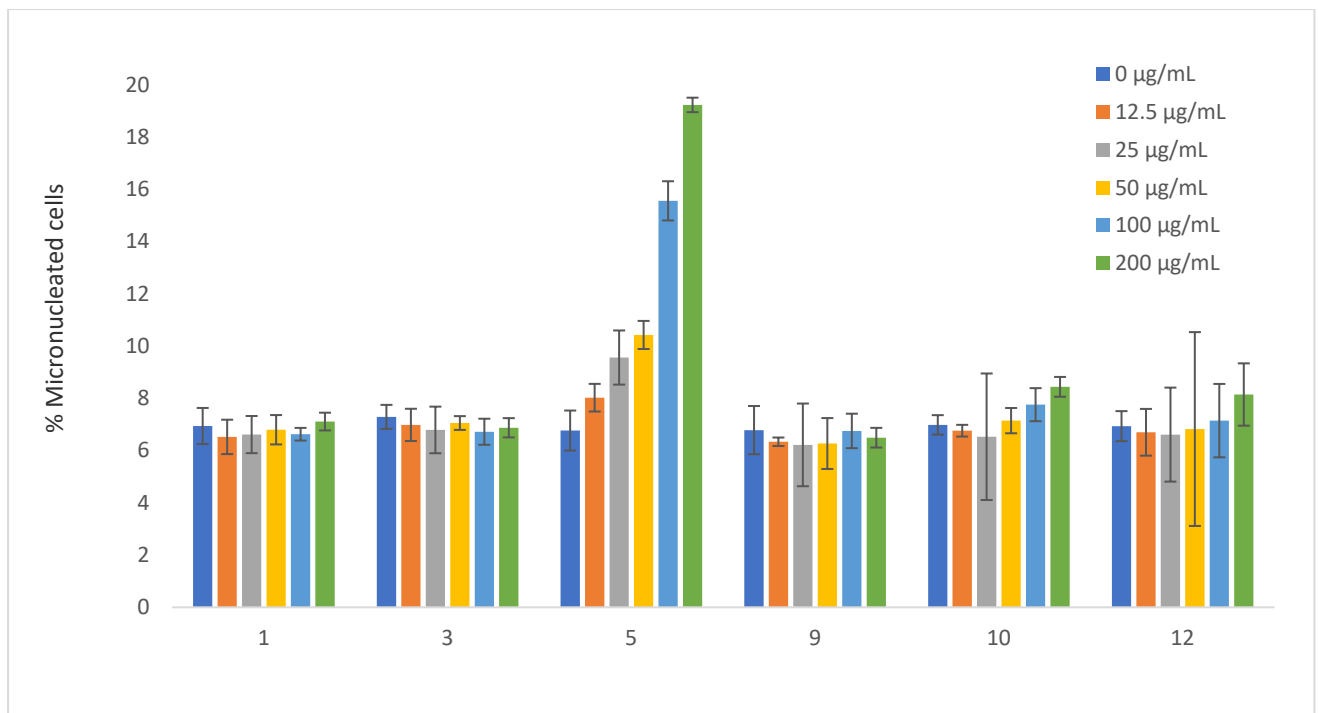
Figure 3.1: Formation of micronuclei in response to treatment of Vero cells with varying concentrations (as indicated) of extract. Error bars indicate standard deviation from quadruplicate values.

In Figure 3.1 there were 7% micronucleated cells that falsely indicated an induced micronucleus even at 0 μg/ml. This could be attributed to the incubation conditions. In the water extracts of the leaf and bulb there was a 1% increase to the negative control, which could also be ignored as one takes error bars into consideration. The DNA damage attributed to all extracts except methanol/dichloromethane on leaf is of less significance. Comparing water leaf extract, which showed no genotoxicity, with the methanol/dichloromethane extract on leaf, it can be concluded that methanol/dichloromethane contributed to the genotoxicity. The results of this study correlated well with the findings of the study by Madike *et al*. (2019) that reported a higher percentage of micronucleated cells after testing with the stem extracts compared to the leaf and root extracts. These results support the use of *T. violacea,* as it failed to induce DNA damage to the Vero cells, but it cannot be ignored that Madike *et al*. (2019) indicated the importance of plant parts when dealing with *T. violacea*.

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3.6 Conclusion

The aqueous and methanol/dichloromethane extracts of *T. violacea* were non-toxic when tested against the WI-38 cell line. According to the criteria of the CSIR, the sample can be estimated as posing a low hazard if the value of IC_{50} is more than 100 µg/ml. Furthermore, the aqueous and methanol/dichloromethane extracts of *T. violacea* were not genotoxic, as they failed to induce the formation of micronuclei and all could not be considered genotoxic. This finding indicates that further testing can be performed to determine the antioxidants and total phenols of *T. violacea*. The results of genotoxicity and cytotoxicity correlated and supported the safety of the plant for human consumption. This further confirms the safety of *T. violacea* when used by people for the treatment of diseases.

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CHAPTER 4

Antioxidant and total polyphenol analysis of *Tulbaghia violacea* plant extracts

4.1 Introduction

The preservation of pro-oxidant and antioxidant homeostasis in living cells is the primary focus of current research in the field (Olorunnisola *et al.*, 2011). Many studies of plant phytochemicals evidenced that phytochemicals with antioxidant activity improve health and combat disease (Kaneria *et al.*, 2009; Fawole *et al.*, 2012). The imbalance in pro-oxidant and antioxidant homeostasis caused by excessive free radical generation or inadequate antioxidants is implicated in the development of several human disease conditions, such as atherosclerosis, high-tension ischaemic diseases, Alzheimer's disease, Parkinsonism and cancer (Tlili *et al.*, 2013). Pro-oxidants are free radicals (superoxide, nitric oxide and hydroxyl radicals) produced in normal or pathological cell metabolism and through exogenous causes, such as human exposure to ionising radiation, injury, oxidative drugs and pollutants (Olorunnisola *et al.*, 2011; Elisha *et al.*, 2016; Takaidza *et al.*, 2018).

Medicinal plants have bioactive non-nutrient and biologically active compounds identified as phytochemicals, which contain a broad spectrum of chemical structures and protective/disease preventative properties (Madike et al., 2017). Phytochemicals are natural and important bioactive compounds formed by plants. They act as safety agents against external stress and pathogenic attack (Pandey and Kumar, 2013). Phytochemicals can be important natural antioxidants that can save the human body from free radicals (Soni and Sosa, 2013). Phenolics are some of the important antioxidants of phytochemicals produced by plants (Naczk and Shahidi, 2004). They are more effective because they have the potential to chelate metal ions, prevent lipid peroxidation and free radical scavenger properties (Tlili *et al.*, 2013). Studies have reported that phenolic compounds such as caterchin and quercetin were enough to stabilise phospholipid bilayers against peroxidation induced by ROS (Perkins *et al.*, 2012; Soni and Sosa, 2013).

The determination of the bioactivity of plant extracts or natural products, as well as synthetic compounds, whether antioxidant, antimicrobial, cytotoxic etc., is a crucial element in the discovery of new and improved therapeutic entities (Pisoschi and Negulescu, 2011). Many health benefits are associated with antioxidants, which provide protection against the development of diseases caused by oxidative stress

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(Masoko, 2017). In addition to antioxidants, polyphenols have been emerging as one of the major natural products of importance to human health (Zhang *et al.*, 2006; Longhini *et al.*, 2017). Antioxidant and total phenolic compounds can remove free radicals, chelate metal catalysts, activate antioxidant enzymes, reduce alpha-tocopherol radicals and inhibit oxidases (Brasileiro *et al.*, 2015). Evidence has shown that polyphenols are good antioxidants and are effective in preventing cardiovascular and inflammatory diseases, as well as serving as anticancer agents (Adebayo *et al.*, 2015).

African people occasionally visit public health facilities, where medication and treatments are frequently very expensive for their standard of living (Gismondi *et al.*, 2013). Numerous researchers and dietary organisations recommend an increase in the use of medicinal products of plant origin (Rates, 2001). Currently, about 64% of the total worldwide population remain reliant on herbal medicine for their healthcare needs (Tlili *et al.*, 2013). There has been an increase in interest in potential antioxidant activity, as well as the total phenolic content, of plant and natural products (Atanassova *et al.*, 2011). Hence, in this study the antioxidant potential and total phenolic constituents of the *T. violacea* plant will be determined. The antioxidant potential of the extract demonstrated by 1,1-diphenyl-2-picrylhydrazyl (DPPH) is designated by their IC50 and compared with that of the standard. The total phenolics and antioxidant screening of the plant is a prerequisite for authentication and utilisation of new sources of drugs of natural origin (Otang *et al.*, 2012).

4.2. Methodology

4.2.1 Preparation of plant material

The plant material of *T. violacea* was authenticated by scientists at the National Botanical Garden in Bloemfontein, South Africa. The collected material was washed to remove soil and then separated into leaves, bulbs and roots. The plant parts were oven-dried at 30-60 ºC for five days. The dried plant parts were weighed for extraction, ground to a coarse powder using a hammer mill and stored at ambient temperature prior to extraction. The dried and ground plant materials were blended in part of the extraction solvent (water and methanol/dichloromethane 1:1) using the Waring blender. After blending the rest of the solvent was added and solutions were allowed

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to steep for 24 h. After 24 h the solids were removed from each solution by filtration with a Millipore funnel with medium filter paper (Bright sign no 102) connected to a Millipore vacuum pump. The organic extracts were concentrated by rotary vacuum at 50-60ºC, then further dried *in vacuo* at ambient temperature. The aqueous extracts were concentrated to dryness using a freeze-dryer. The plant extracts generated were stored in a cold room at -20 ºC until further use.

4.2.2 Assessment of antioxidant activity using the DPPH assay

In a 96-well plate 5 µL sample, 120 µL Tris-HCL buffer (50 mM, pH 7.4) and 120 µL freshly prepared DPPH (0.1 mM in ethanol) were added and allowed to incubate for 20 minutes in the dark at room temperature. The absorbance was measured at 513 nm using a BioTek® PowerWave XS spectrophotometer (Winooski, VT, USA) and the percentage radical scavenging activity was calculated as follows:

% DPPH scavenged = (blank-sample)/blank   x 100.

For the blank/control, buffer was substituted as the 5 µL sample. Plant extracts were tested at final concentrations of 250 and 500 µg/mL. Quercetin was used as a positive control at final concentrations of 6.25 - 25 µM.

4.2.3 Total phenolic determination using the Folin-Ciocalteu assay

A standard curve was prepared using gallic acid at a concentration range of 6.25 µg/mL – 100 µg/mL. It is important to note that all extracts and the standard control (gallic acid) were prepared at 10 times the desired concentrations in consideration of the dilution factor of 1:10. Sample extracts (stock = 100 mg/mL) were prepared at a concentration of 10 mg/mL and centrifuged at 12 000 X g for 10 minutes. Thereafter, 20 µL of supernatant was added to wells of 96-well plates, in triplicate. Dilutions of 1:1 and 1:10 of the supernatant of each extract were also prepared. Then 100 µL of FC reagent was added to each well and the plate was incubated for 5 minutes at room temperature. An aliquot of 80 µL of 7.5 % Na_2CO_3 was added slowly to each well and the plate was left to incubate at room temperature in the dark for 2 h. The absorbance

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was then read at 750 nm using a BioTek® PowerWave XS spectrophotometer (Winooski, USA).

4.3 Results and discussion

4.3.1 Assessment of antioxidant activity using the DPPH assay

The results of this assay are displayed in Table 4.2, clearly showing the percentage DPPH scavenging activity of each extract.

Table 4.1 List of plant extracts used in the DPPH and total phenolic assay against Vero cell

Sample No.	Part of plant	Extraction solvent
1	Bulb	Methanol/dichloromethane
3	Root	Methanol/dichloromethane
5	Leaf	Methanol/dichloromethane
9	Roots	Water
10	Leaf	Water
12	Bulb	Water

DPPH is a stable free radical at room temperature. It produces a violet solution in ethanol. When the free radical reacts with an antioxidant, it loses its free radical property as a result of the reaction, as it obtains an electron or hydrogen from the antioxidant to become a stable molecule and it changes colour to pale yellow (Olutayo *et al.*, 2013). In the present study, extracts that produced a yellow colour were considered to have antioxidant properties. The antioxidant activities of extracts from the leaf, bulb and root extracts from *T. violacea* were evaluated using the percentage scavenging activities (Table 4.2).

Table 4.2: Antioxidant activity of extracts expressed as percentage of DPPH scavenged

Extract	Concentration (µg/mL)	% DPPH scavenged*	Std Dev
1	250	56.56	10.20
	500	75.34	4.76
3	250	82.62	1.82
	500	85.91	4.30
5	250	71.59	8.64
	500	73.62	20.56
9	250	58.44	4.45
	500	66.19	5.14
10	250	88.9	0.30
	500	88.49	1.07
12	250	81.53	5.78
	500	76.05	9.47
Quercetin (positive control)	6.25 µM	48.65	9.71
	12.5 µM	84.35	1.08
	25 µM	86.43	0.65

1 – Methanol/dichloromethane bulb extract; 3 – Methanol/dichloromethane root extract; 5 – Methanol/dichloromethane leaf extract; 9 – Water root extract; 10 – Water leaf extract; 12 – Water bulb extract.

Among the extracts, the leaf water extracts of *T. violacea* had the highest scavenging activity, with the bulb methanol/dichloromethane extracts displaying the lowest scavenging activity. The crude extract of *T. violacea* seemed to be as effective as quercetin, with a maximum inhibition of 88.96% at 25 µg/ml, which is comparable to 86.43% for quercetin at 25 mM concentration. The extract of *T. violacea* displayed high antioxidant activity compared to the standard. These results correlate with a study by Takaidza *et al.* (2018), which showed that *T. violacea* had the highest scavenging activities with both DPPH [0.01 (43%) to 0.5 mg/mL (57%)] and ABTS [0.01 (46%) to 0.5 mg/mL (70%)], compared to other Tulbaghia species.

4.3.2 Total phenolic determination

The absorbance of the standard compound (gallic acid) at λmax =750 nm in *T. violacea* is presented in Table 4.3 and the standard calibration curve for quantification of total phenolic content is shown in Figure 4.1.

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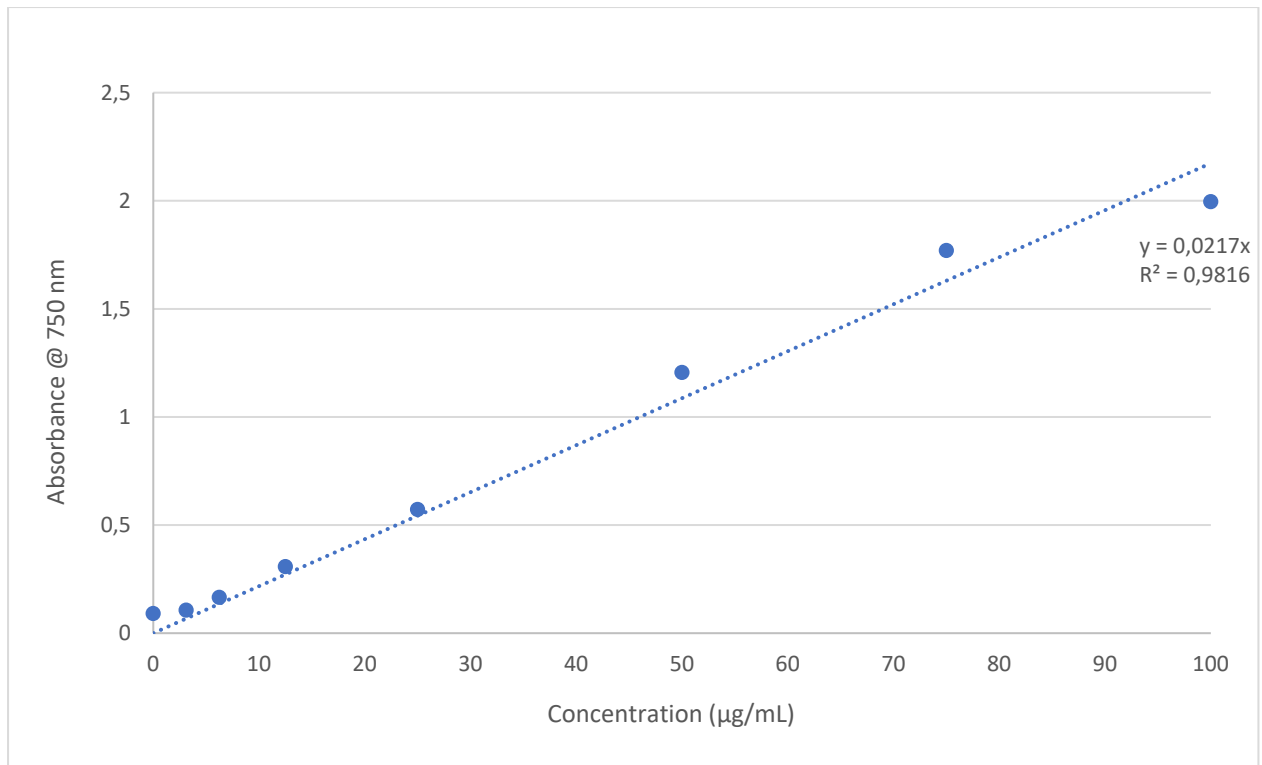
Figure 4.1: Standard curve for total phenolics determination using the FC assay and gallic acid as the standard control**.**

Table 4.3 shows the content of total phenols that were measured by Folin-Ciocalteu reagent in terms of gallic acid equivalent (standard curve equation: y = 0.0217, R2 = 0.9816; Figure 4.1). The total phenolic content for the water extracts was found to be 21.75, 10.94 and 10.94 µg/mL in the leaf, bulb and root extracts, respectively. In the methanol/dichloromethane extracts, the total phenolic content was found to be 20.28, 7.93 and 13.36 µg/mL in the leaf, stem and root extracts, respectively.

Table 4.3: Total phenolics determination using the FC method

Extract	Ave. Abs @ 750 nm	Resulting phenolic concentration (µg/mL)	Std dev of concentration (µg/mL)
1	0.17	7.93	0.12
3	0.29	13.26	1.09
5	0.44	20.28	0.98
9	0.24	10.94	1.46
10	0.47	21.75	0.88
12	0.24	10.94	0.83

1 – Methanol/dichloromethane bulb extract; 3 – Methanol/dichloromethane root extract; 5 – Methanol/dichloromethane leaf extract; 9 – Water root extract; 10 – Water leaf extract; 12 – Water bulb extract.

The amount of total phenolics varied in different accessions and ranged from 7.93-10.94 µg/ml. Total phenolic determination is indicated in Table 4.3, showing that aqueous leaf extract possessed the highest concentration of phenolics and methanol/dichloromethane bulb extract the lowest. According to Madike *et al.* (2017), the total phenolic content for the water extracts was 3.59, 2.38 and 1.91 mg/g in the leaf, stem and roots. The total phenolic content was 0.98, 0.34 and 0.15 mg/g in the leaf, stem and root extracts when 70% ethanol was used as one of the extracting solvents. The study by Madike et al. (2017) indicated that pharmacologically active compounds such as tannins, terpenoids, flavonoids, saponins, proteins, steroids, cardiac glycosides, phenols and coumarins were present in some parts of *T. violacea.* However, phlobatannins, leucoanthocyanins, alkaloids, carbohydrates and anthocyanins were absent in all plant parts. The leaves of the plant contained more active compounds than those present in the stems and roots in cases where water and 70% ethanol extracts. It can therefore be assumed that *T.violacea* extracts used in this study contain pharmacological compounds and water leaf extract can be assumed to have more, as it has the highest antioxidant and phenolics concentration, as shown in Tables 4.2 and 4.3, respectively.

4.4 Conclusion

The study showed that both the aqueous and methanol/dichloromethane extracts of *T. violacea* possessed antioxidant activity properties. Furthermore, for total phenolic determination, both the aqueous and methanol/dichloromethane extracts have potential. It is well recorded that phenolic compounds contribute to quality and nutritional value in terms of adapting colour, taste, aroma and flavour and in providing benefits to health. They also play a role in plant defence mechanisms to counteract ROS, to endure and avoid molecular damage and damage by microorganisms, insects, and herbivores (Sengul *et al.*, 2009). Further testing to determine the antimicrobial effect of *T. violacea* will be performed on organisms that are highly successful opportunistic pathogens and thus involved in various diseases.

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CHAPTER 5

Antibacterial activity of *Tulbaghia violacea* extracts

5.1 Introduction

South Africa is an emerging nation with a big rural population that depends on plant-based resources for many purposes, for example building, craftwork, food, cosmetics and remedies. The diversity of cultural groups in South Africa has given rise to a melting pot of cultural indigenous knowledge, from the 'boererate' of the Afrikaans community and the cultural knowledge of the Khoikhoi and San tribes in the Karoo region, to the cultural knowledge of the Zulu, Xhosa, Basotho and Venda people, across the Republic (Corrigan *et al.*, 2011). Medicinal herbs are now globally known as the basis for several critical human health, social, and economic support systems and benefits (Street and Prinsloo, 2013). Traditional herbs are commonly used by South African traditional healers to treat a range of diseases, such as malaria and its related symptoms. South Africa boasts great biodiversity and rich cultural traditions of plant use (Clarkson *et al.*, 2004). It is estimated that 60% of the South African population use herbal plant as medicines for primary health care treatments (Ncube *et al.*, 2011).

Naturally occurring constituents of plant, animal and mineral sources have provided an ongoing source of medicine to man since ancient times, but it is the plant kingdom, specifically, which has proven to be of most use for treating several diseases (Ahmad *et al.*, 2001; Mahesh and Satish, 2008). Throughout history, investigation has succeeded in distinguishing those plants that have valuable effects from those that are toxic or merely ineffective (Heyman *et al.*, 2009). Through trial and error, human beings have discovered ways of relieving pain and sickness, and of living in harmony with nature (Nielsen *et al.*, 2012). Medicinal plants have been used as a remedy for many years, playing an important role in drug discovery and development (Rabe and van Staden, 1997; Heyman *et al.*, 2009; Madureira *et al.*, 2012). Medicinal plants play a significant role in the primary health system in South Africa, with an estimation of about 80% of the black population still consulting traditional healers for treatment (Heyman *et al.*, 2009; Adebayo *et al.*, 2015 Ghuman *et al.*, 2016; Madikizela *et al.*, 2017). The significance of plant products is evident in the treatment of infectious ailments, where over 60% of antimicrobial agents are of plant origin (Mishra *et al.*, 2017).

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Antibiotics have been broadly accessible for many years; they have had a huge impact on medical practitioners' capability to treat bacterial diseases globally. Nevertheless, in the past 40 years only three new classes of antibiotics have been developed, mostly for medicines used in winter (lipopeptides, oxazolidinones and streptogramins), all geared towards Gram-positive bacterial infections. A deficiency of new antibiotics for the treatment of Gram-negative infections, combined with emerging multi-drug resistance problems, stresses that new antibacterial approaches need to be discovered for treating these infections (Marr *et al.*, 2006).

The great number of plant product-derived antibiotics may be due, in part, to the development of secondary metabolites as a defence strategy against environmental stresses (Madureira *et al.*, 2012). This feature, coupled with their enormous structural diversity and great biodiversity, makes plant products an important and interesting subject in drug discovery and development (Bisi-Johnson *et al.*, 2017). It is estimated that infectious ailments are directly causing 26% of annual deaths globally (Masoko, 2017). The influence of bacterial ailments is important in Africa, were drugs are inadequate and the development of drug resistance has made numerous currently available drugs ineffective (Dzoyem *et al.*, 2016). Bacterial resistance to antibiotics is a serious and increasing problem of public health, representing a worldwide threat (Levy *et al.*, 2004). Certainly, infectious diseases are becoming more problematic to treat because of multi-drug-resistant bacteria, especially Gram-positive pathogens (Engler *et al.*, 2012).

It is projected that by the year 2050, infections by antimicrobial-resistant bacteria will be the leading cause of death globally (van Vuuren and Muhlarhi, 2017). Significant bacterial strains against which transmission resistance is found include among others *S. aureus*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *K. pneumoniae* (Magiorakos *et al.*, 2012). In South Africa, antibacterial resistance has reached a disturbing scope (van Vuuren and Muhlarhi, 2017). The usage of medicinal plants has lately fascinated researchers. This has involved the isolation and identification of secondary metabolites produced by herbs and their usage as active principles in therapeutic preparations (Das *et al.*, 2010). Studies have shown that more than 50% of all hospital-acquired *S. aureus* infections were of methicillin resistance origin (Mulaudzi *et al.*, 2010). In various parts of the globe, herbal plants are used for the treatment of antibacterial, antifungal and antiviral diseases. These plant extracts are

used as the basis of therapeutic agents to treat urinary tract infections, cervicitis vaginitis, gastrointestinal illnesses and skin infections such as herpes simplex virus type I (Essawi and Srour, 2000). Therefore, there is a great need to screen medicinal plants for potential and effective antibacterial drugs against resistant strains (Masoko, 2017).

Antibacterial assays are important to test and screen inhibitory effects on test microorganisms. In this study, a representative of a Gram-positive and one of a Gram-negative bacterium were used, namely *S. aureus* and *K. pneumoniae,* respectively. It is important to include a known antibacterial agent as a positive control for these assays to enable direct comparison of extracts and to ensure that the assay was performed successfully.

5.2 Methodology

5.2.1 Preparation and extraction of plant material

The plant material of *T. violacea* was authenticated by scientists at the National Botanical Garden in Bloemfontein, South Africa. The collected material was washed to remove soil and then separated into leaves, bulbs and roots. The plant parts were oven-dried at 30-60ºC for five days. The dried plant parts were ground to a coarse powder using a hammer mill and stored at ambient temperature prior to extraction.

The dried and ground plant materials were blended in part of the extraction solvent (water and methanol/dichloromethane 1:1) using the Waring blender. After blending the rest of the solvent was added and solutions were allowed to steep for 24 h. After 24 h the solids were removed from each solution by filtration with a Millipore funnel with medium filter paper (Bright sign no 102) connected to a Millipore vacuum pump. Organic extracts were concentrated by rotary vacuum at 50-60ºC, then further dried *in vacuo* at ambient temperature. The aqueous extracts were concentrated to dryness using a freeze-dryer. The plant extracts generated were stored in a cold room at -20ºC until further use (Table 5.1).

Table 5.1 List of plant extracts used in the antibacterial activity against *S. aureus* and *K. pneumonia*

Sample No.	Part of plant	Extraction solvent
1	Bulb	Methanol/dichloromethane
3	Root	Methanol/dichloromethane
5	Leaf	Methanol/dichloromethane
9	Roots	Water
10	Leaf	Water
12	Bulb	Water

5.2.2 Determination of antibacterial activity of extracts using the p-Iodonitrotetrazolium chloride assay

The antibacterial activity of plant extracts was tested on *S. aureus* (ATCC 11632) and *K. pneumoniae* (ATCC 10031), representatives of Gram-positive and Gram-negative bacteria, respectively. Bacterial cultures were grown on Mueller-Hinton (MH) agar plates at 37°C. An overnight streak plate was used to inoculate MH broth (Merck, USA) and this was allowed to grow for 16 h (log phase) at 37°C. Gentamicin sulphate and vancomycin hydrochloride (Sigma, USA) were used as positive controls against *K. pneumoniae* and *S. aureus,* respectively. Antibiotics were dissolved in double-distilled water at stock concentrations of 2 mg/mL and filter-sterilised (0.2 µm filter). Working concentrations of the antibiotics were prepared in MH broth, depending on the minimum inhibitory concentration (MIC) value.

Thereafter 40 µL of MH broth was added to the wells of a sterile 96-well plate and 50 µL of test extracts were added to the relevant wells. This was followed by a serial dilution of extracts to achieve the concentration range, as indicated in Figures 5.1 and 5.2. The cultures were assessed and adjusted to a 0.5 McFarland standard [1.175% $BaCl_2$ and 1% H_2SO_4 with absorbance 600 nm = 0.08-0.1 to achieve ±1.5 x 10^8 cells/mL]. A 50 µL aliquot of the relevant bacteria was added to each test well. Plates were sealed and incubated at 37˚C for 24 h; p-Iodonitrotetrazolium chloride (INT) was prepared at a working concentration of 0.2 mg/mL in ddH2O and filter-sterilised (0.2

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μm filter). Then, 50 μL of INT was added to each well and the plates were further incubated for 30–60 minutes at 37˚C until a colour change was observed (yellow to pink/purple to indicate the reduction of the dye by viable bacteria). No colour change indicated the inhibition of bacterial growth. Absorbance (abs) was measured at 600 nm using a BioTek® PowerWave XS spectrophotometer (Winooski, USA).

Percentage inhibition was defined as: Percentage inhibition = 1 – (test well abs/mean abs triplicate bacteria only well) x 100.

5.3 Results and discussion

The antibacterial activity of indicated extracts was tested against a Gram-negative and Gram-positive bacterium. Figures 5.1 below show the antibacterial activity of six extracts from *T. violacea* against *S. aureus* for a concentration range of 0.0156 mg/ml – 2 mg/ml. Vancomycin was used as a positive control at its MIC of 2 μg/ml. As shown in Figure 5.1, dichloromethane extract 1 showed that MIC activity was dependent at extract concentrations against *S. aureus*. Dichloromethane extract no. 3 and water extract no. 9 did not inhibit bacterial growth at concentration ranging between 0.0125 mg/mg-0.5 mg/ml, then did inhibit bacterial growth at concentration ranging between 1 mg/ml and 2 mg/ml against *S. aureus*. Dichloromethane extract no. 5 and water extract no. 12 showed variation against *S. aureus*. Water extract no. 10 showed no MIC at all concentrations against *S. aureus.* Concerning extract no. 1, the antibacterial activity was dependent on the concentration. Extract no. 10 showed that the activity against *S. aureus* was dependent on the concentrations and type of solvent used. The antibacterial activity of six extracts from *T. violacea* against *K. pneumoniae* for a concentration range of 0.0156 mg/ml – 2 mg/ml .
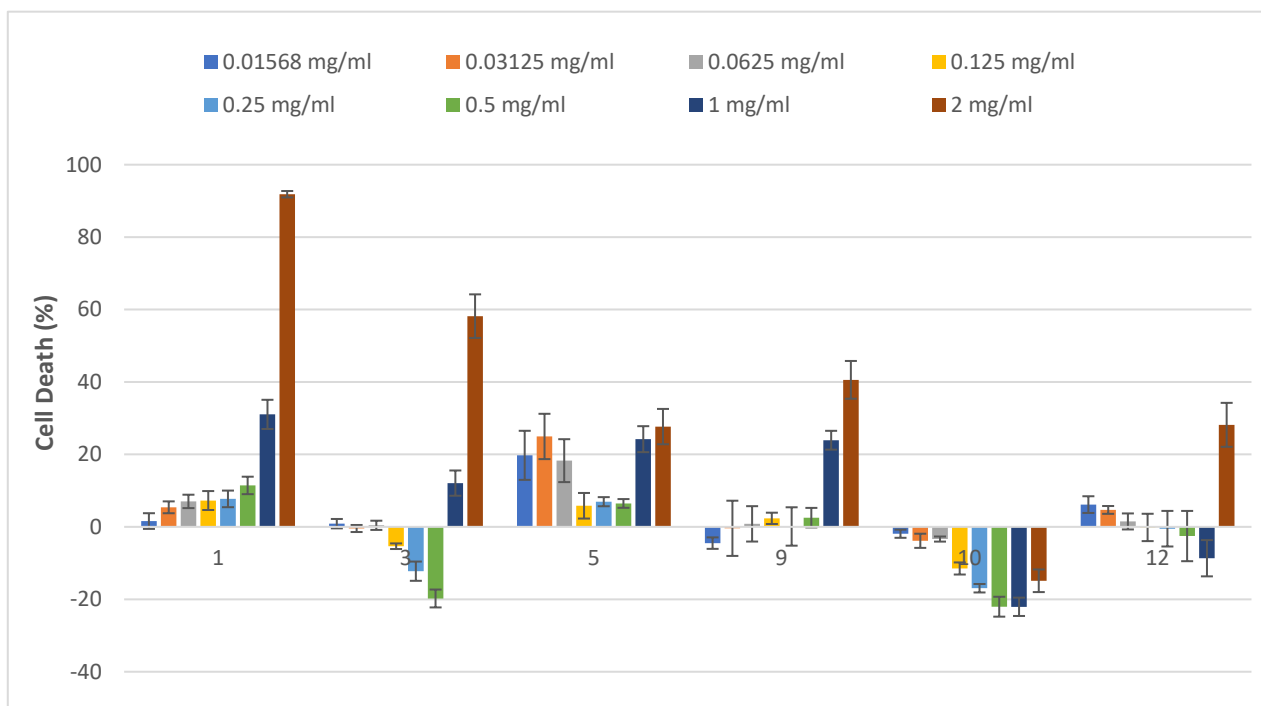
Figure 5.1: Screening for antibacterial activity of six extracts (as indicated) against *S. aureus* for a concentration range of 0.0156 mg/ml – 2 mg/ml. Vancomycin was used as a positive control at its MIC of 2 µg/ml. Error bars indicate standard deviation of quadruplicate values determined in a single experiment.

Gentamicin was used as a positive control and inhibited bacterial growth at MIC of 2 µg/ml as shown in Figure 5.2. All extracts showed unreliable fluctuating activities, except no. 10, which exhibited more concentration-dependent activity. This indicates that extract no. 10 did inhibit *K. pneumoniae* and did not exhibit any activity against *S. aureus*. The study that was done by Ncube *et al*. (2011) showed that the best antibacterial activities were reported in the winter season from the dichloromethane bulb extracts of *T. violacea* against *S. aureus* and *K. pneumoniae* having an MIC value of 0.2 mg/ml. This correlates with dichloromethane extract no. 1 and extract no. 10.

Effective results of botanical compounds from plant materials are greatly reliant on the type of solvent used in the extraction procedure. Traditional healers use mainly water as the solvent, but in this and other studies, the researcher observed that the organic solvent extracts exhibited a greater antibacterial activity than water extract. These remarks can be rationalised in terms of the polarity of the compounds being extracted by each solvent and in addition to their intrinsic bioactivity, their capability to dissolve or diffuse in the different media used in the assay (Das *et al*., 2010).
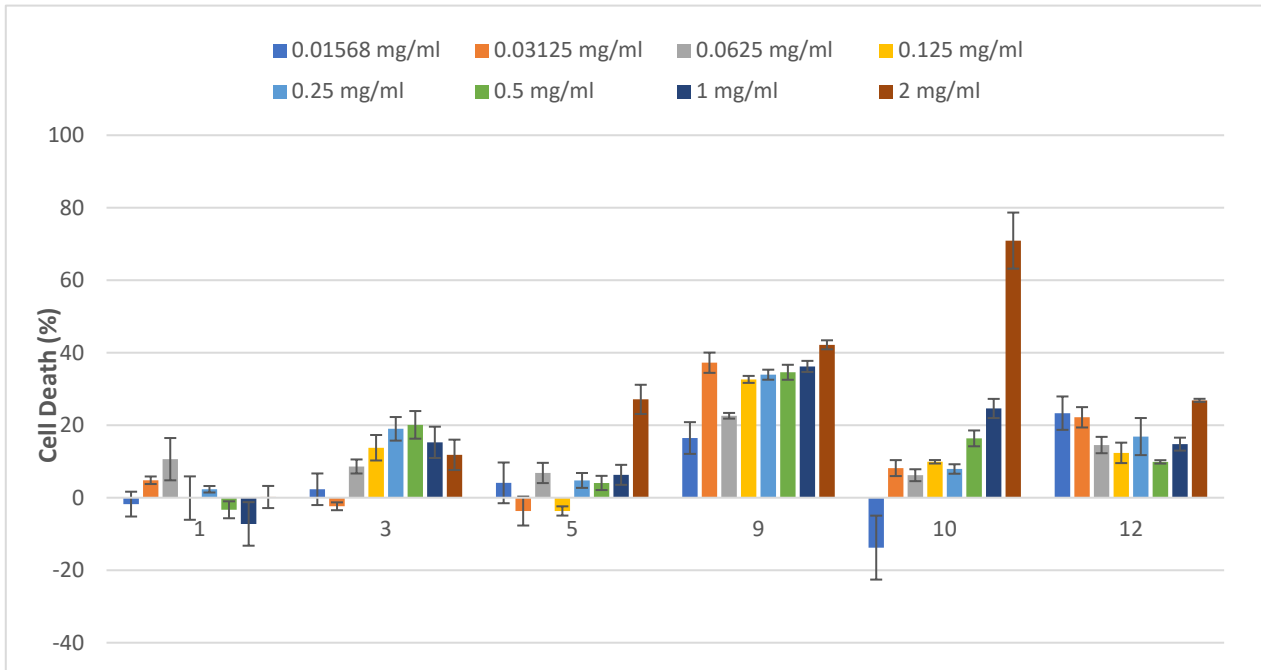
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Figure 5.2: Screening for antibacterial activity of six extracts against *K. pneumoniae* for a concentration range of 0.0156 mg/ml–2 mg/ml. Gentamicin was used as a positive control at its MIC of 2 µg/ml. Error bars indicate standard deviation of quadruplicate values determined in a single experiment.

5.4 Conclusion

The extracts from *T. violacea* were active against *S. aureus* and *K. pneumoniae* at the concentrations used in this study. The antibacterial activity showed interesting results, with certain plant extracts indicating Gram-specificity. Extracts 10 and 12 from *T. violacea* both showed activity against *K. pneumoniae* (Gram-negative) but not against *S. aureus* (Gram-positive). Extract 9 showed a similar result at lower concentrations. The high concentrations of extract 3 showed a more drastic increase in the percentage cell death of *S. aureus* than of *K. pneumoniae*. Extract 1 also seemed to be effective against *S. aureus.* The extracts of *T. violacea* have the potential to be developed as an antimicrobial agent, but the standard displays more antimicrobial activity.

The investigated plant extract of *T. violacea* showed that the results obtained confirm the therapeutic potency of some plants used in herbal therapy. In addition, these results form a good foundation for the selection of candidate plant species for further phytochemical and pharmacological investigation. The results of the present investigation support the usage of the studied extract and suggest that some plant extracts contain compounds with antibacterial properties that can be used as antimicrobial agents in new drugs for the treatment of infectious ailments caused by pathogens. The most active extracts can be exposed to isolation of therapeutic antimicrobials and undergo further pharmacological assessment.

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CHAPTER 6

Overview of the phytochemistry and biological activity of *Tulbaghia violacea*

6.1. Introduction

This chapter is a summative assessment of the conclusions drawn from the research study, as well as the answers to the research aim and objectives. Recommendations were also drawn from the data obtained; these conclude the thesis.

6.2. Conclusion

The evaluation of the phytochemistry and biological activity in plant extracts is a primary phase essential for finding and developing new remedies with improved efficacy. The phytochemistry and biological activity presented in *T. violacea* suggest that the plant is a potential source of chemotherapeutic agents. In this study, most of the phytochemistry and biological activity were found in the leaves of *T. violacea,* authenticating their traditional use in the treatment of various ailments such as fever, colds, asthma, tuberculosis, oesophagus cancer, high blood pressure and stomach problems such as gastroenteritis, as well as abdominal pains. The two solvents used in this research were able to extract different bioactive compounds.

The leaves, bulb and root extracts from *T. violacea* were evaluated against the human cell lines TK-10 (renal), PC3 and HeLa. All extracts were inactive. A higher concentration of aqueous and methanol/dichloromethane extracts were chosen and used against these three human cell lines. The aqueous solvent proved to be the solvent of choice to perform extraction on the *T. violacea* plant. Furthermore, the leaf extract would be the best part of the plant to test against TK-10, PC-3 and HeLa human cell lines. The values of IC_{50} for two to three cell lines showed inactivity (>100 μg/ml) of *T. violacea* plant extracts. Water was a much safer extraction solvent than dichloromethane solvent because the crude water extracts did not exhibit an undesirable IC_{50} value at a low concentration. The aqueous extract of the leaf supports the claims made by traditional healers for their use of the *T. violacea* plant for the treatment of cancer and other diseases.

The findings of the study demonstrated that crude aqueous and methanol/dichloromethane extracts prepared from the leaves, bulbs and roots of *T. violacea* were not toxic at all concentrations. This further confirms the safety of

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T. violacea when used for the treatment of diseases. This research provided preliminary data on the toxicity of crude extracts from *T. violacea* plant parts towards WI-38 cell lines. The results demonstrated that all extracts are not cytotoxic at concentrations higher than 100 µg/ml. According to this study, a concentration of 10 to 100 µg/ml from the water extracts of *T. violacea* plant parts may be considered the optimal dosage for preparation of traditional remedies. The study also supports the traditional use of only *T. violacea* leaves for the preparation of remedies to treat various ailments owing to the reported high percentage cell viability, as well as the desirable IC$_{50}$ value. There is thus no need to harvest the other parts of *T. violacea* for preparation of traditional medicines. This is important for the conservation of species. This study supports the use of water as an extraction solvent, which is in line with the traditional method of extraction. Furthermore, aqueous and methanol/dichloromethane extracts of *T. violacea* were not genotoxic, as they failed to induce the formation of micronuclei. The results of genotoxicity and cytotoxicity correlated with and supported the safety of the plant for human consumption.

The study presented that both the aqueous and methanol/dichloromethane extracts of *T. violacea* possessed antioxidant activity potential. Furthermore, for total phenolic determination, both the aqueous and methanol/dichloromethane extracts displayed potential. It is well recorded that phenolic compounds contribute to quality and nutritional value in terms of adapting colour, taste, aroma and flavour and in having effects beneficial to health. They also play a role in plant defence mechanisms to counteract ROS, to endure and avoid molecular damage and damage by microorganisms, insects, and herbivores (Sengul *et al.*, 2009).

This study indicated that water and methanol/dichloromethane extracts from *T. violacea* were active against *S. aureus* and *K. pneumoniae* at different concentrations used. The water leaf extract and water bulb extract from *T. violacea* both showed activity against *K. pneumoniae* (Gram-negative) but not against *S. aureus* (Gram-positive). The water root extract showed a similar result at lower concentrations. The high concentrations of methanol/dichloromethane root extract showed a more drastic increase in the percentage cell death of *S. aureus* than of *K. pneumoniae*. Methanol/dichloromethane bulb extract also seemed to be effective against *S. aureus*.

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The extracts of *T. violacea* have the potential to be developed as an antimicrobial agent, but the standard displays more antimicrobial activity.

The *T. violacea* extracts studied yielded results confirming the treatment effectiveness of some plants used in medicinal remedies. In addition, these results lay the essential groundwork for the selection of candidate plant species for further phytochemical and pharmacological investigation. The results of the present study support the usage of the studied extract and suggest that some plant extracts contain compounds with antibacterial properties that can be used as antimicrobial agents in new drugs for the treatment of infectious ailments caused by pathogens. The most active extracts can be exposed to isolation of therapeutic antimicrobials and undergo further pharmacological assessment. This study proved that different factors affect the quantity and composition of the phytocompounds present in an extract, such as the nature of the solvent, the concentration and the polarity of the solvent used. Overall, this research study also supports the ancient and frequent use of only leaf water extracts of *T. violacea* for research purposes and for the preparation of herbal remedies.

6.3. Recommendations

The quantitative/semi-quantitative analysis of the present phytochemistry and biological activity in the *T. violacea* plant parts will be an interesting area for further study (Madike *et al*., 2017). Further investigation is required to exploit the biomedical applications of *T. violacea*. Further testing using other cancer cell lines is required, also to identify the active compounds of *T. violacea* plant extracts. Further testing to determine the antimicrobial effect of *T. violacea* will be performed on organisms that are highly successful opportunistic pathogens and thus involved in various diseases. *T. violacea* plant extracts are rich in cardiac glycosides, which have been described to increase sodium ion levels in the myocytes, thus leading to a rise in the level of calcium ions, promoting an increase in calcium ions available for contraction of the heart muscle, which improves cardiac output and decreases distention of the heart (Takaidza *et al*., 2018). The anti-HIV activity of saponins (Ncube *et al*., 2011) is an interesting area for further research, as their presence was found in all the plant parts of *T. violacea.* Research should be conducted too to isolate, identify, characterise and
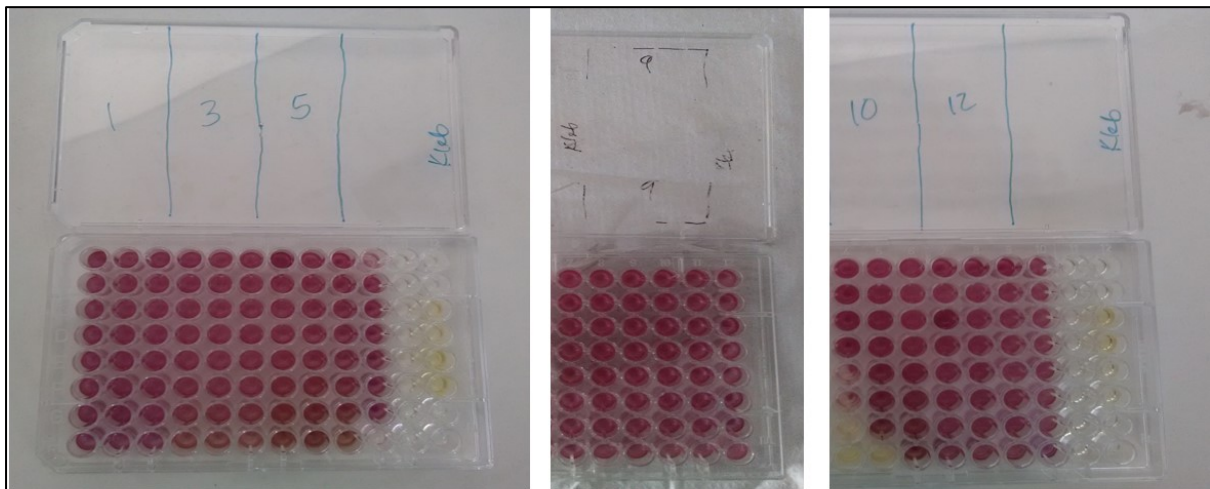
eluidate the structure of the identified bioactive compounds from *T. violacea*. To broaden this research, different extraction methods should be considered for further verification of the results obtained in this study. These may include infusion, percolation, digestion, decoction, hot continuous extraction (Soxhlet), counter-current extraction, microwave-assisted extraction, ultrasound extraction (sonication), supercritical fluid extraction, etc. (Patel *et al.*, 2018). *In vitro* studies have stated that flavonoids possess antiviral activity against several viruses, among others poliovirus. This emphasises the need to research *T. violacea* leaf extracts for the likely remedy of polio, as they are rich in flavonoids (Longhini *et al.*, 2017). The use of different solvents may also be considered, as solvents are selective for the extraction of specific compounds. Extraction solvents such as acetone, chloroform, ether, butanol or methanol may be used. Furthermore, it would be significant to distinguish the effects of the non-toxic concentrations on the cytokine and chemokine profiling of the macrophages (Perkins *et al.*, 2012).

Appendices

Appendix 1:

Antibacterial activity against selected micro-organisms

The antibacterial activity of indicated extracts were tested against a Gram-negative and Gram-positive bacterium, as discussed in the report. Figures 1 and 2 below show photographs of the result, as well as the plate layout of the experiment. From Figure 2 it is clear that Extract 1 has an MIC value of 2 mg/mL against *S. aureus*. No other extracts exhibit MIC values in the concentration range tested.
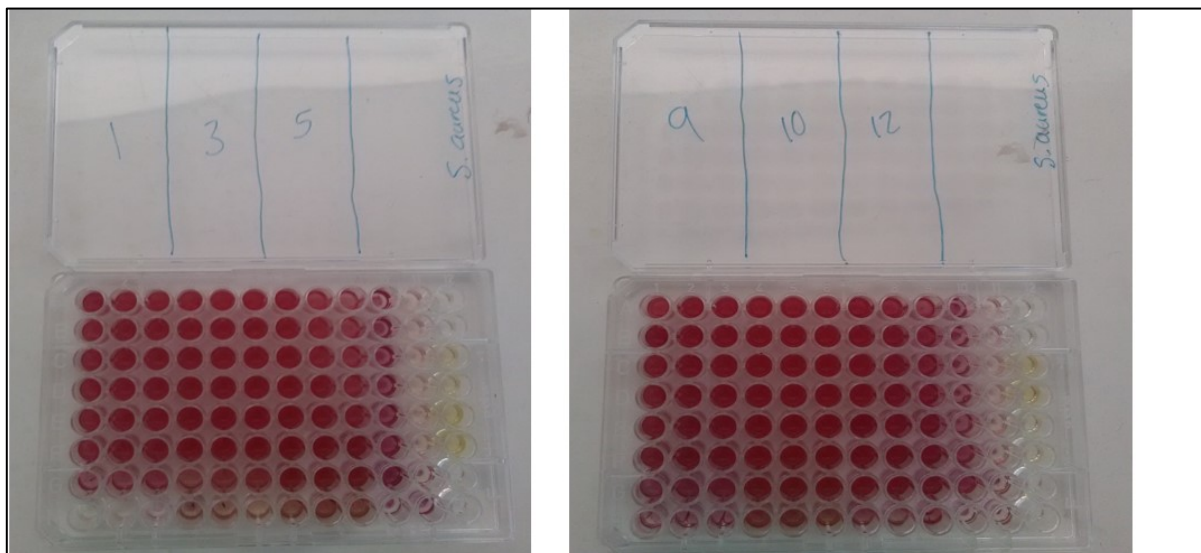

	extract			extract			extract			GC	PC	
0.0156												
0.03125												
0.0625												
0.125												
0.25												
0.5												
1												
2												

PC - refers to respective positive control. Note these were only tested at their MIC values

GC - refers to growth control which only contained medium and respective micro-organism

All extracts were tested at the concentrations indicated in the table above.

Figure 1: Screening for antibacterial activity of extracts 1, 3, 5, 9, 10 and 12 against *K. pneumoniae* at a concentration range of 0.0156 mg/ml – 2 mg/ml. Vancomycin was used as a positive control at its MIC of 2 μg/ml.

	extract			extract			extract			GC	PC	
0.0156												
0.03125												
0.0625												
0.125												
0.25												
0.5												
1												
2												

PC - refers to respective positive control. Note these were only tested at their MIC values

GC - refers to growth control which only contained medium and respective micro-organism

All extracts were tested at the concentrations indicated in the table above.

Figure 2: **Screening for antibacterial activity of extracts 1, 3, 5, 9, 10 and 12 against *S. aureus* at a concentration range of 0.0156 mg/ml – 2 mg/ml. Vancomycin was used as a positive control at its MIC of 2 µg/ml.**