CYTOTOXIC AND GENOTOXIC STUDIES OF CRUDE EXTRACTS FROM THE LEAVES, STEMS AND ROOTS OF *TULBAGHIA VIOLACEA*

BY

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DECLARATION

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

Signed …….. Date ……...

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This dissertation is being submitted in partial fulfilment of the requirements for the degree of Magister Technologiae Biotechnology.

Signed..... Date …….

STATEMENT 2

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

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DEDICATION

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ABSTRACT

Tulbaghia violacea Harv. (wild garlic) has been used in traditional medicine in Southern Africa for the treatment of various ailments. Despite the widespread use and popularity of this medicinal plant as a herbal medicine, there is contradictory evidence regarding the safety and toxicity of the plant. The phytochemical profiling of the plant has also been neglected in research. The determination of chemical constituents present in plant material as well as the potential toxicity found in plants are preliminary steps necessary for the discovery and development of novel therapeutic agents with improved efficacy. The aim of this study was to evaluate the cytotoxic and genotoxic potential of crude extracts from the leaves, stems and roots of *T. violacea.* This was performed *in vitro* using aqueous and ethanol extracts of the leaves, stems and roots. The aim of the study was achieved by three major objectives; (1) to identify the active phytocompounds present in the leaves, stems and roots, (2) to assess the cytotoxicity using the MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) cell proliferation assay, and (3) to evaluate the genotoxic potential of the leaf, stem and root water extracts using the *Allium cepa* assay.

A total of 14 phytochemicals were each extracted separately with distilled water and 70% ethanol by maceration from the leaves, stem and roots of *T. violacea*. The results of the qualitative phytochemical analysis showed that pharmacologically active compounds such as tannins, terpenoids, flavonoids, saponins, proteins, steroids, cardiac glycosides, phenols and coumarins were present in some organs of *T. violacea.* However, phlobatannins, leucoanthocyanins, alkaloids, carbohydrates and anthocyanins were absent in all plant parts. Overall, the leaves of the plant contained more active compounds than those present in the stems and roots when both water and 70% ethanol were used as the extractants. The quantitative phytochemical analysis for the Total Flavonoids Content (TFC) and Total Phenolic Contents (TPC) was also assessed. The water (0.027 mg/g) and 70% ethanol (0.053 mg/g) were most effective in extracting flavonoids from the leaves while the least amounts were obtained from the stems and roots. This observation was similar to the TFC were the water extracts of the leaves were the most effective in extracting phenols followed by the stems and roots.

The MTT assay was conducted using two cell lines RAW 264.7 and C2C12. The experiment was conducted in triplicates for the leaf, stem and root extracts (water and ethanol) of *T. violacea*. The experimental design employed a 2^3 factorial design where three independent variables (concentration, incubation time and type of extracts) were selected using two levels for each variable (high (+) and low (-)). The results illustrated that both the water and ethanol

extracts only showed a significant reduction in the number of viable cells at the concentration higher than 250 µg/ml treatment for both RAW 264.7 and C2C12 cells. The ethanol extracts from the leaves, stems and roots were found to be toxic towards the RAW 264.7 cells even at lower concentrations at both 24 and 48 h incubation periods (% cell viability < 50%). The water extracts were non-toxic to RAW 264.7 cells except for the water stem extract which showed toxicity after 48 h incubation (IC_{50} = 9.475 (4.061 to 23.39)). For the C2C12 cells, the lowest potent toxic concentration was 250 µg/ml for the ethanol extract of the stem after 48 h incubation. Overall, the *T. violacea* plant extracts were non-toxic as percentage cell viability greater than 50% was noted for both extraction solvents in all the plant parts of *T. violacea*. No cytotoxic activity was observed in all *T. violacea* plant parts with the C2C12 cell line (IC_{50} > 30 µg/ml).

For the *Allium cepa* assay, only the water crude extracts of the leaves, stems and roots of *T. violacea* were used. A similar trend of potent genotoxic activity in the water stem extracts compared to the leaf and root extracts at the concentration ranges studied. Similar to the MTT assay, it is clear from the study that at higher concentrations, the water crude extracts from the leaves, stems and roots of *T. violacea* is toxic.

From this study, it can be concluded that the extraction of compounds using water is more efficient than using ethanol. Overall, the *T. violacea* leaf extracts extracted the most phytocompounds and showed the highest percentage of viable cells as well as desirable IC_{50} values. However, preparation of herbal remedies using *T. violacea* plant extracts should be done with caution due to their possible genotoxic and cytotoxic potential at higher concentrations. This study raises a need to further conduct *in vivo* cytogenetic studies to ascertain the possible toxic effects of *T. violacea* crude extracts.

Key words: 2^3 factorial design, *Allium cepa* assay, C2C12 cells, MTT cell proliferation assay, Phytocompounds, RAW 264.7 cells, Total Flavonoids Content (TFC), Total Phenolic Contents (TPC) and *Tulbaghia violacea*.

ABBREVIATIONS

ANOVA	Analysis of Variance
APG	Angiosperm Phylogeny Group
CA	Chromosomal aberrations
BSLT	Brine Shrimp Lethality test
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
FBS	Fetal bovine serum
HAI	Health Action International
HIV/AIDS	Human Immunodeficiency Virus/ Acquired Immunodeficiency Syndrome
GAE	Gallic acid equivalent
IC_{50}	Inhibitory concentration
LC_{50}	Lethal dose
MI	Mitotic index
MN	Micronucleus
MTT	3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide
NA	Nuclear Abnormalities
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide Adenine Dinucleotide Phosphate Hydrogen
QE	Quercetin equivalent
RNA	Ribonucleic acid
TFC	Total Flavonoid content
TPC	Total Phenolic content
UV	Ultraviolet light
WHO	World Health Organization

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CHAPTER 1

1. GENERAL INTRODUCTION

1.1. Introduction and overview of the study

This chapter provides a general background on why this research was conducted. The classification of the genus *Tulbaghia* is outlined. It highlights the role of medicinal plants in toxicology studies. The chapter also reports on the advantages and disadvantages of using medicinal plant as herbal medicine.

1.2. General background of the study

Tulbaghia, a genus of about 30 species belonging to the family Amaryllidaceae (formerly Alliaceae), is native to the Southern Africa region. In general, species of *Tulbaghia* are described as modest, unassuming plants with small flowers, grassy foliage, sometimes with a pungent, skunky or alliaceous scent to the rhizomatous rootstalks (Vosa *et al.* 2006; Vosa 2007). Some *Tulbaghia* species have been cultivated in countries as far as Europe and America (Benham 1993). Certain species of the genus *Tulbaghia* are known to have medicinal properties, while others have been used as food. However, most species are grown as ornamentals (Williamson 1955; Tredgold 1986; Van Wyk *et al.* 2000; Lyantagaye 2011). Species of *Tulbaghia* are closely related to *Allium sativum* (sweet garlic) and hence are commonly known as wild garlic (Dahlgren *et al.* 1985). Of the 30 species currently identified in the genus *Tulbaghia*, only 3 have been identified in the literature as being ethnobotanically useful or investigated phytochemically (Lyantagaye 2011).

For centuries, the use of traditional medicine for the treatment of infectious and non-infectious diseases such as fever, colds, rheumatism, etc. has been a part of human culture (Diallo *et al.* 2003; Gurib-Fakim 2006). Despite the widespread use and popularity of medicinal plants as herbal medicine, their use and safety are still a concern. It has been reported that less than 10% of herbal products in the world market are standardized to known active components and strict quality control measures are not diligently adhered to (Winston *et al.* 2007). For most of the products in use, there is insufficient information available about their active and toxic constituents. Reports have shown that drugs made from some medicinal plants can be extremely harmful to human health (Di Stasi *et al.* 2002; Melo-Reis *et al.* 2011).

Many plants are known to produce toxic secondary metabolites as a natural defence against pathogens. Toxicity tests can be used to reveal some of the risks concerned with the use of medicinal plants (Kennedy *et al.* 2011). For the last 30 years, studies were conducted to develop

different methodologies, strategies and approaches to assess chemicals that could demonstrate genotoxic and/or carcinogenic effects of plant metabolites (Dearfield *et al.* 1991; Waters *et al.* 1999). Genotoxicity assays are generally used for the identification of extracts/substances with the ability to interact with nucleic acid at low concentrations. When a toxic agent interacts with DNA, it may result in chromosomal aberrations and alter the DNA structure. This may result in the loss, addition, or replacement of bases, thus altering the sequence in the DNA, affecting the fidelity of the genetic message and lead to irreversible changes in the cell (Varanda *et al.* 2002). There is thus a need to assess genotoxicity during the preclinical evaluation of herbal extracts/substances to verify their mutagenic potential for both safety and economic reasons. This is mainly because medicinal plants are extensively used in folk medicine and as a primary resource for the development of new drugs (Di Stasi *et al.* 2002; Melo-Reis *et al.* 2011). With previous research reporting uncertainties regarding the safety of some species of the genus *Tulbaghia,* it is necessary that further studies be conducted to provide more insight on their safety and toxicity (Jäger *et al.* 2012). Hence this study is undertaken to evaluate the toxicology of crude extracts from the leaves, stems and roots of *Tulbaghia violacea.*

1.3. Problem statement

There are over 1.5 million medicinal plants that have been investigated, and most of them are reported to contain toxic substances (Ishii *et al.* 1984) including some secondary metabolites. Plants produce a variety of secondary metabolites which have been classified based on their molecular structure. These include: saponins, terpenoids, cyanogenic compounds, tannins, toxic amino acids and alkaloids (Dai *et al.* 2010). Tulay (2012) reported that the toxicity of plants can be influenced by several factors such as soil and climate, the strength of the secondary metabolites, the quantity consumed, the time of exposure to the toxin, the part of the plant consumed (root, oil, leaves, stem bark or seeds), the state of the individual's health, stereochemistry which can be positive or negative, lack of polarity in structure (lacking hydroxyl group and N atoms) and genetic variation within the species. With this information in mind, medicinal plants should be used with caution. This raises the need for more research in the toxicology of medicinal plants and the way they are prepared to build up reliable information on their safety for the development of appropriate guidelines for safe and effective use (Mann *et al.* 2002).

1.4. Motivation

In recent years, there has been a significant increase globally in the demand for herbal medicinal products. It is estimated that the world's population will be more than 7.5 billion in

the next 10 to 15 years primarily in the Southern hemisphere where approximately 80% of the population still relies on a traditional system of medicine based on herbal drugs for primary healthcare (Chan 2003; Ramawat *et al.* 2008; Muhammad *et al.* 2011). Therefore, medicinal plants and their bioactive molecules are always in demand and are a central point of research (Ramawat *et al.* 2008). Herbal medicine is also inexpensive as compared to commercial medicine which is becoming more expensive and out of reach especially for people in rural areas (David 1996; Fasola *et al.* 2005; Obi *et al.* 2006). The human population is primarily interested in easy access to safe and efficient drugs, as well as in animal welfare. Since medicinal plants have been used for centuries, one might expect them to have low toxicity. But research has indicated that many medicinal plants applied in traditional medicine showed adverse health effects (Ertekin *et al.* 2005; Koduru *et al.* 2006). Therefore, it should be stressed that the use of any plant for medicinal purposes, by no means, guarantees its safety. This therefore raises concerns about the possibility of toxic effects resulting from the short and long-term use of such medicinal plants (Ukwuani *et al.* 2012).

Several species of *Tulbaghia* have shown great medicinal importance such as a remedy for pulmonary tuberculosis, asthma and stomach problems (Dyson 1998). Regardless of the extensive research that has been conducted on *Tulbaghia* species for various purposes, reports of toxicity of the species are a concern, raising a need for a more thorough evaluation of the species (Jäger *et al.* 2012). Therefore, in this study, the toxicity of the leaves, stems and roots of *Tulbaghia violacea* was evaluated using various tests such as the MTT cell proliferation assay and the *Allium cepa* assay.

1.5. Aim

To assess the cytotoxicity and genotoxicity of crude extracts from the leaves, stems and roots of *Tulbaghia violacea.*

1.6. Objectives

1. To prepare the crude extracts of the leaves, stems and roots of *T. violacea.*
2. To perform phytochemical analysis of these crude extracts using standard methods by Harborne (1973).
3. To evaluate the cytotoxicity of the crude extracts using the MTT cell proliferation assay.
4. To screen for genotoxicity of the extracts by using the *Allium cepa* assay for the evaluation of different end points (chromosomal aberrations) (CA), Micronucleus (MN) and calculate the mitotic index (MI).

CHAPTER 2

2. LITERATURE REVIEW

2.1. Introduction

This chapter introduces the history of medicinal plants and their value in modern medicine. The chapter presents the history and origin of the genus *Tulbaghia.* This chapter also explains the classification of *Tulbaghia violacea* and its medicinal values. Finally, the chapter introduces toxicity of medicinal plants and the types of assays that can be conducted to identify their presence in plants.

2.2. Medicinal Plants

Preceding the era enforced by Western colonization, medicinal plants were the major agents for primary health care accessible to millions of people throughout Africa. There is documented evidence for the use of herbs in the treatment of different ailments; as was seen with Mesopotamian, Indian ayurveda, ancient traditional Chinese medicine and Greek unani medicine (Bhatnagar *et al.* 1994; Shafqat 1994; O'Brien *et al.* 2003; Biggs 2005; Aoelsoud 2010). According to the World Health Organisation (1978), about 80% of the world's rural population currently relies on medicinal plants as their complementary or alternative source of health care (Chan 2003; Muhammad *et al.* 2011). The indigenous act of using medicinal plants to treat and deal with various diseases is an ancient one that has now been accepted and recognized as one of the surest means to achieve total health care coverage of the world's population (World Health Organisation 1978; Abdullahi 2011). Recent advancements and numerous studies that have been conducted in the fields of environmental science, immunology, medical botany and pharmacology of medicinal plants are the contributing factors to the general acceptance of medicinal plants in modern medicine, primarily to facilitate the production of new medicine (Abdullahi 2011; Takaidza *et al.* 2015)

The widespread use of traditional medicine in middle and low-income countries, especially in Africa may be influenced by the limited access to modern medicine and drugs to treat and manage diseases. The WHO/Health Action International (2008) recently conducted a study in 36 low and middle-income countries and proved that modern medicine is indeed inaccessible to large sections of the population (Cameron *et al.* 2009). This then proves that in addition to being inexpensive, the use of traditional medicine in Africa is on the increase because it can be easily accessed by a large number of people. Chatora (2003) reported that the ratio of traditional healers to the population in Africa is 1: 500 compared to 1: 40 000 for medical doctors (Table 1). This is probably due to the fact that most medical doctors are based in urban areas and cities

as opposed to rural areas, making traditional healers the primary source of health care providers in the latter areas (Abdullahi 2011).

Table 1: The ratio of Traditional Healers to the population compared to Medical Doctors in Africa (Chatora 2003; Maoela 2005).

Countries	**Ratios of Traditional Practitioners to Population**	**Ratio of Medical Doctors to Population**
Kenya, Urban (Mathare)	1: 833	1: 987
Rural (Kilungu)	1: 143-345	1: 70 000
Zimbabwe	1: 600	1: 6 500
Swaziland	1: 100	1:10 000
Nigeria (Benin City)	1: 110	1: 16 400
National Average	No data	1: 15 74
South Africa (Venda area)	1: 700-1 200	1: 17 400
South Africa	1: 1,639 (overall)	No data
	1: 17,400 (homeland)	No data
Ghana	1: 200	1: 20 000
Uganda	1: 700	1: 25 000
Tanzania	1: 400	1: 33 000
Mozambique	1: 200	1: 50 000
Namibia	1: 1,000 (Katutura)	
	1: 500 (Cuvelai)	No data
	1: 300 (Caprivi)	

2.3. Extraction

The separation of medicinally active portions of plant (and animal) tissues using solvents is pharmaceutically termed as extraction (Ncube *et al.* 2008; Remington 2006). The end product is usually in a liquid, semisolid or powder form among which are decoctions, infusions, fluid extracts, tinctures, pilular (semisolid) extracts or powdered extracts depending on the intended use (Remington 2006). The type of extract used is a vital aspect to consider to ensure successful determination of biologically active compounds from plant material. It is essential to use a solvent with low toxicity, ease of evaporation at low heat, promotion of rapid physiologic absorption of the extract, preservative action and inability to cause the extract to complex or

dissociate. There are different types of methods that have been used for extraction, among which are: infusion, percolation, digestion, decoction, hot continuous extraction (Soxhlet), aqueous-alcoholic extraction by fermentation, counter-current extraction, microwave-assisted extraction, ultrasound extraction (sonication), supercritical fluid extraction, etc. (Bentley 2010).

The use of different solvents may also be considered as they are used to selectively extract specific compounds. Extraction solvents such as water, ethanol, acetone, chloroform, ether, dichloromethanol, butanol or methanol may be utilized (Tiwari *et al.* 2011). For centuries, water has been used as an extraction solvent for the preparation of remedies because it is readily available (Eloff 1998; Grierson *et al.* 1999; Shale *et al.* 1999; Kelmanson *et al.* 2000; Inngjerdingen *et al.* 2004). Ethanol is also a popular solvent among practitioners because it is cost effective and free for practitioners (Louw *et al.* 2002).

2.4. The Family Amaryllidaceae

The family Amaryllidaceae (formerly: Alliaceae) consists of herbaceous perennial flowering plants and has 900 species in 30 genera growing worldwide (Figure 1) (Vosa 2000; Angiosperm Phylogeny Group 2003; Fay *et al.* 2006; Vosa 2007; Chase *et al.* 2009). The Amaryllidaceae is widely distributed in the Mediterranean, Europe, Asia, North and South Americas and Southern Africa. The Southern African genera include *Tulbaghia* and *Allium* (Dahlgren *et al.* 1984; Angiosperm Phylogeny Group 1998; 2003). When the leaves or rhizomes of members of the Amaryllidaceae family are bruised, an onion/garlic-like-odour is released due to cysteine-derived sulphur compounds present in the plant (Van Wyk *et al.* 1997; Van Wyk *et al.* 2000; Kubec *et al.* 2002; Maoela 2005; Lyantagaye 2011).

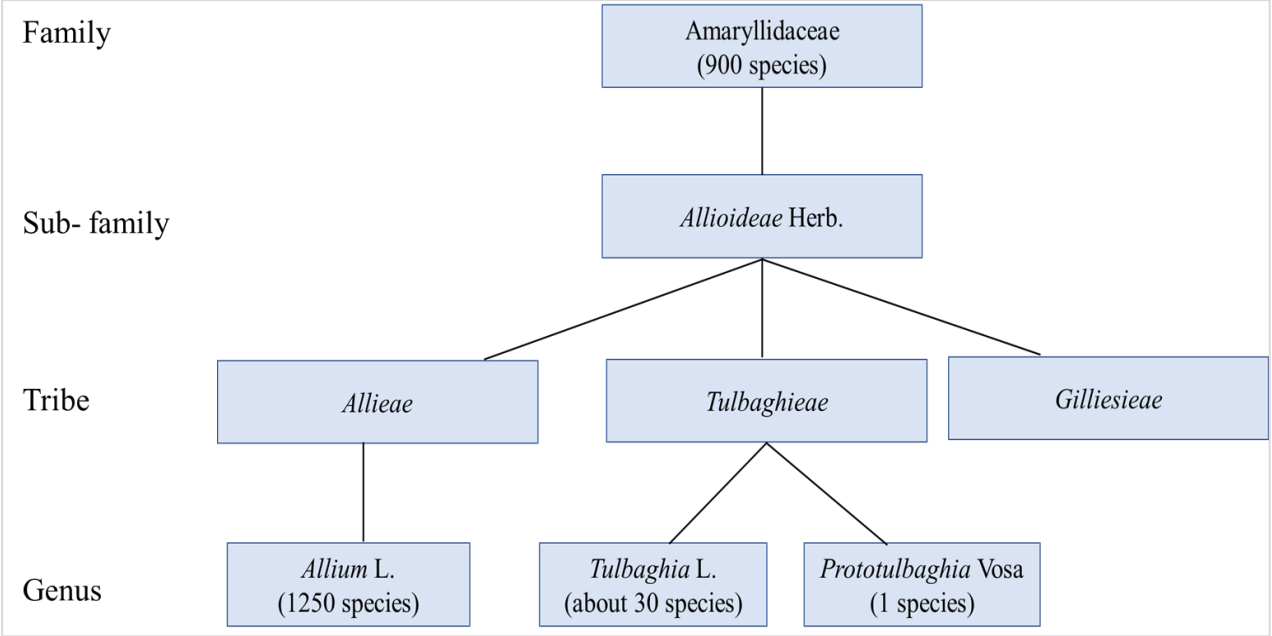

Figure 1: The systematic classification of the family Amaryllidaceae showing treatment of the subfamily *Allioideae.* Adapted from Species 2000: Catalogue of life 2017.

2.5. The genus *Allium*

The genus *Allium* belongs to the tribe *Allieae*, subfamily *Allioideae*, family Amaryllidaceae, order Asparagales, class Liliopsida and phylum Tracheophyta. It has about 1250 species, making it one of the largest plant genera in the world (Dahlgren *et al.* 1985). The plants in this genus are diverse and can grow in height between 5 to 150 cm, with a leafless stalk and an umbel formed from the flowers. Besides the well-known garlic (*Allium sativum* and *A. scordoprasum*) and onion (*Allium cepa*), several other species are widely grown for culinary use. *Allium sativum* and *A. cepa* are widely utilized throughout the world for medicinal purposes (Ross 2003).

2.5.1. *Allium cepa* L.

Allium cepa L. (onion) is believed to be native to Central Asia (between Turkmenistan and Afghanistan) (Grubben 2004; Bewuketu *et al.* 2016; Messele 2016). They have since migrated throughout the world with important production bulb onion areas being Senegal, Mali, Burkina Faso, Ghana, Niger, Nigeria, Chad, Sudan, Ethiopia, Kenya, Tanzania, Uganda, Zambia and Zimbabwe (Grubben 2004). *Allium cepa* L. is a diploid and has the chromosome number 2*n* = 16 (Mayer 1990; Gorsuch *et al.* 1993; Lower *et al.* 1997; Grubben 2004; Duarte *et al.* 2015). The leaves of the plant are cylindrical with subglobose umbels of trimerous flowers which initially appear covered by a membranous spathe (Figure 2). The *A. cepa* bulbs have different colors, shape and sizes depending on the variety of the species. In traditional medicine, *A. cepa*

has been used externally to treat boils, felons, wounds, anthrax or whitlows and stings. Internally onion has been used to relieve coughs, bronchitis, asthma, gastrointestinal disorders, and headaches (Maoela 2005). *Allium* cepa has gained popularity in research as a suitable model organism for the determination of potential genotoxic agents in environmental samples (Rank 2003).

Figure 2: Typical example of *Allium cepa* L. species (www.plantillustration.org).

2.5.2. *Allium sativum* L.

Garlic (*Allium sativum*) is the most widely used herb in the world and has long been used both for flavoring and for the potential benefits of preventing and curing ailments in many cultures (Amagase 2006). Although *A. sativum* is indigenous to Asia, it has been grown in most of tropical and subtropical regions (Hyams 1971). *Allium sativum* has been cultivated as a food plant for over 10, 000 years. Egyptian records dating back to about 1550 BC makes references to garlic as a remedy for a variety of diseases (Block 1985). *Allium sativum* has a chromosome number of $2n = 3x = 24$. The plant has linear sheathing leaves with globose umbels of white or reddish flowers. The bulbs of the plant consist of "cloves", wrapped in a shared whitish papery coat. The garlic smell only grows stronger upon damage of the cloves (Dahlgren *et al.* 1985). More recently, epidemiological studies showed that the enhanced consumption of garlic is

closely related to reduced risk of cancer incidence (Haenszel *et al.* 1972; Buiatti *et al.* 1989; You *et al.* 1989; Steinmetz *et al.* 1994; Fleischauer *et al.* 2001; Hsing *et al.* 2002). Dietary garlic has been acknowledged for its beneficial health effects. In particular, garlic has been affiliated with (i) reduction of risk factors for cardiovascular diseases and cancer, (ii) stimulation of immune function, (iii) enhanced detoxification of foreign compounds, (iv) hepatoprotection, (v) antimicrobial effects, (vi) antioxidant effects, and most importantly (vii) its hypoglycemic and anticoagulant properties. These properties are responsible for the widespread use of garlic and its closely related genera which includes *T. violacea* for the treatment of various ailments (Banerjee *et al.* 2002; Benavides *et al.* 2007).

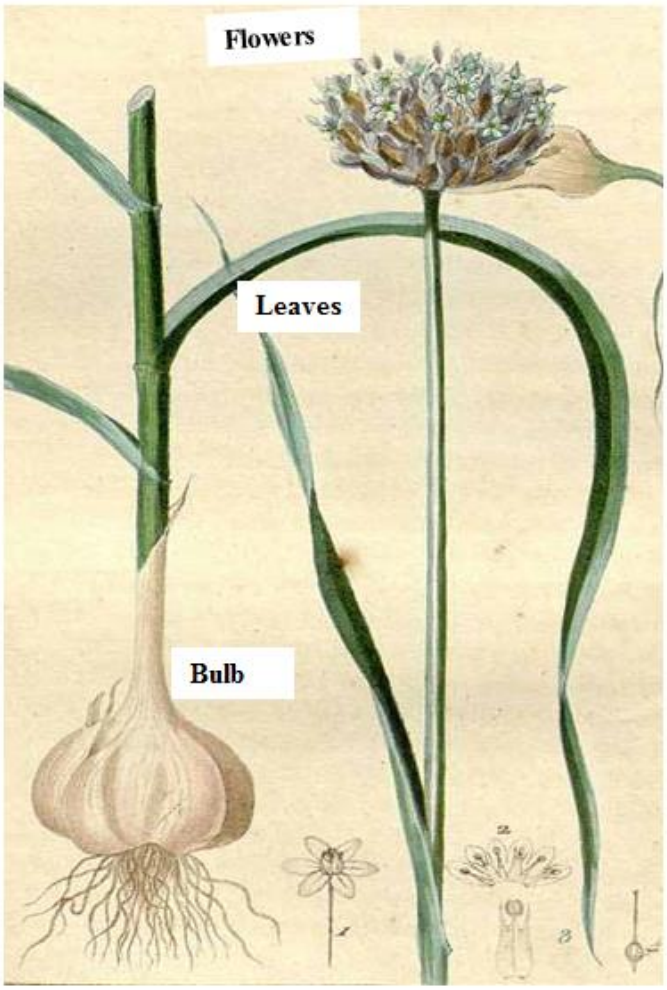

Figure 3: Typical phenotype of *Allium sativum* species (www.plantillustration.org).

2.6. The genus *Tulbaghia*: Origin and distribution

The genus name for wild garlic is *Tulbaghia*; it was named by Linnaeus after Ryk or Rijk Tulbagh (1699-1771) who was then the governor of The Cape Province (1751-1771) and based on material sent to Europe in 1769 by Rijk. Linnaeus first called the genus Tulbagh after Rijk

Tulbagh but later this was corrected to *Tulbaghia* by P.D. Giseke (Vosa 2000). The genus *Tulbaghia* belongs to the *Tulbaghieae* tribe, subfamily *Allioideae*, family Amaryllidaceae, order Asparagales, class Liliopsida and phylum Tracheophyta (Fig. 1). *Tulbaghia* is a plant genus of about 30 species and is entirely African in distribution (Benham 1993). This distribution extends from Namibia and the Western Cape to the southern parts of Tanzania in Southern Tropical Africa. There are two species in the genus that are commonly grown as ornamentals, namely, *Tulbaghia violacea* and *T. simmleri*. Indigenous people used several species of *Tulbaghia* as food and medicine (Vosa 1975; Kubec *et al.* 2002).

Figure 4: Different types of food obtained from *Tulbaghia* species: A: Pesto, B: Salt, C: Pickled *T. violacea* leaves, D: *Tulbaghia* infused vinegar, E: Wild garlic oil, F: Wild garlic mayonnaise, G: Wild garlic soup, H: Wild garlic butter and I: Wild garlic pasta (https://za.pinterest.com)

The genus is mainly distributed in the Southern hemisphere with majority of the species distributed in Southern Africa (Figure 5) (Vosa 2007). Their natural distribution extends from southern Tanzania to Malawi, Botswana, Lesotho and South Africa (Lyantagaye 2011). The South African distribution of the genus *Tulbaghia* is indigenous to the Eastern Cape, southern KwaZulu-Natal and the former Transvaal (Gauteng, Limpopo, Mpumalanga) with the Eastern Cape region being reported as the center of specification (Vosa 2000). A majority of the species in the genus grow in semi-desert to wet and boggy areas (summer-rain area) like dry or damp rocky grasslands and in marshy stream banks (Pooley 1998). However, other species like *T.*

capensis and *T. alliacea* are adapted to grow in areas with the winter-rain type of climate (Vosa 2003).

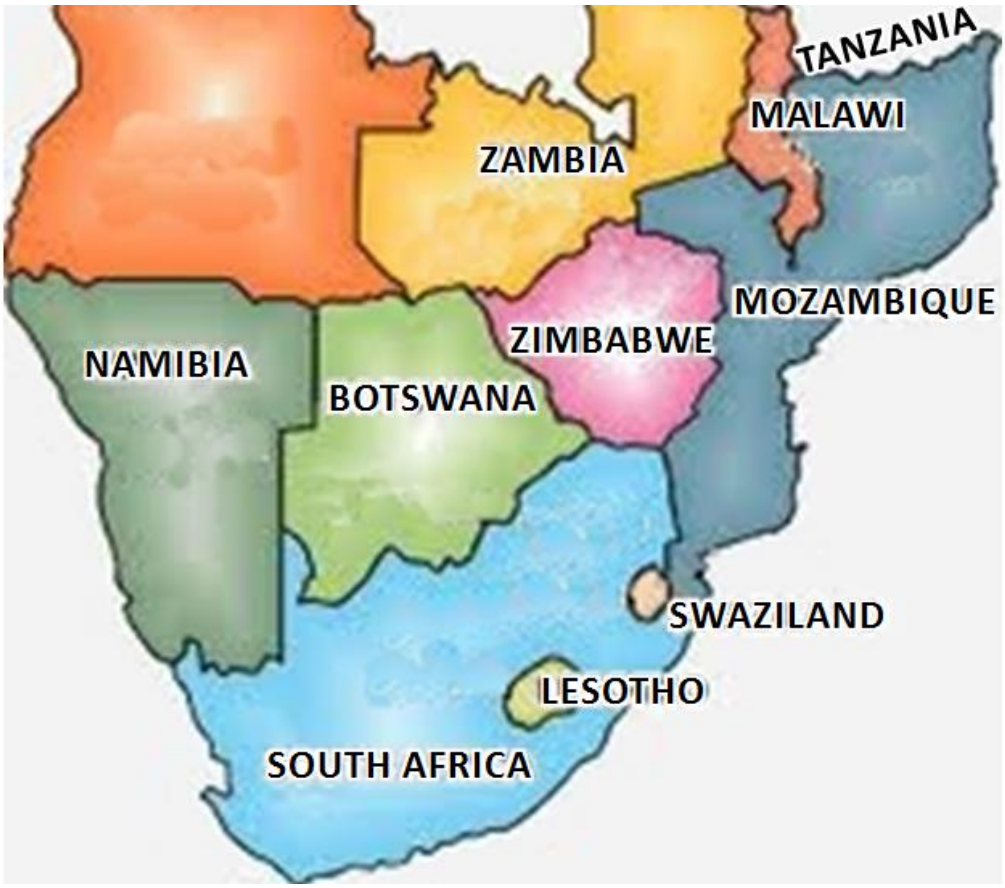

Figure 5: A map showing countries (Southern Africa) where *Tulbaghia* species are indigenous (Lyantagaye 2011).

2.6.1. Taxonomy and morphology

An evaluation into the phenotype of the genus *Tulbaghia* revealed that the species are fairly uniform with relatively small differences among them apart from the size and type of the corona as well as other morphological characters (Vosa 1966). The genus *Tulbaghia* is further subdivided into seven distinct groups based on the different karyotypes that have been identified in the genus (Vosa 2000). A majority of the species in the genus *Tulbaghia* are diploid with twelve chromosomes ($2n = 2x = 12$). There are reports of some polyploidy forms (*T. alliacea* with $4x = 24$ and *T. capensis* with $6x = 36$) which are restricted to few locations (Vosa 1966; Vosa 2007) with no reported true polyploid species identified (Belewa *et al.* 2011). The genus *Tulbaghia* consists of monocotyledonous herbaceous perennial bulbs, bulb-like corms or rhizomes predominantly in South African species (Pooley 1998). Species in this genus are mainly acaulescent geophytes and can grow to a height ranging from 15–60 cm. The roots

of the plants appear to be swollen, irregularly shaped and mostly covered by dry, fibrous leaves with a short, closed sheath that is formed at the base. The plant has basal and strap-shaped leaves. Plants in the genus *Tulbaghia* can be identified by the presence of a corona which is a raised crown or crown-like structure which appears as a fleshy ring at the centre of the flower tube (Figure 6). The number and colour of the flowers in the genus depends on the species and usually range from 3 to 40 in number and could be purple violet, pink, white or orange in colour (Fabian *et al.* 1997; Pooley 1998; Manning 2009; Vlok *et al.* 2010). Most species in the genus are pollinated by insects (Kubitzki 1998).

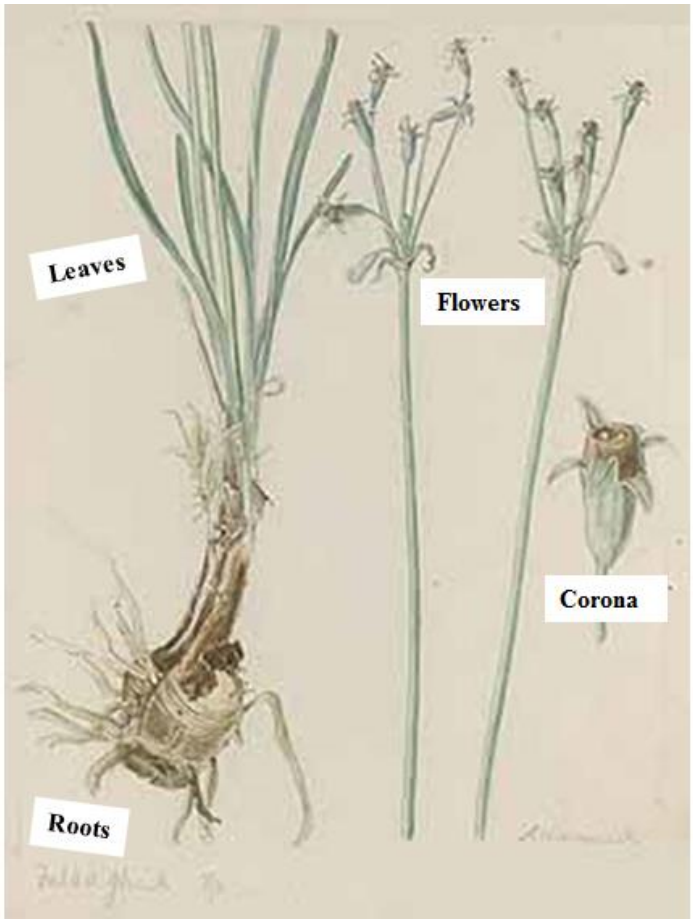

Figure 6: Typical phenotype of *Tulbaghia* species (www.plantillustration.org).

2.6.2. *Tulbaghia violacea*

The common English name for *T. violacea* Harv. is "wild garlic or society garlic". This name apparently originated from the belief that, in spite of its garlic-like flavor, the consumption of *T. violacea* is not accompanied by the development of bad breath, as is the case with consumption of commercial garlic (*A. sativum*) even though it is believed to possess similar bioactive compounds as garlic (Van Wyk *et al.* 1997; Van Wyk *et al.* 2000; George *et al.* 2010). *Tulbaghia violacea* has narrow, hairless, strap-shaped, dark green and leathery textured leaves

that can grow to 30 cm in length and 1.5 cm wide, arising from several white bases (Van Wyk *et al.* 2000). The flowers of *T. violacea* are produced from summer to autumn and are held above the leaves on a tall flower stalk with umbels of up to 20 mauve-purple flowers. *Tulbaghia violacea* is currently the most popular as well as the most highly investigated species in the genus *Tulbaghia* (Aremu *et al.* 2013).

Tulbaghia violacea is found originally in the Eastern Cape, KwaZulu-Natal, and Northern Gauteng in South Africa, and even as far north as Zimbabwe and grows in rocky grasslands to a height of 50 cm. The evergreen, leathery textured leaves of *T. violacea* which exhibit a garlic-like smell when bruised has been used in some cultures as a substitute for garlic and chives (Dyson 1998). *Tulbaghia violacea* has non-bimodal karyotype of $2n = 2x = 12$ (Fay *et al.* 1996). The plant is also known by several indigenous names, Wildeknoffel (Afrikaans), Icinsini (Zulu), Itswele lomlambo (Xhosa) and Mothebe (Sotho) (Dyson 1998; Van Wyk *et al.* 2000).

Tulbaghia violacea has traditionally been used extensively in South African traditional medicine for Human Immunodeficiency Virus/ Acquired Immunodeficiency Syndrome (HIV/AIDS) patients for the treatment of oral fungal infections. It has also found diverse use in the treatment of gastrointestinal ailments, asthma, fever, colds, pulmonary tuberculosis (Burton 1990; Dyson 1998; Kubec *et al.* 2002; Ncube *et al.* 2011a), constipation/purgative and use as anti-helmintics (Watt *et al.* 1962). It has been reported that *T. violacea* deters moles, due to its odour. The Zulu nation of South Africa grows this plant around their homes, as it is believed to repel snakes and they use the bulb to make an aphrodisiac (Burton 1990; Dyson 1998; Kubec *et al.* 2002). Communities in the Eastern Cape use *T. violacea* for colic, wind, restlessness, headache and fever, largely for young children. Some Rastafarian communities eat copious amounts of it during winter allegedly "to keep the blood warm" and stop aches and pains (Dyson 1998). The leaves on *T. violacea* can be crushed on the skin and the residue can be used to cure sinus headaches, repel fleas, ticks and mosquitoes (Lim 2013). In other reports, soaking the bulbs and leaves in water for a day can be used for rheumatism, arthritis and to reduce fever. Other uses include the treatment of infant and mother in the case of depressed fontanelle (Dyson 1998).

However, like any other drug, extensive consumption of medication prepared from *T. violacea* has been affiliated with a variety of undesirable symptoms such as abdominal pain, inflammation, gastroenteritis, acute inflammation and sloughing of the intestinal mucosa, cessation of gastrointestinal peristalsis, contraction of the pupils, subdued reactions to stimuli

and even some fatalities. This is assumed to be due to the high sulphur (2, 4, 5, 7-tetrathiaoctane-2, 2-dioxide and 2, 4, 5, 7-tetrathiaoctane) and the steroidal saponin content of the plant. *Tulbaghia simmleri* may act as a substitute for *T. violacea* where the latter is not available (Burton 1990; Van Wyk *et al.* 2000; Maoela 2005). A typical example of the *T. violacea* plant, flowers and a single plant after harvesting are illustrated in Figure 7.

Figure 7: Typical examples of the (a) *T. violacea* plants (http://floridaaquatic.com) (b) flowers (Mountain Valley Growers Inc. 2009) (c) single plants after harvesting (http://kebunmalaykadazangirls.blogspot.co.za)

2.7. Chemistry of *Tulbaghia* in relation to *Allium*

Garlic has been reported to possess a wide range of biological activities due to the bioactive compounds present in the plant (Kallel *et al.* 2014). Among those are antimicrobial, anticancer, antioxidant, immune boosting, antidiabetic, hepatoprotective, antifibrinolytic and antiplatelet aggregatory activity and its potential role in preventing cardiovascular diseases (Santhosha *et al.* 2013). However, there is insufficient research on the biological activities of *Tulbaghia* (Ncube *et al.* 2011a*).* Lyantagaye (2011) reported that *T. violacea* is rich in sulphur-containing compounds which can also be attributed to the characteristic garlic-like-odours, flavour and the medicinal properties of both the *Tulbaghia* and *Alluim* species.

The main compound present in *Tulbaghia* spp. is marasmin (Kubec *et al.* 2002; Aremu *et al.* 2013). Thamburan *et al.* (2006) reported that the fungicidal effect of *T. violacea* was due to the presence of the sulphur-containing compound marasmicin. When the plant tissue is damaged, marasmin reacts with the enzyme C-S lyase which converts it to marasmicin (Figure 8) which is further degraded into various sulphur containing compounds (Kubec *et al.* 2002; Kubec *et al.* 2013). Marasmicin is an unstable thiosulphinate present in *T. violacea* which is further degraded to generate various degradation products, such as 2,4,5,7-tetrathiaoctane-2,2-dioxide, 2,4,5,7-tetrathiaoctane-4,4-dioxide and 2,4,5,7-tetrathiaoctane-2,2,7,7-tetraoxide (Kubec *et al.* 2002). The beneficial effect of *Tulbaghia* spp. may also be attributed to the

presence of saponins (Watson *et al.* 1992), flavonoids, tannins (Ncube *et al.* 2011b) and kaempferol (Aremu *et al.* 2013)

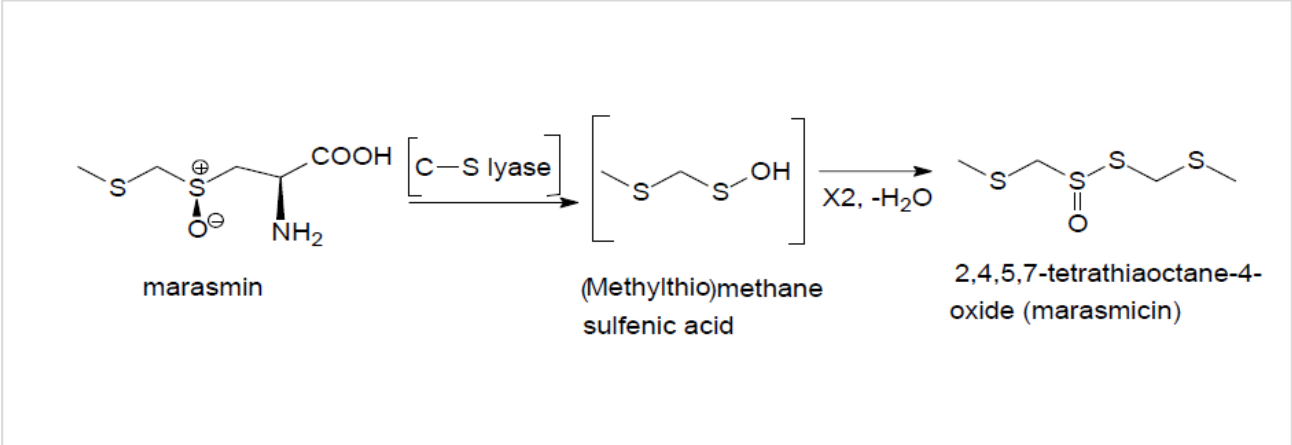

Figure 8: Formation of marasmicin. Adapted from Van Wyk *et al.* (2013).

2.8. Phytochemical screening

Phytochemicals are bioactive non-nutrient and biologically active compounds present in medicinal plants which contain a broad spectrum of chemical structures and protective/disease preventative properties (Peteros *et al.* 2010; Yadav *et al.* 2014; Shalini *et al.* 2017). Phytochemicals are primary and secondary compounds with chlorophyll, proteins, common sugars included in the primary constituents while terpenoids, alkaloids and phenolic compounds included as the secondary compounds (Krishnaiah *et al.* 2007). The secretion of these compounds varies from plant to plant; some produce more while others produce very minimal quantities and they could be harmful or helpful to the plant (Tariq *et al.* 2013). Plants produce secondary metabolites as part of their defence against various pathogenic microbes (Phan *et al.* 2001). Secondary metabolites are known to be chemically and taxonomically diverse compounds with obscure functions (Trease *et al.* 1978). Thus, conducting preliminary phytochemical screening of plants is an important aspect in determining the chemical constituents in plant materials. Preliminary phytochemical screening of plants is also necessary for the discovery and development of novel therapeutic agents with improved efficacy (Chopra *et al.* 1969; Bharath Kumar *et al.* 2014).

Despite the ancient use and preliminary findings about therapeutic benefits of medicinal plants, some of their constituents may be potentially cytotoxic, genotoxic, mutagenic, carcinogenic, or teratogenic (Gadano *et al.* 2006). Paracelsus (‘The father of toxicology’) reported that all compounds have the capacity to be poisonous depending upon dosage (Rozman *et al.* 2001).

This then raises a need to conduct toxicity tests to affirm the safety thresholds and efficiency of all new potential chemotherapeutics (Simaan 2009).

2.9. Cytotoxicity

Cytotoxicity is defined as the cell-killing property of a chemical compound like food, cosmetic, or pharmaceutical or rather a mediator like a cytotoxic T cell (Roche Diagnostics GmbH 2008). Compounds or treatments are regarded to be cytotoxic if they prevent cellular attachment, cause dramatic morphological changes, adversely affect replication rate, or lead to a reduction in overall viability (Horvath 1980). There are different factors that can cause cytotoxicity, among which are different types of agents, including drugs, pathogens, immune cells and external stress factors such as heat. There are different mechanisms of cell death namely; necrosis which is known as the "accidental" cell death that occurs when cells are exposed to a serious physical or chemical insult; apoptosis known as the "normal" cell death that removes unwanted or useless cells; autophagic cell death, which causes massive accumulation of double-membrane containing vacuoles known as autophagosomes and necroptosis which is a newly discovered pathway (Roche Diagnostics GmbH 2008). Morphological abnormalities in cells include nuclear fragmentation, cell shrinkage, membrane blebbing or loss of membrane integrity, leakage of cell content as well as swollen nuclei (O'Brien 2014).

2.10. Genotoxicity

Shah (2008) defined genotoxicity as a destructive effect on a cell's genetic material (DNA, RNA) thus affecting its integrity. Any substance that has the property of genotoxicity is known as a genotoxin. Genotoxins could be due to chemicals and radiation and can be carcinogens (cancer-causing agents) mutagens (mutation-causing agents) or teratogens (birth defect-causing agents). Genotoxicity has been reported to lead to mutations in various cells and other body systems. There are different genotoxicity tests that have been used to identify gene mutations, chromosome changes and alterations in the DNA sequence. These tests are also essential for the production of new drugs and for the validity of medicinal plants as potential chemotherapeutic agents. Genotoxicity assays can be conducted in various species including whole animals, plants, microorganisms and mammalian cells (Oliveira *et al.* 2010). There are currently about 24 genotoxicity assays that are used to assess the genotoxicity of herbal extracts (Sponchiado *et al.* 2016).

Different genotoxicity assays have been conducted to determine the potential toxic effects of *Tulbaghia* species. Van Huyssteen *et al.* (2011) observed cytotoxicity at 62.5 and 125 µg/ml

from the ethanol extracts (whole plant material) of *T. violacea* on Chang liver cells. This was followed by Olorunnisola *et al.* (2012) who used the Brine Shrimp Lethality test (BSLT), and reported that the oil extract from the roots of *T. violacea* was cytotoxic and that the level of toxicity depended on the concentration used. The study concluded that the significant lethality of the oil extracts (LC_{50} value less than 100 µg/ml) against brine shrimp *nauplii* might have been due to the presence of polysulfides which have been implicated as cytotoxic agents with potential anticancer, antimicrobial and antifungal activities. Using the bacterial reverse mutation (Ames) assay, Resende *et al.* (2012) reported that Kaemferol which is one of the compounds that has been isolated from *Tulbaghia*, showed mutagenicity towards *Salmonella typhimurium* strains TA98, TA100 and TA102.

Although the above studies appear to have demonstrated toxicity of some of the *Tulbaghia* species, other similar studies have disagreed with such conclusions. Ncube *et al.* (2011b) reported that the leaves and flowers of *T. violacea* are edible as vegetables. Using the Ames and VITOTOX tests Elgorashi *et al.* (2003) reported that these parts (leaves and flowers) are non-toxic. Soyingbe *et al.* (2013) investigated the acute and sub-chronic toxicity of methanolic extract of *T. violacea* roots in wista rats and reported that a single oral dose of 5 g/kg had no significant effect on their behaviour and did not cause mortality within 14 days of observation. They also reported that the essential oil of *T. violacea* had low (1218 and 1641 µg/ml) cytotoxicity levels against HEK293 and HepG2 cell lines. With the current disputes in research regarding the potential safety and toxicity of *Tulbaghia* species, there is thus a need to conduct different toxicity assays. For the sake of this study, only a few relevant assays will be discussed.

2.10.1. *Allium cepa* assay

Allium cepa (onion) is one of the higher plants that has been recognized as an excellent genetic model to detect environmental mutagens and is frequently used in monitoring studies. *Allium cepa* has been used for the evaluation of DNA damages, such as chromosome aberrations and disturbances in the mitotic cycle. The use *of A. cepa* as a test system to detect mutagens is a procedure that has been used for decades and is still employed today to assess a great number of chemical agents. Some of the advantages of using the *A. cepa* assay are that it is cost effective and it is easily handled (Rank 2003; Leme *et al.* 2009). The assay has been widely used to assess the impacts caused by xenobiotics, characterizing an important tool for environmental monitoring studies and has advantages over other short-term tests that require previous preparations of tested samples, as well as the addition of an exogenous metabolite (Leme *et al.* 2009). Figure 9 is an illustration of an *A. cepa* experimental setup.

Figure 9: Experimental setup of the onions grown in distilled water before treatments.

The *A. cepa* assay also enables the evaluation of different genetic endpoints. These endpoints include, chromosome aberrations (CA) which has been the one most used to detect genotoxicity along the years. The Mitotic Index (MI) which is used as a parameter to assess cytotoxicity levels (Fernandes *et al.* 2007). Nuclear Abnormalities (NA) are characterized by morphological alterations in the interphasic nuclei resulting from the agents tested (Fernandes *et al.* 2007; Migid *et al.* 2007; Caritá *et al.* 2008; Leme *et al.* 2008). Lastly, Micronucleus (MN) assay which has been reported as the most effective and simplest endpoint to analyze the mutagenic effects promoted by chemicals (Ribeiro 2003). The *A. cepa* assay has been widely used by researchers mainly as a bioindicator of environmental pollution (Bagatini *et al.* 2009; Leme *et al.* 2009), testing crude extracts of cyanobacteria (Laughinghouse 2007), as well as to evaluate the genotoxic potential of medicinal plants (Camparoto *et al.* 2002; Knoll *et al.* 2006; Fachinetto *et al.* 2007; Lubini *et al.* 2008; Fachinetto *et al.* 2009). A typical example of the experimental design used for the *Allium cepa* assay is illustrated in Figure 10.

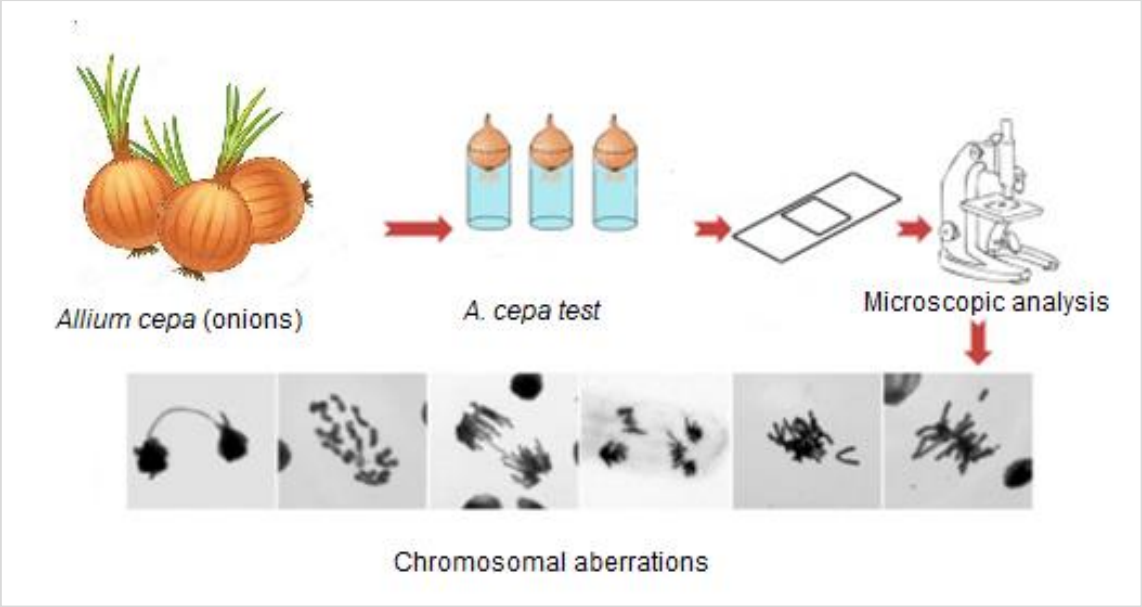

Figure 10: Experimental design for the *Allium cepa* assay. Adapted from Neves *et al.* (2016).

2.10.2. MTT cell proliferation assay

The MTT cell proliferation and variability assay is a basic colometric *in vitro* assay used to assess cytotoxicity, cell viability, and proliferation studies in cell biology or when metabolic events lead to apoptosis or necrosis, and a reduction in cell viability (Freimoser *et al.* 1999; Berridge *et al.* 2005; van Meerloo *et al.* 2011). This procedure was described by Mosmann (1983) to be timesaving, simple, reliable and cost effective when compared to other conventional hemocytometer counting methods. The yellow tetrazolium salts and MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) are reduced by the respiratory chain in metabolically active cells, in part by the action of dehydrogenase enzymes, to generate reducing equivalents such as NADH and NADPH (Figure 11). The resulting intracellular non-water-soluble violet formazan crystals can be solubilized and quantified by spectrophotometric means to represent an estimation for the number of mitochondria (Altman 1976; Denizot *et al.* 1986). The results obtained from the MTT Cell Proliferation Assay can be manipulated in cell biology, immunology, and toxicology studies (Sieuwerts *et al.* 1995).

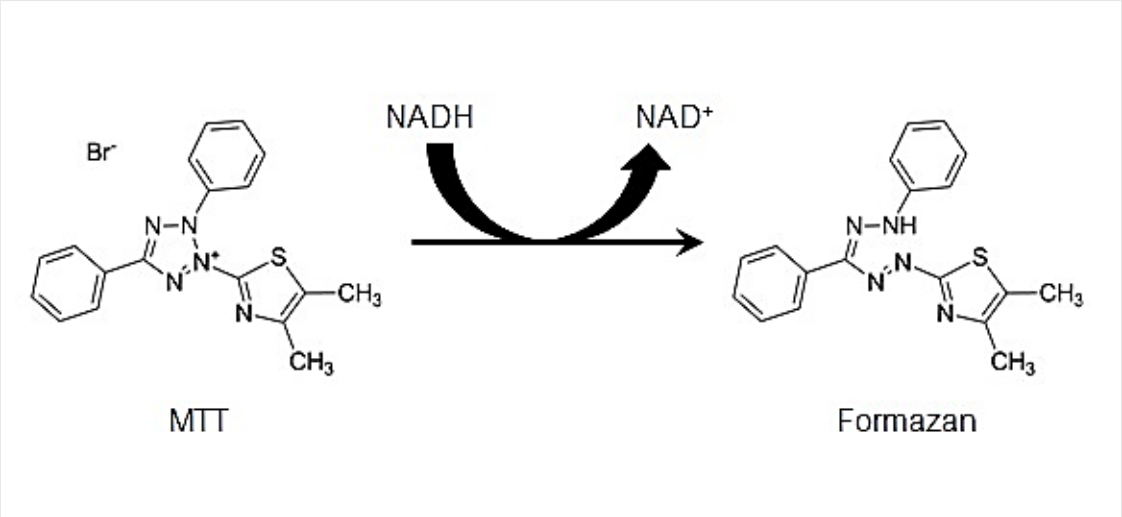

Figure 11: Structures of MTT and coloured formazan product (Riss *et al.* 2004).

2.11. Factorial design

In this study, a factorial design was used to assess the cytotoxic potential of *T. violacea* plant extracts on cells. The use of design of experiment (DOE) for statistical analysis enables a significant reduction in the total number of experiments while the quality and the standard of the experiment remains unchanged. Employing the traditional one factor at a time approach makes it difficult to observe the optimum value of the working parameters as no interaction among them is considered. The DOE in this case offers a solution by studying the effect of variables and their responses while using minimum number of experiments (Montgomery 2001; Silva *et al.* 2011). Some studies involve the evaluation of more than one factor at different levels for each of these factors. These studies involve a full investigation of all possible combinations of the levels of the factors that should be implemented (AlcheikhHamdon *et al.* 2015). A factorial design is the best way to analyse and conclude on which of the experimental factors are the most important to further investigate and also to evaluate which factors do not have a significant effect on the experimental results (Ferreira *et al.* 2007). For an experiment involving many variables, factorial design experiments are able to screen significantly important factors from the less important once. The main effect is defined as the change in (average) response produced by a change in the (average) level of the factor. As an example, consider an experiment including two factors (A and B) each at two levels (low (−1) and high (+1)), the main effect of A is the difference between the average response value at the low level of factor A (Y_A-) and the response at the high level of factor A (Y_A+). The interaction of the independent factors occurs as a result of one factor's response levels depending on the levels of the other factor. The interaction effect for the different factors

can be used to plot a graph for the response data against factor A for example for both levels of factor B. A non-significant interaction is indicated by parallel lines whereas, non-parallel lines are an indication of an interaction between the two factors. For a 2^3 factorial designs, three factors of interest, each at two levels (low (−1) and high (+1)) are evaluated (AlcheikhHamdon *et al.* 2015).

2.12. Conclusion

There is currently an increase in the development of physical and chemical agents used to facilitate human life. However, some of these agents pose health risks due to their potential toxic effects on living organisms. For centuries, medicinal plants have been used in folklore medicine as the basic material for alternative medicine due to their medicinal properties on different organisms. Regardless of their extensive use and therapeutic advantages, the use of medicinal plants is not without risk as some of their components may be potentially mutagenic, carcinogenic, or teratogenic (Gadano *et al.* 2006). With the current disputes in research regarding the safety and toxicity of *T. violacea,* there is thus a need to employ toxicity assays with regard to quality, safety and efficiency (Simaan 2009; Jäger *et al.* 2012)

CHAPTER 3

3. MATERIALS AND METHODS

3.1. Introduction

This chapter reports on how the study was carried out from the research design, collection and preparation of the *T. violacea* plant parts powdered materials, phytochemical screening from 70% ethanol and water extracts, MTT cell proliferation assay from the ethanol and water extracts and *Allium cepa* assay from the water extracts.

3.2. Research design

Figure 12 illustrates the research design that was followed to carry out the study.

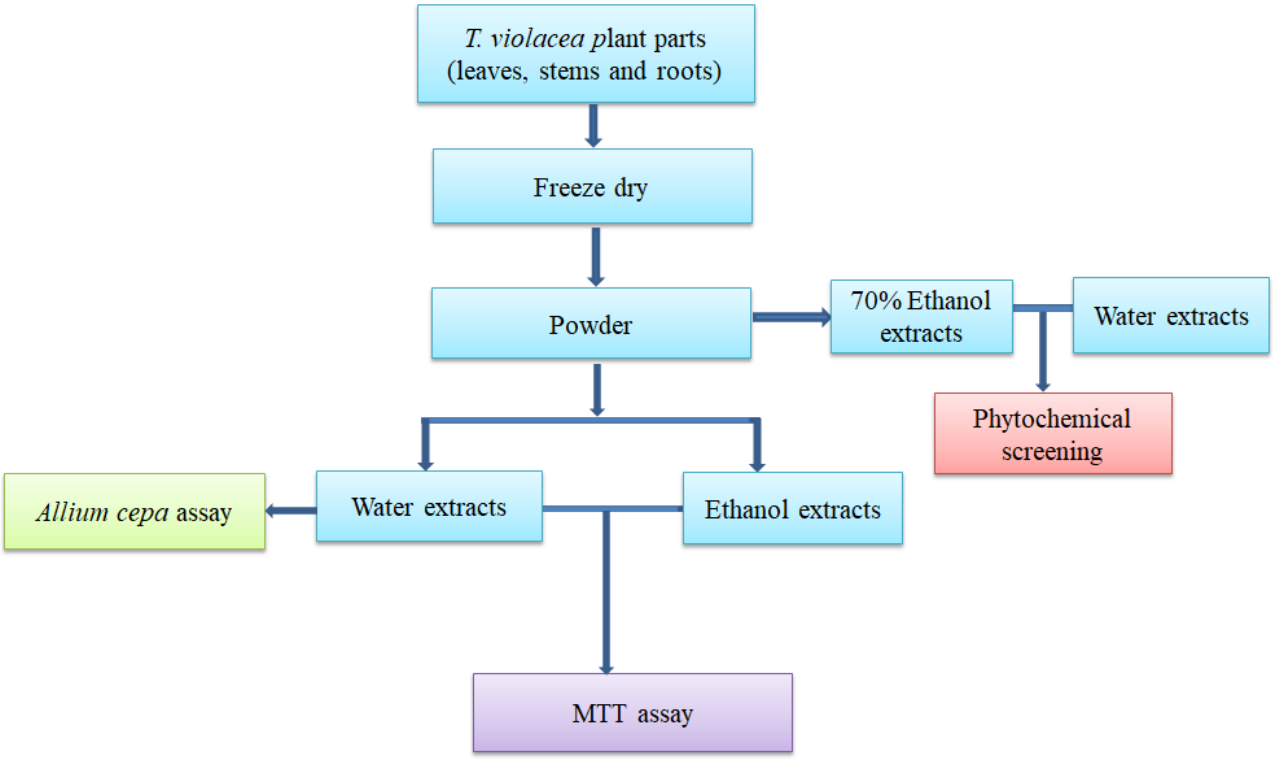

Figure 12: Research design of the study

3.3. Plant collection

Tulbaghia violacea was collected from Vanderbijlpark, South Africa and grown inside a greenhouse at the Vaal University of Technology. Identification of this plant was done with the assitance of Professor Stefan Siebert, a botanist at AP Goosen Herbarium, North-West University where a unique voucher specimen number ST0008 was deposited.

3.4. Preliminary phytochemical screening

3.4.1. Preparation of plant extracts

Five grams of the plant samples (leaves, stem, and roots) were extracted separately with distilled water and 70% ethanol by maceration (24 h for each solvent) with constant shaking. The homogenates were then filtered through Whatman® filter paper (0.45 µm pore size) and the extracts (0.05 g/ml) were all stored at 4ºC.

3.4.2. Reagents and chemicals

- Gallic acid and Quercetin solution: Gallic acid and Quercetin 10 mg were accurately weighed into 10 ml volumetric flasks and dissolved in 10ml methanol. The solutions were each made up to 10 ml with the same solvent (1 mg/ml).
- Wagner's reagent: 2 g of iodine and 6 g of KI were dissolved in 100 ml of distilled water.
- Mayer's reagent: Solution A: 0.355 g of mercuric chloride was dissolved in 60 ml of distilled water. Solution B: 5.0 g of potassium iodide was dissolved in 20 ml of distilled water. Both solutions were mixed and the volume was made up to 100 ml with distilled water.
- Fehling's solution A: 6.3 g of copper sulphate crystals were dissolved in distilled water and made up to 100 ml.
- Fehling's solution B: 35.2 g of potassium sodium tartrate (Rochelle Salt) and 15.4 g of sodium hydroxide were dissolved in distilled water and made up to 100 ml.
- Dragendorff's reagent: Solution A: 1.7 g of basic bismuth nitrate and 20 g of tartaric acid were dissolved in 80 ml of distilled water. Solution B: 16 g of potassium iodide was dissolved in 40 ml of distilled water. Both solutions (A and B) were mixed in 1:1 ratio.
- Tween 80, methanol, ethanol, glacial acetic acid, sulphuric acid, isoamyl alcohol, nitric acid 2N HCl, 1% HCl, 2N Ammonia, 70% Ethanol, 10% NaOH, Ferric chloride (5% and 10%), Folin-Ciocalteu's phenol reagent, 7.5% Sodium carbonate, 20 g/l Aluminum chloride and chloroform

3.4.3. Qualitative Phytochemical Analysis

All the extracts (0.05 g/ml) were subjected to preliminary phytochemical screening following standard methods (Harborne 1973; Evans 2002; Godghate *et al.* 2012) for detection of the following constituents.

Steroids

Five milliliters of chloroform and 5 ml of H_2O_4 were added to 500 µl of the prepared plant extracts. The presence of steroids was indicated by a color change from violet to blue or green

or a ring of blue/green or if the upper layer turns red and the sulphuric layer was yellow with a green fluorescence.

Saponins

About 3 ml of plant extracts were added to 3 ml of distilled water and shaken vigorously. The formation of a stable persistent froth was taken as a positive test for saponins.

Alkaloids

Approximately 3 ml of extracts were added to 3 ml of 1% HCl and heated for 20 min. The mixtures were then cooled and used to perform the following tests:

1. *Mayer's test:* To the filtrate in test tube I, 1 ml of Mayer's reagent was added drop by drop. The formation of a greenish colored or cream precipitate indicated the presence of alkaloids.
2. *Dragendoff's test:* To the filtrate in test tube II, 1 ml of Dragendoff's reagent was added drop by drop. The formation of a reddish-brown precipitate indicated the presence of alkaloids.
3. *Wagner's test:* To the filtrate in tube III, 1 ml of Wagner's reagent was added drop by drop. The formation of a reddish-brown precipitate indicated the presence of alkaloids.

Protein

Xanthoproteic test: A few drops of nitric acid were added to 2 ml of plant extracts and a color change to yellow was observed.

Anthocyanin

Approximately 2 ml of the prepared plant extracts were added to 2 ml of 2N HCl and ammonia. The appearance of a pink red coloration that turned blue violet indicated the presence of anthocyanin.

Coumarin

About 3 ml of 10% NaOH were added to 2 ml of plant extracts. The formation of a yellow color was an indication for the presence of coumarins.

Carbohydrates

Fehling test: Two milliliters of each plant extract were hydrolyzed with dilute HCl, neutralized with alkali, and then heated with Fehling's solution A and B. The formation of a red precipitate was an indication for the presence of a reducing sugar.

Flavonoid

Alkaline reagent test: Three milliliters of plant extract was treated with 1 ml of 10% NaOH solution. The formation of an intense yellow color was an indication of the presence of flavonoids.

Leucoanthocyanins

Approximately 5 ml of isoamyl alcohol were added to 5 ml of plant extracts. The appearance of a red upper layer indicated the presence of leucoanthocyanin.

Cardiac Glycosides

Keller-Killani Test: Two milliliters of plant extract were treated with 2 ml glacial acetic acid containing a drop of $FeCl_3$. A brown colored ring or brown-violet under a brown greenish layer indicated the presence of cardiac glycosides.

Phlobatannins

Two milliliters of 1% HCl were added to 3 ml of plant extracts and boiled. The deposition of a red precipitate was taken as evidence for the presence of phlobatannins.

Terpenoids

Approximately 2 ml of chloroform and 3 ml of H_2SO_4 were added to 5 ml of plant extracts. A reddish-brown coloration was taken as positive test for terpenoids.

Test for phenols and tannins

Ferric chloride test: Two milliliters of 5% solution of $FeCl_3$ were added to 1 ml crude extracts. A black or blue-green color indicated the presence of tannins and phenols.

3.4.4. Quantitative Phytochemical Analysis

3.4.4.1. Determination of total phenolic content (TPC)

The concentration of TPC in all the plant extracts was measured using a UV spectrophotometer, based on oxidation/reduction reaction (Škerget *et al.* 2005) using Folin-Ciocalteu reagent (Annals of Applied Statistics (AOAS) 1990). To 500 µl of diluted extracts (10 mg in 10 ml solvent), 2.5 ml of Folin-Ciocalteu reagent (diluted 10 times with distilled water) and 2 ml of Na_2CO_3 (7.5%) were added. The samples were incubated for 5 min at 50ºC and then cooled. Distilled water (500 µl) was used as a negative control for the experiment. The absorbance of the standard gallic acid solution (0.5 mg/ml) was measured using 500 µl of 50, 100, 150, 200, 250 and 300 µg/ml methanolic gallic acid solutions. All determinations were performed in triplicate and a standard curve was established. The total phenol value was obtained from the

regression equation: y = 0.0106*x* + 0.1246 and expressed as mg/g gallic acid equivalent using the formula, C = cV/M; where C = total content of phenolic compounds in mg/g GAE, c = the concentration of gallic acid (µg/ml) established from the calibration curve, V = volume of extract (0.5 ml) and m = the weight of pure plant methanolic extract (0.05 g).

3.4.4.2. Determination of total flavonoid content (TFC)

Total flavonoid content was measured according to the Aluminium Chloride colorimetric method (Ordonez *et al.* 2006) with some modification. A 1.5 ml aliquot of 20 g/l $AlCl_3$ ethanol solution was added to 500 µl of the plant extracts (5 g in 100 solvent) and distilled (500 µl) water was used as the negative control. The extracts were evaluated at a final concentration of 0.05 g/ml. The absorbance of the standard quercetin solutions was recorded after 60 min at 420 nm using 20, 40, 60, 80 and 100 µg/ml methanolic quercetin solutions and a calibration curve was established. The total flavonoid content expressed as quercetin equivalent (QE) was calculated based on the calibration curve using the following equation: y = 0.0175*x* – 0.0061, where *x* is the absorbance and y is the concentration (mg QE) of the methanolic quercetin solutions.

3.5. MTT cell proliferation assay

3.5.1. Preparation of plant crude extracts

Tulbaghia violacea was collected from the green house and gently washed with tap water to remove the dust. The leaves, stems and roots were then separated from each other, cut into small pieces, frozen (-20ºC), lyophilized and eventually pulverized into a fine powder. Crude ethanol extracts of the different parts of the plant were prepared by mixing 5 g of the pulverized plant material with 500 ml of 100% ethanol. The mixture was then macerated for 24 hours with constant shaking and then filtered through a Whatman® filter paper (0.45 µm). The filtrates were then evaporated in a fume hood until they were dry. A stock solution of each dried crude ethanol extract at a concentration of 100 mg/ml was prepared using ethanol and stored at -20ºC in opaque vessels until when they were required. The crude water extracts from the different parts of the plant were prepared by mixing 10 g of the pulverized plant material with 200 ml of distilled water. The mixture was then boiled for 10 minutes and allowed to cool down. Thereafter, the mixture was filtered through a Whatman® filter paper (0.45 µm). The resultant filtrate was then frozen and lyophilized. A stock solution of each lyophilized crude water extract at a concentration of 10 mg/ml was prepared using water and stored at -20ºC in opaque vessels until when they were required.

3.5.2. Cell culture

A murine macrophage cell line RAW 264.7 and a skeletal muscle (myoblasts or C2C12) cell line (Cellonex, Johannesburg, South Africa) were each separately grown and maintained in DMEM supplemented with 10% FBS (fetal bovine serum) and Penicillin-streptomycin (10,000U/ml penicillin G and 10 mg/ml streptomycin) at 37ºC in 5 % CO_2 incubator (ESCO, Horsham, PA). The culture medium was replaced with fresh medium every 2-3 days until cells were 80% confluent. The cells were trypsinised and plated in a 96-well plate at a cell density of 2.27 x 10^6 cells/ml before assay.

3.5.3. The 2^3 Factorial design

Three 2^3 full factorial designs were constructed to investigate the effect that the crude extracts obtained from the three different parts (leaves, stems and roots) of the plant had on the percentage cell viability of the RAW 264.7 and C2C12 cells *in vitro*. For the three parts of the plant, three independent variables were selected using two levels for each variable (high (+) and low (-)) as shown in Table 4. The crude plant extracts were prepared and used to stimulate the cells at the concentrations shown in Table 4 in culture media. Fresh basic cell culture media, 50% H_2O_2 and basic cell culture media without cells were used as controls. The experimental runs were randomly executed and the response (percentage cell viability (%)) collected. By randomizing the experimental runs, the effect of extraneous factors or errors that may have been present were "averaged out" thus preventing the violation of independence (Montgomery 2013). To minimize external variation, experimental runs were done in triplicate

3.5.4. Morphological studies and MTT cell percentage cell viability assay

After 24 and 48 h of exposure, images of the cells were captured using an inverted microscope (IX53, OLYMPUS, Tokyo, Japan). The percentage cell viability was determined using the MTT assay (INVITROGEN, Eugene, OR**)** following the manufacturer's instructions with slight modification. In brief, the cells were incubated for 24 and 48 h after which the media was removed and replaced with 100 µl of fresh media. Then 0.5 mg/ml of MTT solution was added into each well and the plate was incubated for 4 h. All but 25 µl of media was removed from the wells and then 50 µl of dimethyl sulfoxide (DMSO) was added into each well. The cell culture plates were then incubated at 37°C for 10 min and the absorbance was read at 560 nm using Glomax Multi-detection system plate reader (PROMEGA, Sunnyvale, CA). The percentage cell viability was calculated using the equation 1 below:

$$Cell\ viability\ (\%) = \frac{OD(optimal\ density)_{560}(Treated\ samples)}{OD_{560}(Untreated\ control)} \times 100 \quad (1)$$

3.5.5. Statistical analysis

After data collection, the statistical significance of the different independent variables on the percentage cell viability was investigated using the analysis of variance (ANOVA) methodology as described by Terblanche *et al.* (2017). ANOVA was conducted using Design Expert software (version 6.0.6, Stat – Ease, Minneapolis, MN) set to a significant level of $p < 0.05$. Regression models for the leaves, stems and roots were then used to evaluate the direction and magnitude of the relationship between a variable and percentage cell viability. The regression formula illustrated in equation 2 below was used to predict the effect of the different variables on percentage cell viability.

$$y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{12} AC + \beta_{13} AB + \beta_{23} BC + \beta_{123} ABC + \varepsilon \quad (2)$$

In equation 2, y represents the percentage cell viability, whereas βn represents the regression coefficient associated with variable *n*. The values for *n* in equation 2 were obtained from the analysis of the experimental results. The letters A (concentration), B (time) and C (extracts) are the main variables, AB (concentration x time), AC (concentration x extracts) and BC (time x extracts) represent the two-way interactions whereas ABC (concentration x time x extracts) is an illustration of the three-way interactions and ε the experimental error. The adequacy of regression models obtained in this study were examined by ANOVA, coefficient of determination (R^2), adjusted R^2 and predicted R^2. R^2, which is known to have a value from 0 to 1, was used to measure the global fit of a model according to the formula shown in equation 3 where SS_T and SS_{within} within are the total sum of squares and error sum of squares, respectively.

$$R^2 = \frac{SS_T - SS_{within}}{SS_T} = 1 - \left(\frac{SS_{within}}{SS_T}\right) \quad (3)$$

In this study, the value of the predicted R^2 as opposed to the calculated and adjusted R^2 values were used to determine the quality of the model and to confirm that the final regression model did not over-fit the observed data points. The resultant model was used to predict the percentage cell viability response of the cells for concentrations 25, 50, 100, 250 and 500 µg/ml using the Design Expert 6.0.6 software. The predicted responses were then used in Graphpad Prims 6 software (GraphPad software, San Diego, CA) to calculate the IC_{50} and 95% CI.

3.6. *Allium cepa* assay

3.6.1. Preparation of plant extracts

Previously prepared water crude extracts of 10 mg/ml (0.1 g in 10 ml) from the leaves, stems and roots of *T. violacea*, were reconstituted by dissolving in distilled water to 100, 250, 500 and 1000 µg/ml.

3.6.2. Solutions required

- Acetocarmine: 0.5 g of carmine was weighed and placed in 55 ml of distilled water in a 200 ml flask and boiled. To the mixture, 45 ml of glacial acetic acid was added. A cotton wool was then pugged on the flask and boiled again. The solution was then cooled, filtered and stored in a dark bottle in the fridge (2- 4ºC).
- Ethanol-glacial acetic acid (3:1): 30 ml of ethanol was added to 10 ml of glacial acetic acid. The solution was prepared fresh each time before use.

3.6.3. Pre-treatment

The *Allium cepa* L. (onion) bulbs were grown in distilled water at room temperature for 2–3 days. When the roots were 2–6 cm in length, the bulbs were treated with different concentrations of the crude extracts (100, 250, 500 and 1000 µg/ml) for 24 h. Another set of the onions were placed in ethidium bromide (100, 250, 500 and 1000 µg/m) to serve as the positive control while a set of *A. cepa* was growing in distilled water to serve as the negative control. The solutions were changed daily and root growth was measured. The onion with the poorest growth was excluded from every concentration and the remaining three (3) onions were prepared for microscopy (Oyedare *et al.* 2009; Cuyacot *et al.* 2014).

3.6.4. Slides Preparation

For each bulb, five root tips at a length of 10 mm were harvested and fixed in ethanol/glacial acetic acid solution 3:1 (v/v) for 10 minutes. After fixation, the root tips were washed a few times with distilled water. They were then hydrolyzed with 1 N HCl at 60-70 ºC for 5 minutes. After hydrolysis, the roots were washed a few times with distilled water. Then, about 1–2 mm of the root tips were cut and placed on a glass slide. The rest of the materials were removed from the slide and the excess liquid was sucked up using blotting paper. A small drop of acetocarmine was placed on the root tip and left for 5-10 minutes. The coverslip was used to squash the root tips to form a smear and excess stain was blotted out to exclude air bubbles and the sides of the slides were sealed with clear fingernail polish. Three (3) slides were prepared per bulb with a total of nine (9) slides per concentration.

3.6.5. Observation of slides

The slides were observed under the light microscope at 200x and 400x magnification. An Olypmus light microscope with a digital camera was used to get a clear image of the chromosome aberrations. Photomicrographs (10 images per slide) were made and minimum of 1000 cells per slide were analysed (nine slides were observed for each treatment). The mitotic index, micronucleus in interphase and chromosome aberrations in mitotic phases were calculated by examining and counting a minimum of 1000 cells per slide (nine slides were observed for each treatment). The experiment was replicated three times with three roots for each replicate, therefore, nine slides were prepared for each treatment group. The mitotic index, percentage of aberrant cells and the percentage micronucleus were obtained using equation 4, 5 and 6 below, respectively.

$$Mitotic\ index = \frac{Number\ of\ cells\ in\ mitosis}{Total\ number\ of\ cells}\ x\ 100 \quad (4)$$

$$\%\ Aberrant\ cells = \frac{Total\ number\ of\ aberrations}{Total\ number\ of\ cells}\ x\ 100 \quad (5)$$

$$\%\ Micronucleus = \frac{Total\ number\ of\ micronucleus}{Total\ number\ of\ cells\ in\ interphase}\ x\ 100 \quad (6)$$

3.6.6. ImageJ analysis

Images obtained from the light microscope were converted to 8-bit grayscale using ImageJ software (version 1.46r, Bethesda, MD). The thresholds of the images were then adjusted to obtain the best fit for different particle aggregates in each cell and the total number of cells was calculated. A total number of nine (9) slides for each concentration of plant extract (100, 250, 500 and 1000 µg/ml) was analysed. Ten (10) images from each slide were assessed amounting to a total of ninety (90) images per concentration of plant extract. The mean data for each concentration was used for further analysis.

3.6.7. Data Analysis

The mean and standard error of the mean (SEM) for each of the treatment groups were calculated. Data obtained from the microscopic and ImageJ analysis was analysed using the multiple *t*-test to determine the significant differences between treatment groups and the negative control ($p < 0.05$).

CHAPTER 4

4. RESULTS

4.1. Introduction

The chapter presents the results obtained from the preliminary phytochemical screening, the MTT cell proliferation assay and *Allium cepa* assay.

4.2. Preliminary phytochemical screening

The aim of the study was to screen for 14 phytocompounds using extracts obtained separately with water and 70% ethanol from the leaves, stem, and roots of *T. violacea. (Please note that water and 70% ethanol extracts were done separately for each of the plant organs).*

4.2.1. Qualitative Phytochemical Analysis and percentage yields

The yields obtained from the leaves, stem and roots with water ranged from 33.94 to 41.67% w/w, while that with ethanol ranged from 15.20 to 26.08% w/w (Table 2). The results of the phytochemical screening tests (strong, weak and negative) obtained from the water and 70% ethanol extracts of the leaves, stem, and roots of *T. violacea* are presented in Table 2.

Table 2: Results of preliminary phytochemical screening of water and 70% ethanol extracts from the leaves, stems, and roots of *Tulbaghia violacea*.

No	Compounds	Leaves		Stem		Roots	
		Water	Ethanol	Water	Ethanol	Water	Ethanol
1	Saponins	+ +	+	+	+	+ +	−
2	Flavonoids	+ +	+ +	+	+ +	+	+
3	Proteins	+	+ +	+	+	+	+
4	Coumarins	+ +	+ +	+ +	+ +	+ +	+
5	Cardiac glycoside	+	+ +	+ +	+ +	+ +	+ +
6	Terpenoids	+ +	+ +	+ +	+	+ +	+
7	Phlobatannins	−	−	−	−	−	−
8	Steroids	−	−	−	+ +	−	+
9	Phenols	+ +	+ +	+ +	+	+	−
10	Tannins	+ +	+ +	+ +	+	+	−
11	Carbohydrates	−	−	−	−	−	−
12	**Alkaloids**						
	• Mayer's reagent	−	−	−	−	−	−
	• Dragendorff's reagent	−	−	−	−	−	−
	• Wagner's reagent	−	−	−	−	−	−
13	Leucoanthocyanins	−	−	−	−	−	−
14	Anthocyanins	−	−	−	−	−	−
Percentage yields (% w/w)		33.94	26.08	41.67	22.33	39.44	15.20

+ + = Strong positive test, + = Weak positive test, − = Negative tests

4.2.2. Quantitative Phytochemical Analysis

The total phenolic and flavonoid content of the three parts of the plant are shown in Table 3. The amount of phytochemicals varied not only among the leaves, stem and roots but also depended on whether water or ethanol was used as the extractant. The highest phenolic content appeared in the leaves in the water and ethanol extracts followed by the stem and roots. The leaves had the highest total flavonoid content compared to the stem and roots. However, the ethanol extracts had a higher amount of flavonoid in the leaves compared to the water extracts. A similar phenomenon was observed for the water extracts.

Table 3: Phenolic and flavonoid contents of the water and 70% ethanol extracts from the leaves**,** stems, and roots of *T. violacea*.

Plant parts	Phenolic Content (mg of GAE/g of dry extract) ± SD		Flavonoid content (mg of QE/g of dry extract) ± SD	
	Water	Ethanol	Water	Ethanol
Leaves	3.59 ± 0.1*a*	0.98 ± 0.06*a*	0.47 ± 0.01*a*	0.66 ± 0.01*a*
Stem	2.38 ± 0.05*a*	0.34 ± 0.02*a*	0.027 ± 0.02*ab*	0.053 ± 0.01*ab*
Roots	1.91 ± 0.1*a*	0.15 ± 0.02*a*	0.025 ± 0.01*ac*	0.038 ± 0.01*ac*

Data represents the mean ± SD mg of Gallic acid equivalent per gram of dry weight (mg GAE/g) and Quercetin equivalent per gram of dry weight (mg QE/g) of the extracts, $n = 3$. Small letter *a* indicates statistically significant groups according to the *t*-test: Two-Sample Assuming Equal Variance ($p < 0.05$). Non-significant groups are represented by a*b* and *ac* ($p > 0.05$).

4.2.2.1. Total Phenolic Content

Table 3 shows the content of total phenol that were measured by the Folin-Ciocalteu reagent in terms of gallic acid equivalent (standard curve equation: $y = 0.0106x + 0.1246$, $R^2 = 0.9949$; Fig. 13). The total phenolic content for the water extracts was found to be 3.59, 2.38 and 1.91 mg/g in the leaf, stem and root extracts, respectively. In the 70% ethanol extracts, the total phenolic content was found to be 0.98, 0.34 and 0.15 mg/g in the leaf, stem and root extracts, respectively.

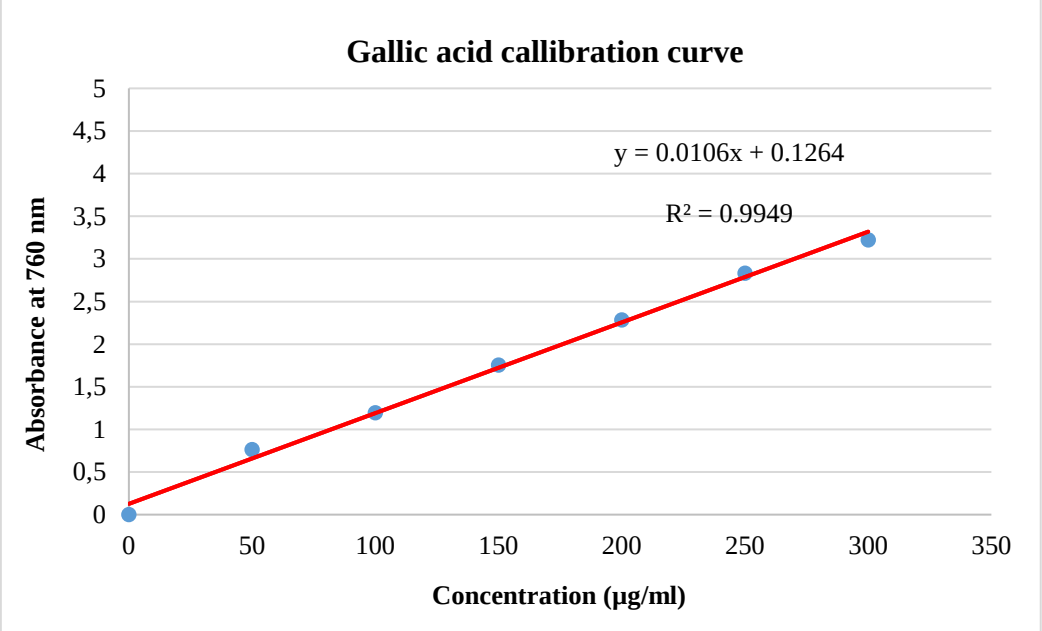

Figure 13: Standard calibration curve for Gallic acid.

4.2.2.2. Total Flavonoid Content

The flavonoid content of the water extracts in terms of quercetin equivalent (the standard curve equation: $y = 0.0175x - 0.0061$, $R^2 = 0.9892$; Fig. 14) were found to be 0.47, 0.027 and 0.025 mg/g for the leaf, stem and root extracts, respectively. In the 70% ethanol extracts, the flavonoid content was found to be 0.66, 0.053 and 0.038 mg/g for the leaf, stem, and root extracts, respectively.

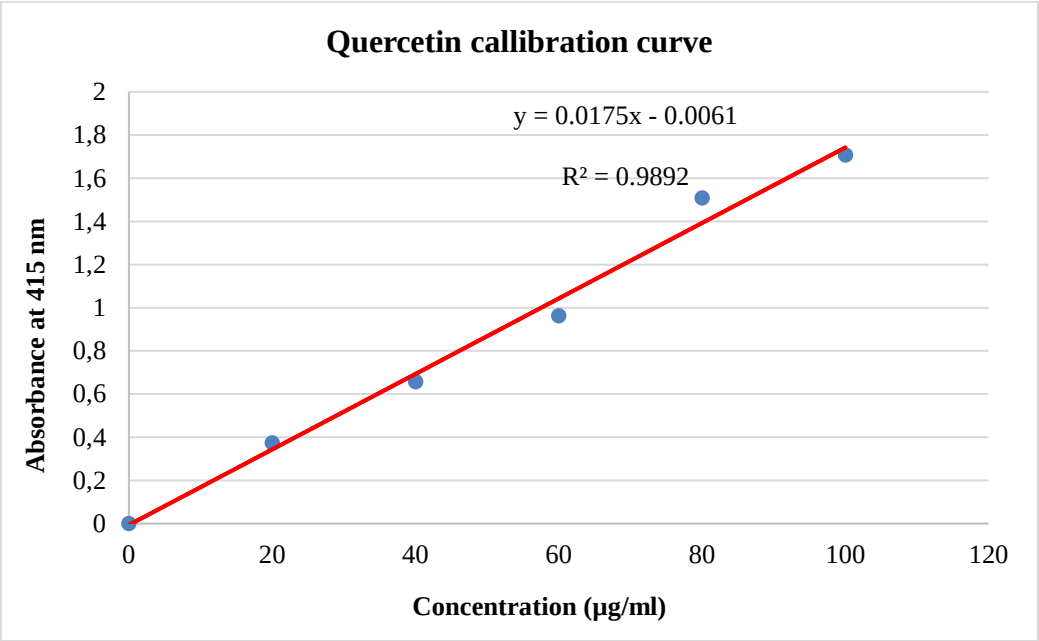

Figure 14: Standard calibration curve of Quercetin.

4.3.MTT cell proliferation assay – RAW 264.7

4.3.1. Experimental observations

This objective of this part if the study was to determine the effect of concentration (A), incubation time (B) and the type of extracts (C) on the percentage cell viability of a murine macrophage cell line *in vitro.* A 2^3 factorial design was used for the study and the experimental design showing the independent variables is illustrated in Table 4. For this study, the following letters were designated: the letters A (concentration), B (time) and C (extracts) are the main variables, AB (concentration x time), AC (concentration x extracts) and BC (time x extracts) represent the two-way interactions whereas ABC (concentration x time x extracts) is an illustration of the three-way interaction.

Table 4: Experimental design range and levels of independent variables to assess the effect of water and ethanol extracts of the leaves, stems and roots of *T. violacea* on the RAW 264.7 cell line.

Independent variables	Range and levels	
	Lower limits (−)	Upper limits (+)
A = Concentration (µg/ml)	10 µg/ml	1000 µg/ml
B = Time (hours)	24 h	48 h
C = Nature of the Extracts	Water	Ethanol

The analysis was conducted in triplicate resulting in 24 observations (mean was calculated resulting in 8 observations) for each plant organ. Table 5 illustrates the actual and predicted mean responses (% yield) per run ± SEM. Data analysis was conducted using Design Expert 6.0.6 software.

Table 5: A 2^3 full factorial design showing average runs in actual factors for independent variables.

							% Cell viability ± SEM					
							Actual			Predicted		
Run	A	B	C	A	B	C	Leaves	Stem	Roots	Leaves	Stem	Roots
01	−	−	−	10	24	Water	79.9±6.99	53.29±10.41	54.39±4.93	79.9±0.00	53.29±0.00	54.39±5.0E-15
02	+	−	−	1000	24	Water	33.06±8.46	12.86±4.42	33.3±11.21	33.06±0.00	12.86±0.00	33.3±0.00
03	−	+	−	10	48	Water	76.5±13.68	40.18±0.92	61.7±10.31	76.5±0.00	40.18±0.00	61.7±5.0E-15
04	+	+	−	1000	48	Water	61.75±7.89	3.38±2.23	27.64±2.97	61.75±0.00	3.38±3.1E-16	27.64±0.00
05	−	−	+	10	24	Ethanol	18.54±2.45	19.03±0.31	17.65±0.16	18.54±0.00	19.03±0.00	17.65±0.00
06	+	−	+	1000	24	Ethanol	2.11±2.11	4.06±1.89	4.93±2.44	2.11±0.00	4.06±0.00	4.93±0.00
07	−	+	+	10	48	Ethanol	30.27±6.07	31.59±0.85	44.88±13.4	30.27±0.00	31.59±0.00	44.88±0.00
08	+	+	+	1000	48	Ethanol	0.00±0.00	0.00±0.00	0.00±0.00	3.6E-14±0.00	1.78E-14±0.00	2.71E-14±2.2E-30

4.3.2. Half normal plots for the leaf, stem and root extracts

Factorial design analysis uses the half normal plot to choose significant and non-significant effects (Natrella 2010). The half normal plots data (Fig. 15) illustrated that for all the plant parts of *T. violacea* (leaves, stems and roots), the main effect of concentration (A) and extracts (C) had a significant effect on cell viability while the main effect of time (B) produced a subminimal effect. There were no common significant interactions among the *T. violacea* leaf, stem and root extracts.

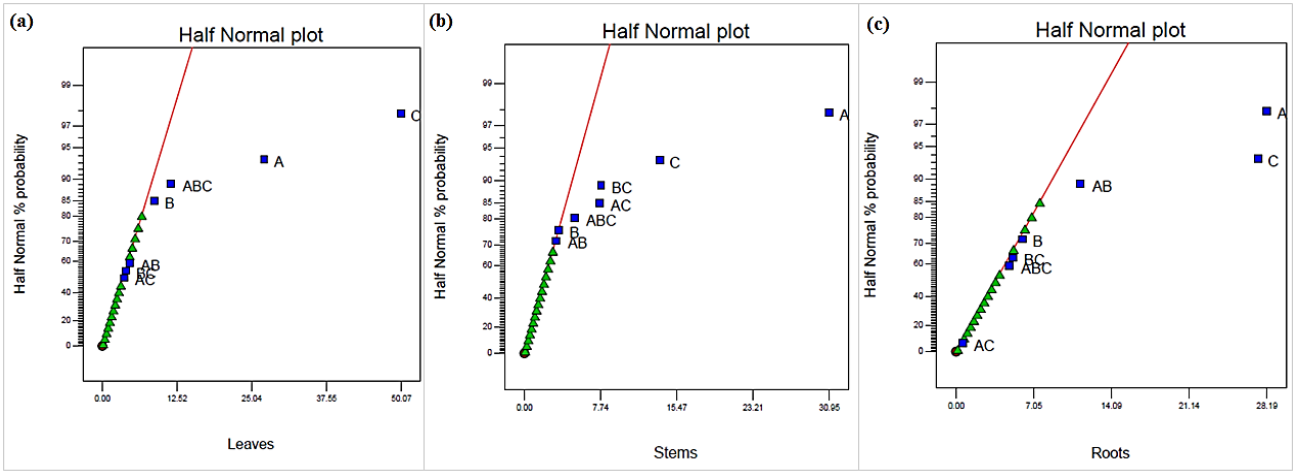

Figure 15: Half normal probability plots showing the effect (% cell viability) for (a) leaf (b) stem and (c) root extracts. The green triangles (▲) are noise effect estimates or 'pure error' and the blue (■) squares are factor estimates.

4.3.3. Crude leaf extracts

4.3.3.1. Main effects and Interaction plots

The main effect and interaction plots are lines resulting from the connection of the mean values from each treatment condition (AlcheikhHamdon *et al.* 2015). As illustrated in Fig. 16 (a, b, c), there were two main effects: concentration (A) and type of extract (C). An increase in each of these variables from lower levels (-) to higher levels (+) resulted in a decrease in the number of viable cells. Incubation time (B) had no effect on percentage cell viability as indicated by the lower gradient line plotted on the main effect plot in Fig. 16 (b). In the interaction plots (Figure 16 d, e, and f), the parallel lines indicate a non-significant interaction between any of the independent variables for crude extracts from the leaves of *T. violacea.*

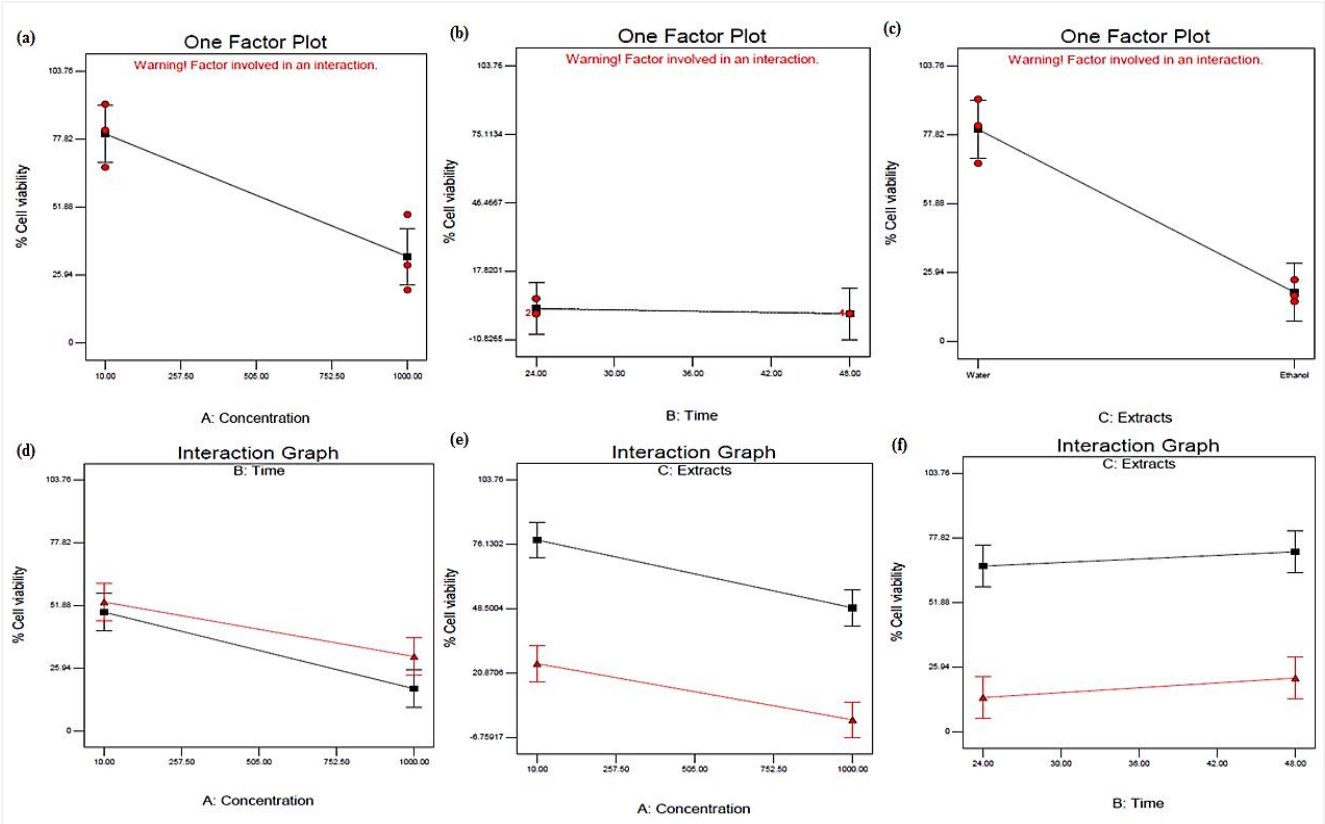

Figure 16: Interaction (a - f) plots on the effect (% cell viability) of the leaf crude extracts on RAW 264.7 cells.

4.3.3.2. Regression model analysis and ANOVA

The full regression model (Table 6) consisted of both the significant ($p < 0.05$ level) and non-significant model terms. The main effect of concentration (A) and extracts (C) were highly significant whereas the effect of time (B) was non-significant. All the two-way interactions AB, AC and BC were non-significant while the three-way interaction ABC was slightly significant.

Table 6: ANOVA report for the full regression model for the leaves of *T. violacea*.

Source	Sum of squares	Degree of freedom	Mean squares	F-values	P-value
Model	20989.26	7	2998.47	19.16	< 0.0001
A	4397.25	1	4397.25	28.10	< 0.0001
B	457.10	1	457.10	2.92	0.1068
C	15044.03	1	15044.03	96.13	< 0.0001
AB	124.76	1	124.76	0.80	0.3852
AC	83.25	1	83.25	0.53	0.4763
BC	92.12	1	92.12	0.59	0.4541
ABC	790.74	1	790.74	5.05	0.0390
Pure Error	2503.89	16	156.49		
Cor Total	23493.16	23			

SD = 11.13; R^2 = 0.7479; R^2(adj) = 0.7101; R^2 (pred) = 0.6370; Adeq precision = 12.854; Mean = 57.95

Equations based on the full regression model to predict the percentage cell viability of crude extracts from the leaves were developed (equation 4, 5 and 6).

$$\hat{y} = \beta_0 - \beta_1 A - \beta_2 B + \beta_{13} AB \quad (4)$$

Extracts -Water

Cell viability = 84.08714−0.079714*Concentration−0.15489*Time + 1.35017E-003* Concentration * Time (5)

Extracts - Ethanol

Cell viability = 6.83279 - 2.61279E-003*Concentration + 0.49457 * Time - 5.82492E-004 * Concentration * Time (6)

In equation 4, $\hat{y}$ represents the predicted percentage cell viability, A represents the concentration, B represent the incubation time, and AC represents the concentration-extract interaction. β_0, β_1, β_2, and β_{12} were the regression coefficients. β_0 was the mean value of responses of all experiments.

4.3.3.3. Percentage cell viability

The results for the MTT assay (Fig. 17) illustrated that for the leaf extracts, cell viability was dependent on both concentration and type of extract. The number of viable cells decreased as the concentration increased and when ethanol was used instead of water. However, incubation time had no significant effect on cell viability. At concentrations higher than 250 µg/ml, there was a significant decrease in the number of viable cells. The leaf ethanol extract was toxic to

the cells even at the lowest concentration of 10 µg/ml at both 24 and 48 h incubation periods (% cell viability < 50%).

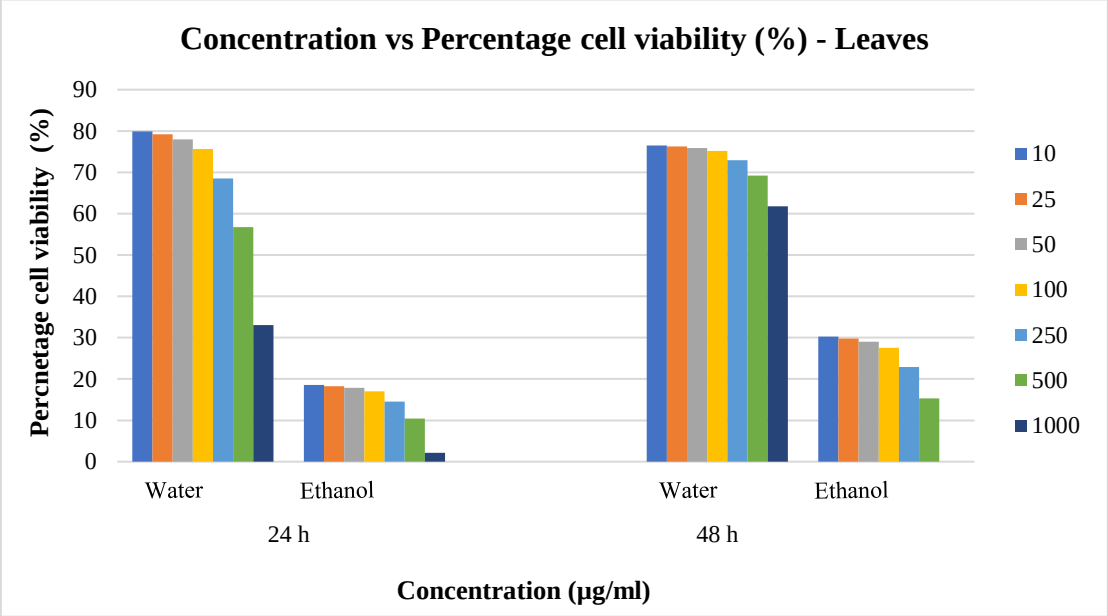

Figure 17: Cell viability (%) of RAW 264.7 cell line after treatment with various concentrations of water and ethanol extracts of the leaves of *T. violacea* for both 24 and 48 h.

4.3.3.4. Morphological characterization

The RAW 264.7 cells were treated with 10 and 1000 µg/ml of the water and ethanol leaf extracts of *T. violacea.* Fresh culture media was used as the negative control (untreated cells) and 50% H_2O_2 was used as the positive control. All treatments were incubated for both 24 and 48 h and the morphology of the cells was viewed under a Nikon microscope at 20x magnification (Fig. 18a – j). Untreated cells appeared smooth and well-rounded with some having a single pointy end showing no sign of abnormalities (Fig. 18a). However, for the cells treated with 50% H_2O_2, there was clumping, growth inhibition, shrinkage, vacuolisation with a dense irregular debris and detachment of cells from the culture flask (Fig. 18f). The cells were completely damaged forming a dense irregular debris-like material mainly in the cells treated with the ethanol extracts after 48 h incubation at both 10 (Fig. 18i) and 1000 (Fig. 18j) µg/ml.

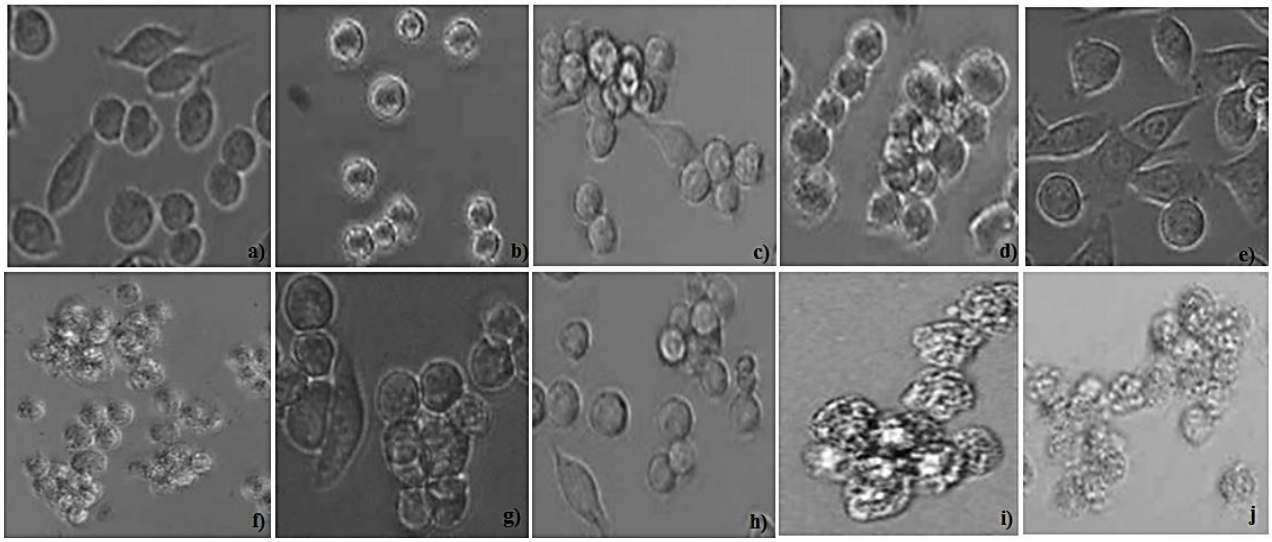

Figure 18: RAW 264.7 were treated with fresh culture media (negative control) (a) and 50% H_2O_2 (positive control) (f). The cells were also treated with 10 and 1000 µg/ml of the water leaf extract incubated for 24 h, (b and c) and 48 h (d and e), respectively. The ethanol extracts results after 24 h incubation for 10 and 1000 µg/ml were (g and h) and after 48 h (i and j), respectively. Magnification 20×.

4.3.4. Crude stem extracts

4.3.4.1. Main effects and Interaction plots

Similar to the leaf crude extracts, there were two main effects; concentration (A) and type of extract (C). An increase in each of these variables from lower levels (-) to higher levels (+) also resulted in a decrease in the number of viable cells. Incubation time (B) had no effect on percentage cell viability as indicated by the lower gradient line plotted on the main effect plot in Fig. 19 (b). The non-parallel lines (Fig. 19e and f) due to the interaction between AC (concentration x extracts) and BC (time x concentration) implies that there were strong two-way interactions between the main effects, concentration (A) and type of extract (C) as well as between time (B) and type of extracts (C).

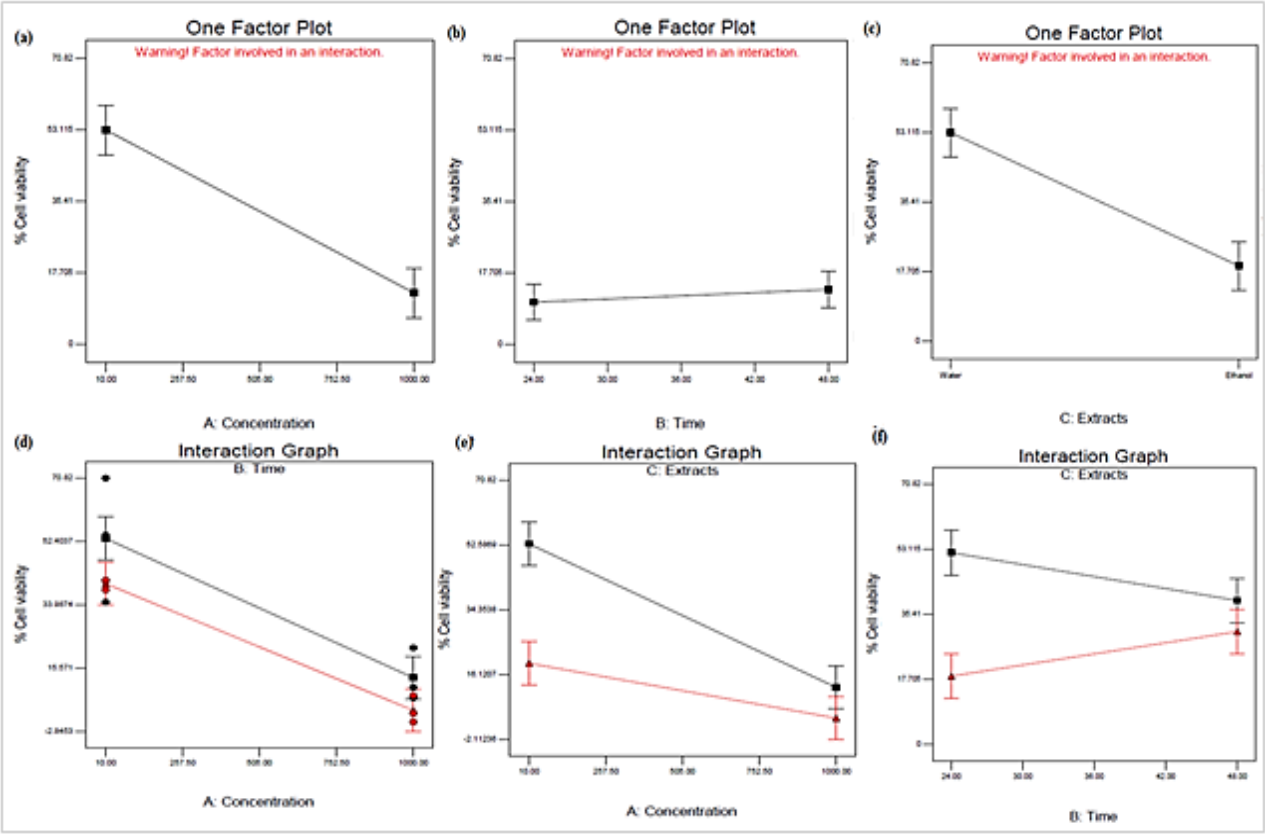

Figure 19: Interaction (a - f) plots on the effect (% cell viability) of the stems crude extracts on RAW 264.7cells.

4.3.4.2. Regression model analysis and ANOVA

The full regression model (Table 7) consisted of both the significant ($p < 0.05$ level) and non-significant model terms. Similar to the leaf extracts, the main effect of concentration (A) and extracts (C) were significant whereas the effect of time (B) was non-significant. For the two-way interactions, concentration x extract (AC) and time x extract (BC) were significant while concentration x time (AB) as well as the three-way interaction concentration x time x extract (ABC) were non-significant.

Table 7: ANOVA report for the full regression model for the stems of *T. violacea*.

Source	Sum of squares	Degree of freedom	Mean squares	F-values	P-value
Model	7888.25	7	1126.89	21.76	< 0.0001
A	5745.87	1	5745.87	110.93	< 0.0001
B	74.45	1	74.45	1.44	0.2480
C	1135.89	1	1135.89	21.93	0.0002
AB	63.21	1	63.21	1.22	0.2856
AC	352.74	1	352.74	6.81	0.0190
BC	362.32	1	362.32	6.99	0.0177
ABC	153.77	1	153.77	2.97	0.1042
Pure Error	828.75	16	51.80		
Cor Total	8717.00	23			

SD = 7.20; R^2 = 0.9049; R^2 (adj) = 0.8633; R^2 (pred) = 0.7861; Adeq precision = 12.825; Mean = 20.55

Equations based on the full regression model to predict the percentage cell viability of crude extracts from the stems were developed (equation 7 and 8).

Extracts - Water

Cell viability = 66.84508−0.044508*Concentration−0.54778 * Time + 1.52918E-004* Concentration * Time (7)

Extracts - Ethanol

Cell viability = 6.45670 + 1.66330E-003 * Concentration + 0.53019 * Time−6.99355E-004 * Concentration * Time (8)

4.3.4.3. Percentage cell viability

The results for the MTT assay (Fig. 20) illustrated that for the stem extracts, cell viability was dependent on both concentration and extract. The number of viable cells decreased as the concentration increased and when ethanol was used instead of water. At concentrations, higher than 250 µg/ml, there was a significant decrease in the number of viable cells. Both the water and ethanol crude extracts of the stem were toxic to the cells even at the lowest concentration of 10 µg/ml at both 24 and 48 h incubation periods (% cell viability ≤ 50%).

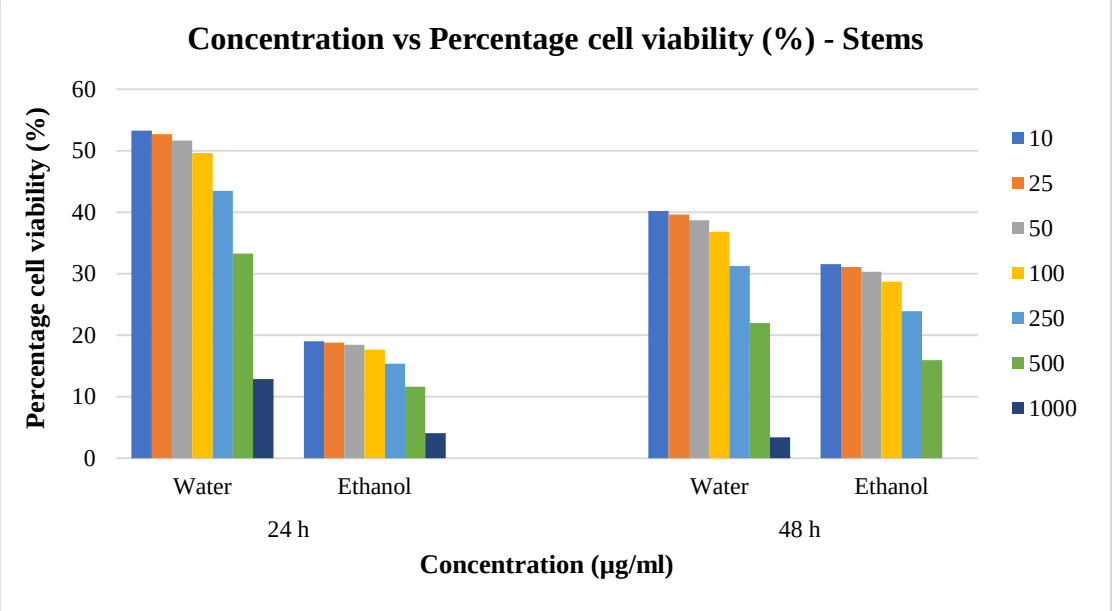

Figure 20: Cell viability (%) of RAW 264.7 cell line after treatment with various concentrations of water and ethanol extracts of the stems of *T. violacea* for both 24 and 48 h.

4.3.4.4. Morphological characterization

Similar to the leaf extracts, the RAW 264.7 cells were treated with 10 and 1000 µg/ml of the water and ethanol stem extracts of *T. violacea*. Fresh culture media was used as the negative control (untreated cells) (Fig. 21a) and 50% H_2O_2 (Fig. 21f) was used as the positive control. All treatments were incubated for both 24 and 48 h and the morphology of the cells was viewed under a Nikon microscope at 20x magnification (Fig. 21a – j). Compared to the controls (Fig. 21a and f), morphological abnormalities were observed in the treated cells after 24 h incubation with both the water and ethanol extracts whereby most of the cells were clumped together. Cells were completely damaged after 48 h incubation forming a dense irregular debris-like material with both the water and ethanol extracts at concentrations.

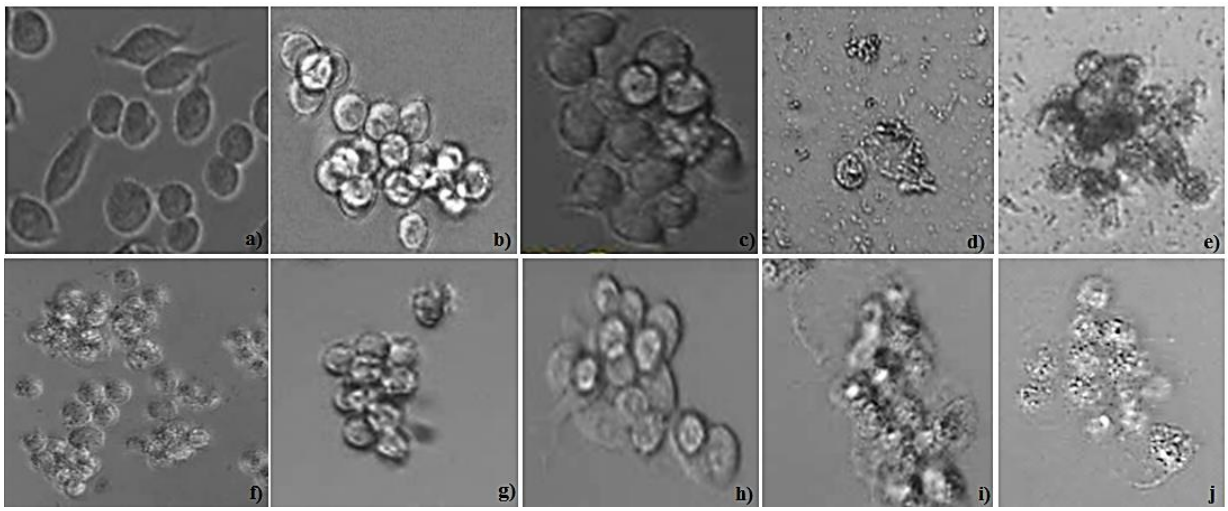

Figure 21: RAW 264.7 were treated with fresh culture media (negative control) (a) and 50% H_2O_2 (positive control) (f). The cells were also treated with 10 and 1000 µg/ml of the water stem extract incubated for 24 h, (b and c) and 48 h (d and e), respectively. The ethanol extracts results after 24 h incubation for 10 and 1000 µg/ml were (g and h) and after 48 h (i and j), respectively. Magnification 20×.

4.3.5. Crude root extracts

4.3.5.1. Main effects and Interaction plots

Lastly, similar to the crude leaf and stem extracts, there were two main effects: concentration (A) and type of extract (C) for the roots. An increase in each of these variables from a lower level (-) to a higher level (+) also resulted in a decrease in the number of viable cells. Incubation time (B) had no effect on percentage cell viability as indicated by the lower gradient line plotted on the main effect plot in Fig. 22 (b). The non-parallel lines between AB (concentration x time) implies that there was a strong two-way interaction between the main effect of concentration (A) and incubation time (B) (Fig 22d). However, the parallel lines observed in Fig. 22 (e and f) are a representation of a non-significant interaction between the AC (concentration x extracts) as well as between BC (time x extracts), respectively.

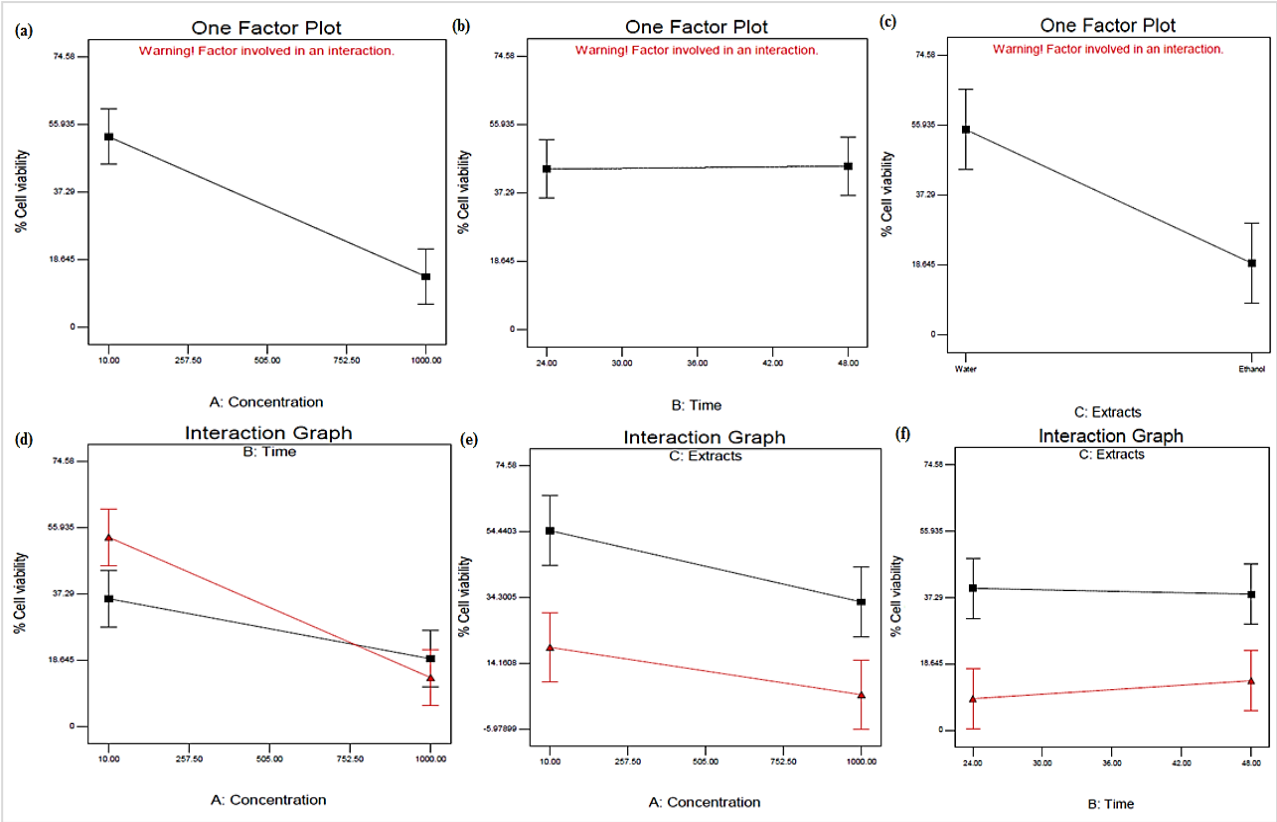

Figure 22: Interaction (a - f) plots on the effect (% cell viability) of the root crude extracts on RAW 264.7 cells.

4.3.5.2. Regression model analysis and ANOVA

Lastly for the root extracts, the full regression model (Table 8) also consisted of both the significant ($p < 0.05$ level) and non-significant model terms. Similar to the leaf and stem extracts, the main effect of concentration (A) and extracts (C) were significant whereas the effect of time (B) was also non-significant. For the two-way interactions, concentration x time (AB) was significant whereas concentration x extracts (AC), time x extract (BC) as well as the three-way interaction, concentration x time x extract (ABC) were non-significant.

Table 8: ANOVA report for the full regression model for the roots of *T. violacea*.

Source	Sum of squares	Degree of freedom	Mean squares	F-values	P-value
Model	10548.38	7	1506.91	8.95	0.0002
A	4767.77	1	4767.77	28.30	< 0.0001
B	215.10	1	215.10	1.28	0.2751
C	4501.55	1	4501.55	26.72	< 0.0001
AB	763.99	1	763.99	4.54	0.0491
AC	2.25	1	2.25	0.013	0.9094
BC	159.81	1	159.81	0.95	0.3445
ABC	137.90	1	137.90	0.82	0.3790
Pure Error	2695.12	16	168.44		

SD = 12.98; R^2 = 0.7479; R^2(adj) = 0.7075; R^2 (pred) = 0.5421; Adeq precision = 8.235; Mean = 30.56

Equations based on the full regression model to predict the percentage cell viability of crude extracts from the roots were developed (equation 9 and 10).

Extracts - Water

Cell viability = 47.15189 −8.18855E-003 * Concentration + 0.31032 * Time −5.46296E-004* Concentration * Time (9)

Extracts - Ethanol

Cell viability = 9.76963 + 0.019630* Concentration + 1.14798 *Time −1.35340E-003* Concentration * Time (10)

4.3.5.3. Percentage cell viability

Similarly, to the leaves and stems, the root extracts also showed that cell viability was also dependent on both concentration and extract (Fig. 23). The number of viable cells decreased as concentration increased and when ethanol was used in the place of water. As per the other plant parts, there was a significant decrease in the number of viable cells at concentrations higher than 250 µg/ml. The roots ethanol extracts were toxic to the cells even at the lowest concentration of 10 µg/ml at both 24 and 48 hr incubation periods (% cell viability < 50%).

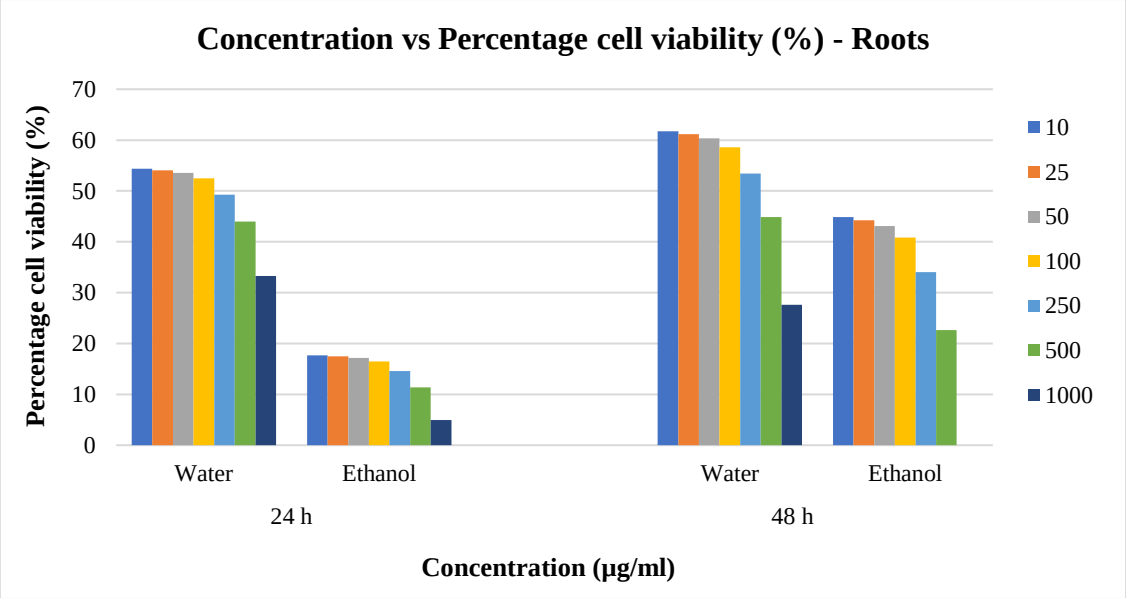

Figure 23: Cell viability (%) of RAW 264.7 cell line after treatment with various concentrations of water and ethanol extracts of the roots of *T. violacea* for both 24 and 48 h.

4.3.5.4. Morphological characterization

The RAW 264.7 cells were also treated with 10 and 1000 µg/ml of the water and ethanol root extracts of *T. violacea.* Fresh culture media was used as the negative control (untreated cells) and 50% H_2O_2 was used as the positive control. All treatments were incubated for both 24 and 48hrs and the morphology of the cells was viewed under a Nikon microscope at 20x magnification (Fig. 24a – j). As previously mentioned, untreated cells appeared smooth and well-rounded with some having a single pointy end showing no sign of abnormalities (Fig. 24a) However, for the cells treated with 50% H_2O_2, there was clumping, growth inhibition, shrinkage, vacuolisation with a dense irregular debris and detachment of cells from the culture flask (Fig. 24f). Similar to the stem extracts, cells were completely damaged after 48 h incubation forming a dense irregular debris-like material with both the water and ethanol extracts of the roots at both 10 (Fig. 24i) and 1000 (Fig. 24j) µg/ml.

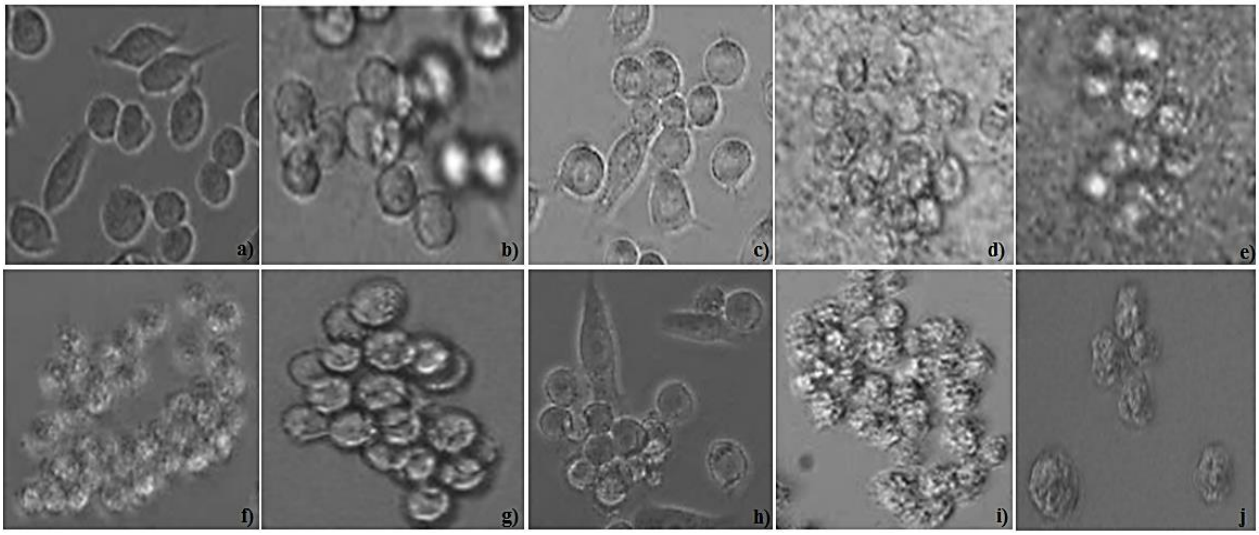

Figure 24: RAW 264.7 were treated with fresh culture media (negative control) (a) and 50% H_2O_2 (positive control) (f). The cells were also treated with 10 and 1000 µg/ml of the water root extracts incubated for 24 h, (b and c) and 48 h (d and e), respectively. The ethanol extracts results after 24 h incubation for 10 and 1000 µg/ml were (g and h) and after 48 h (i and j) respectively. Magnification 20×.

4.3.6. Half maximal inhibitory concentration (IC_{50})

A crude plant extract with an IC_{50} value below 20 µg/ml is considered highly cytotoxic (Mahavorasirikul *et al.* 2010). Table 9 shows the IC_{50} values for the RAW 264.7 cells treated with water and ethanol crude extracts from the leaves, stems and roots of *T. violacea* after 24 and 48 h incubation. The strongest cytotoxic activity was observed where the RAW 264.7 cells were stimulated with crude ethanol extracts from all the different parts of the plant (Table 9). Of the three parts of the plant assessed, the crude extracts (both water and ethanol) from the stems of *T. violacea* were most cytotoxic to the RAW 264.7 cells (Table 9). However, the water extracts prepared from the leaves and roots *T. violacea* were not toxic regardless of the duration of stimulation (both 24 and 48 h).

Table 9: IC_{50} (µg/ml) and 95% CI values of crude extracts from the leaves, stems and roots of *T. violacea* against RAW 264.7 cell line.

	IC_{50} (95% CI)					
Time (hours)	**Leaves**		**Stem**		**Roots**	
	Water	**Ethanol**	**Water**	**Ethanol**	**Water**	**Ethanol**
24	1364 (817 to 2276)	0.010 (0.00077 to 0.1426)	22.49 (13.87 to 36.87)	0.193 (0.2039 to 1.842)	60.67 (38.44 to 95.76)	0.061 (0.0052 to 0.7197)
48	22355 (6252 to 79929)	2.177 (0.6176 to 7.671)	9.475 (4.061 to 23.39)	2.241 (0.6800 to 7.386)	173.8 (112.7 to 268)	13.78 (6.292 to 30.18)

4.4. MTT cell proliferation assay – C2C12 cell line

4.4.1. Experimental observations

The aim of this objective was to determine the effect of concentration (A), incubation time (B) and the type of extracts (C) on percentage cell viability of a skeletal muscle (myoblasts or C2C12) cell line *in vitro.* A 2^3 factorial design was used for the study and the experimental design showing the independent variables is illustrated in Table 10.

Table 10: Experimental design range and levels of independent variables for inhibition of C2C12 by ethanol and water extracts of the leaves, stems and roots of *T. violacea*.

Independent variables	**Range and levels**	
	Lower limits (−)	**Upper limits (+)**
A = Concentration (µg/ml)	10µg/ml	1000µg/ml
B = Time (hours)	24hrs	48hrs
C = Nature of the Extracts	Water	Ethanol

The analysis was conducted in triplicate resulting in 24 observations (mean was calculated resulting in 8 observations) for each plant part. Table 11 illustrates the actual and predicted mean responses (% yield) per run ± SEM. Data analysis was conducted using Design Expert 6.0.6 software.

Table 11: A 2^3 full factorial design showing average runs in actual factors for independent variables.

							% Cell viability ± SEM					
							Actual			Predicted		
Run	A	B	C	A	B	C	Leaves	Stem	Roots	Leaves	Stem	Roots
01	−	−	−	10	24	Water	118.58±4.80	92.49±6.70	82.22±7.96	118.58±0.00	92.49±0.00	82.22±0.00
02	+	−	−	1000	24	Water	67.23±4.80	44.81±5.69	19.44±12.75	67.23±0.00	44.81±0.00	19.44±0.00
03	−	+	−	10	48	Water	69.2±4.47	81.70±1.78	56.99±5.72	69.20±0.00	81.70±0.00	56.99±0.00
04	+	+	−	1000	48	Water	33.63±19.21	9.08±8.42	43.01±5.36	33.63±0.00	9.08±0.00	43.01±0.00
05	−	−	+	10	24	Ethanol	130.84±10.63	105.11±18.31	61.93±7.51	130.84±0.00	105.11±0.00	61.93±0.00
06	+	−	+	1000	24	Ethanol	0.00±0.00	18.60±2.93	14.79±14.80	3.5E-14±0.00	18.60±0.00	14.80±1.3E-15
07	−	+	+	10	48	Ethanol	65.06±18.93	56.10±11.34	53.87±6.72	65.06±0.00	56.10±0.00	53.87±0.00
08	+	+	+	1000	48	Ethanol	0.00±0.00	0.00±0.00	0.00±0.00	2.6E-14±0.00	2.9E-14±0.00	3.3E-14±0.00

4.4.2. Half normal plots leaf, stem and root extracts.

This part of the study (Fig. 25) illustrated that for all the plant parts of *T. violacea* (leaves, stems and roots), the main effect of concentration (A) had a significant effect on cell viability while the main effect of time (B) was only significant for the leaf and stem extracts. The time x extract (BC) interaction was non-significant in all plant parts. The concentration x time (AB) and the concentration x extract (AC) interactions were non-significant only in the stem and root extracts.

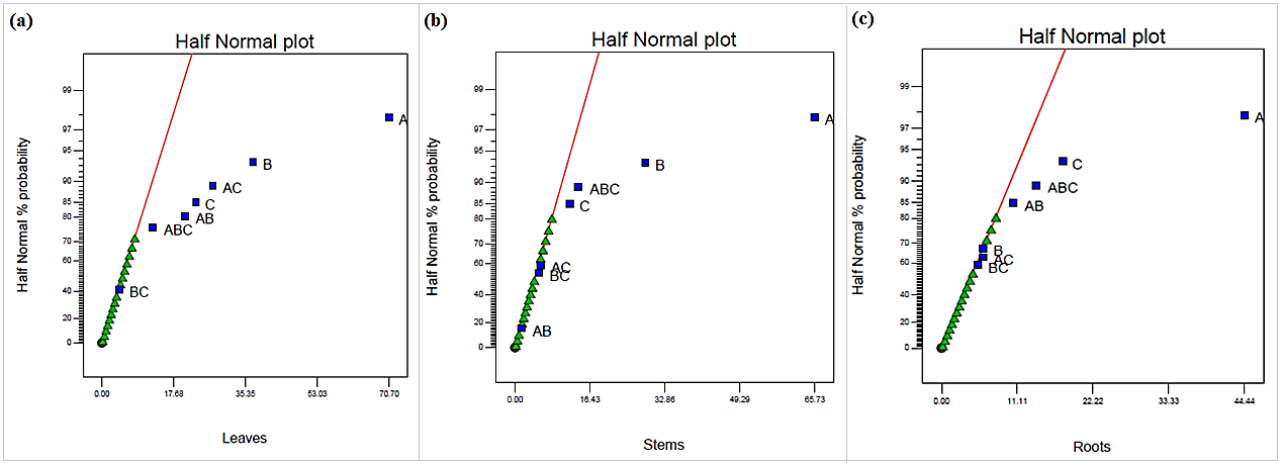

Figure 25: Half normal probability plots showing the effect (% cell viability) for (a) leaf (b) stem and (c) root extracts. The green triangles (▲) are noise effect estimates or 'pure error' and the blue (■) squares are factor estimates.

4.4.3. Crude leaf extracts

4.4.3.1. Main effects and Interaction plots

Main effect and interaction plots are lines resulting from the connection of the mean values from each treatment condition. As illustrated in Fig. 26 (a, b, c), there were three main effects: concentration (A), incubation time (B) and type of extract (C). An increase in each of these variables from lower levels (-) to higher levels (+) resulted in a decrease in the number of viable cells. The non-parallel lines (Fig. 26d and e) due to the interaction between concentration x time AB and concentration x extracts(AC) implies that there were strong two-way interactions between the main effect of concentration (A) and incubation time (B) as well as between concentration (A) and type of extracts (C). The time x extracts (BC) interaction was non-significant.

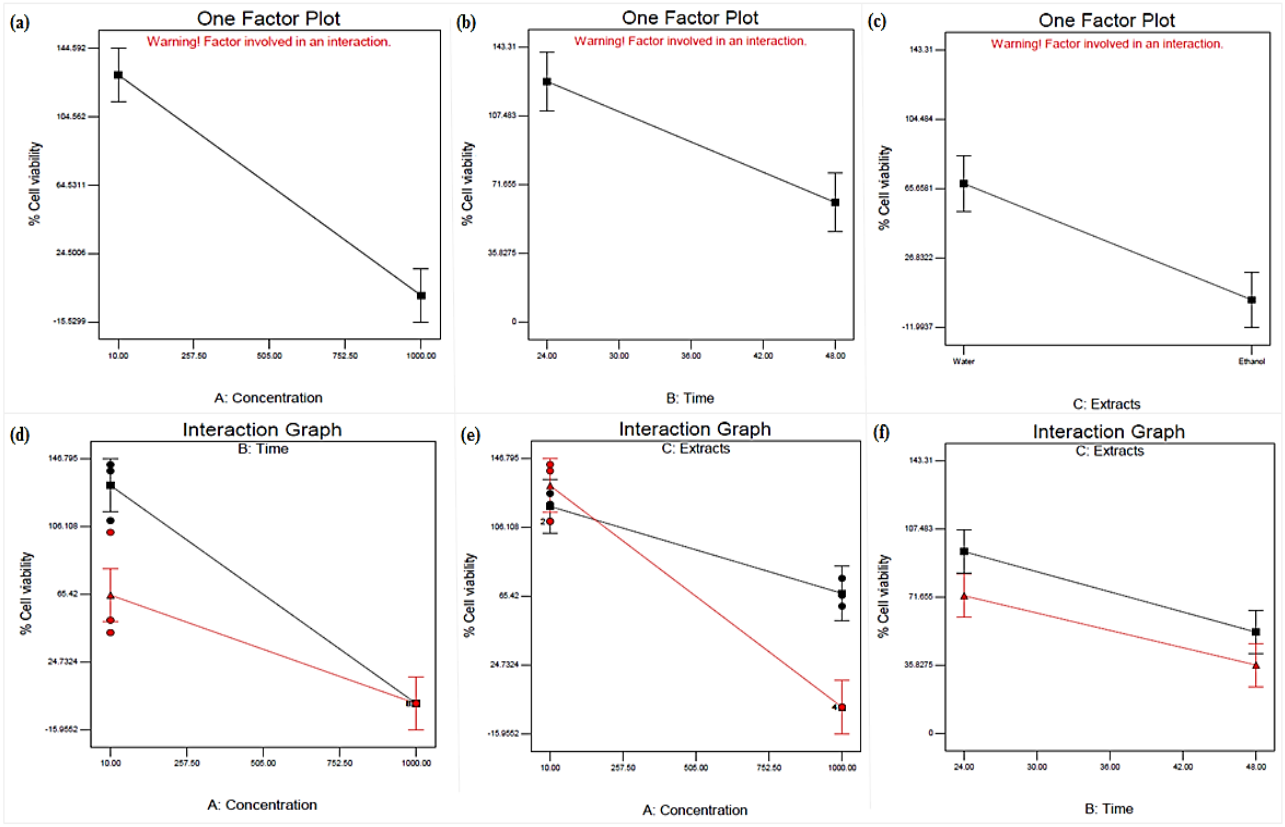

Figure 26: Interaction (a - f) plots on the effect (% cell viability) of the leaf crude extracts on C2C12 cells.

4.4.3.2. Regression model analysis and ANOVA

The full regression model for the leaf extracts (Table 12) consisted of both the significant ($p <$ 0.05 level) and non-significant model terms. The three main effects of concentration (A), time (B) and extracts (C) were all significant. For the two-way interactions, the concentration x

extract (AC) and the concentration x time (AB) interactions were significant whereas the time x extract (BC) interaction as well as the three-way interaction of concentration x time x extract (ABC) were non-significant.

Table 12: ANOVA report for the full regression model for the leaf extracts of *T. violacea*.

Source	Sum of squares	Degree of freedom	Mean squares	F-values	P-value
Model	49515.23	7	7073.60	20.81	< 0.0001
A	29993.77	1	29993.77	88.25	< 0.0001
B	8298.58	1	8298.58	24.42	0.0001
C	3225.27	1	3225.27	9.49	0.0072
AB	2494.51	1	2494.51	7.34	0.0155
AC	4454.83	1	4454.83	13.11	0.0023
BC	111.03	1	111.03	0.33	0.5756
ABC	937.25	1	937.25	2.76	0.1163
Pure Error	5438.02	16	339.88		
Cor Total	54953.25	23			

SD = 18.44; R^2 = 0.9010; R^2 (adj) = 0.8577; R^2 (pred) = 0.7773; Adeq precision = 12.293; Mean = 60.57

Equations based on the full regression model to predict the percentage cell viability of crude extracts from the leaves were developed (equation 4, 5 and 6).

$$\hat{y} = \beta_0 - \beta_1 A - \beta_2 B + \beta_{13} AB \quad (4)$$

Extracts -Water

Cell viability = 168.64141−0.067808*Concentration−2.06428*Time + 6.64282E-004* Concentration * Time (5)

Extracts – Ethanol

Cell viability = 198.60269−0.19860*Concentration−2.76838*Time + 2.76838E-003* Concentration * Time (6)

In equation 4, $\hat{y}$ represents the predicted percentage cell viability, A represents the concentration, B represent the incubation time, and AC represents the concentration-extract interaction. β_0, β_1, β_2, and β_{12} were the regression coefficients. β_0 was the mean value of responses of all experiments.

4.4.3.3. Percentage cell viability

The results for the MTT assay (Figure 27) illustrated that for the leaf extracts, cell viability was dependent on both concentration and time. The number of viable cells decreased with an

increase in concentration and incubation time. However, the type of extract (ethanol and water) had a non-significant effect on cell viability. At concentrations higher than 250 µg/ml, there was a gradual decrease in the number of viable cells for both the water and ethanol extracts at 24 and 48 h, respectively. The 48 h incubation period showed a lower percentage of viable cells when compared to that of 24hrs for both the water and ethanol extracts. The ethanol extract was lethal (no viable cells) to the C2C12 cells at 1000 µg/ml for both 24 and 48 h.

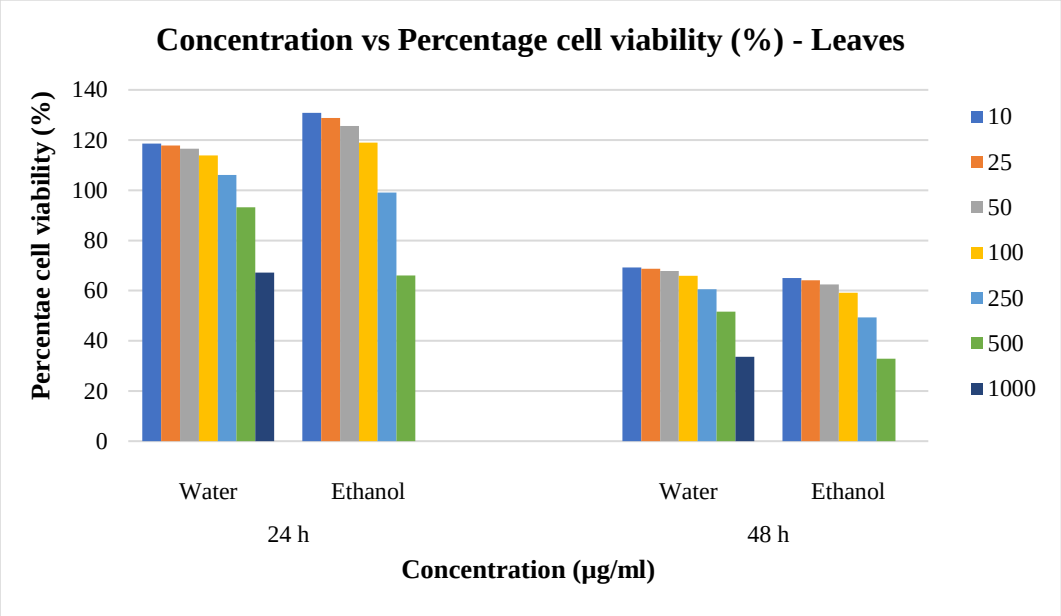

Figure 27: Cell viability (%) of C2C12 cell line after treatment with various concentrations of water and ethanol extracts of the leaves of *T. violacea* for both 24 and 48 h.

4.4.3.4. Morphological characterization

The C2C12 cells were treated with 10 and 1000 µg/ml of the water and ethanol leaf extracts of *T. violacea.* Fresh culture media was used as the negative control (untreated cells) and 50% H_2O_2 was used as the positive control. All treatments were incubated for both 24 and 48 h and the morphology of the cells was viewed under a Nikon microscope at 20x magnification (Fig. 28a – j). The untreated cells appeared thin and elongated with two tapering ends (Fig. 28a); however, cells treated with 50% H_2O_2 were roughly rounded with variable size, and had aggregated dense irregular cellular debris with no recognition of intact cells (Fig. 28f) Morphological abnormalities were observed in the cells treated with 1000 µg/ml (48 h) of the water extract as well as severe growth inhibition and shrinkage of cells in the cells treated with 1000 µg/m of the ethanol extracts (both 24 and 48 h). The damage due to ethanol extracts resembled that of the cells treated with 50% H_2O_2 (positive control).

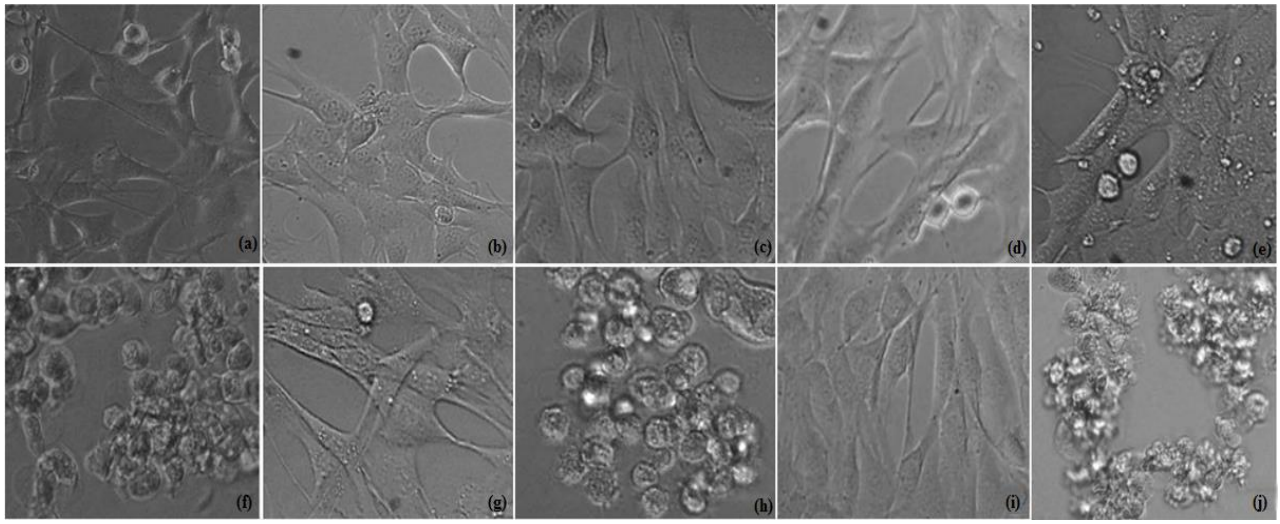

Figure 28: C2C12 were treated with fresh culture media (negative control) (a) and 50% H_2O_2 (positive control) (f). The cells were also treated with 10 and 1000 µg/ml of the water leaf extracts incubated for 24 h, (b and c) and 48 h (d and e), respectively. The ethanol extracts results after 24 h incubation for 10 and 1000 µg/ml were (g and h) and after 48 h (i and j) respectively. Magnification 20×.

4.4.4. Crude stem extracts

4.4.4.1. Main effects and Interaction plots

For the stem extracts, there were two main effects: concentration (A) and incubation time (B) (Fig. 29a and b). An increase in each of these variables from lower levels (-) to higher levels (+) also resulted in a decrease in the number of viable cells. The main effect of the extracts (C) had no effect on percentage cell viability as indicated by the lower gradient line plotted on the main effect plot in Fig. 29 (c). In the interaction plots (Fig. 29d, e, and f), the parallel lines observed represented a non-significant interaction between all of the independent variables for the stem crude extracts of *T. violacea*.

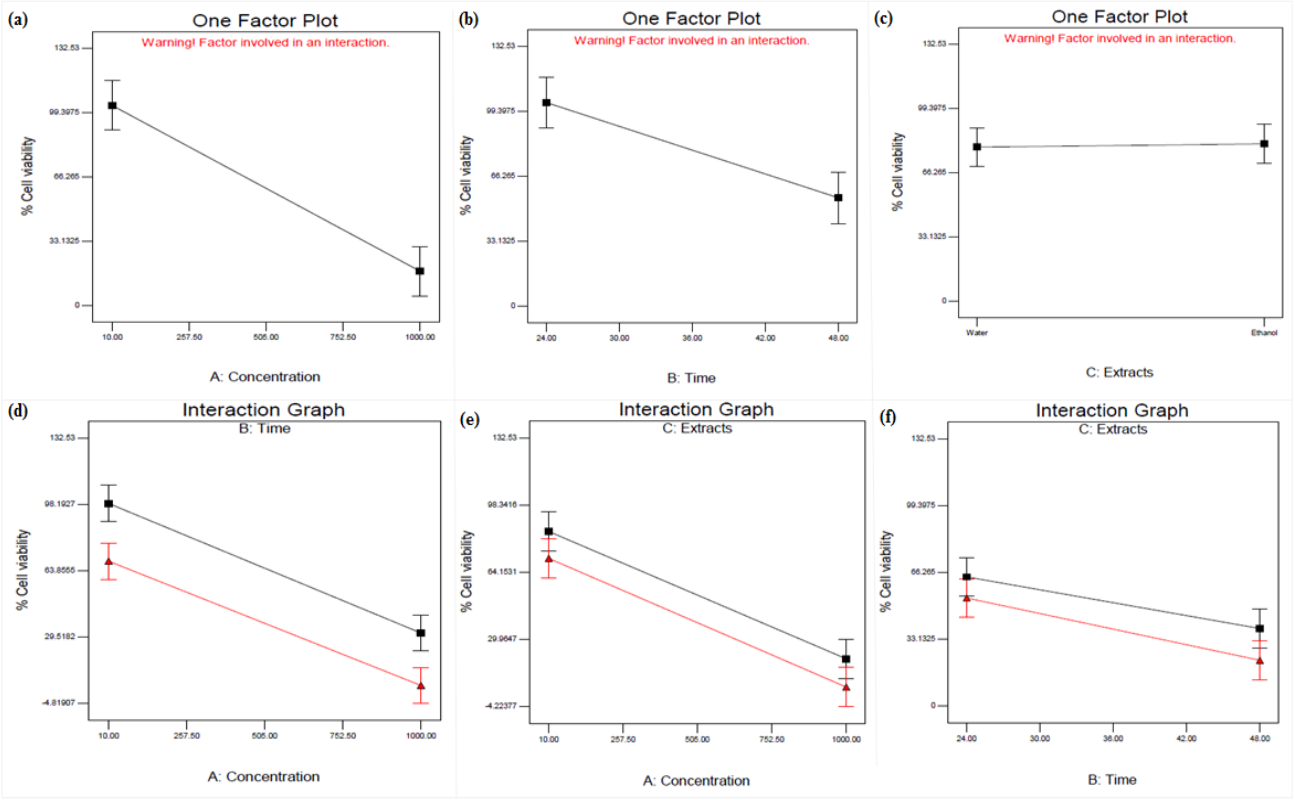

Figure 29: Interaction (a - f) plots on the effect (% cell viability) of the stem crude extracts on C2C12 cells.

4.4.4.2. Regression model analysis and ANOVA

The full regression model (Table 13) consisted of both the significant ($p < 0.05$) and non-significant model terms. The main effects of concentration (A) and time (B) were significant whereas the effect of the extracts (B) was non-significant. All the two-way interactions concentration x time (AB), concentration x extracts (AC) and time x extracts (BC) were non-significant while the three-way interaction concentration x time x extracts (ABC) was slightly significant.

Table 13: ANOVA report for the full regression model for the extracts of the stem of *T. violacea*.

Source	Sum of squares	Degree of freedom	Mean squares	F-values	P-value
Model	33190.83	7	4741.55	20.28	< 0.0001
A	25919.31	1	25919.31	110.86	< 0.0001
B	4884.62	1	4884.62	20.89	0.0003
C	873.51	1	873.51	3.74	0.0712
AB	11.19	1	11.19	0.048	0.8296
AC	186.65	1	186.65	0.80	0.3848
BC	166.69	1	166.69	0.71	0.4109
ABC	1148.86	1	1148.86	4.91	0.0415
Pure Error	3740.92	16	233.81		
Cor Total	36931.75	23			

SD = 15.29; R^2 = 0.8987; R^2 (adj) = 0.8544; R^2 (pred) = 0.7721; Adeq precision = 11.906; Mean = 50.98

Equations based on the full regression model to predict the percentage cell viability of crude extracts from the leaves were developed (equation 7 and 8).

Extracts -Water

Cell viability = 103.50630−0.022963*Concentration−0.43909*Time + 1.04980E-003* Concentration * Time (7)

Extracts – Ethanol

Cell viability = 155.29428−0.11809*Concentration−2.05474*Time + 1.27974E-003* Concentration * Time (8)

4.4.4.3. Percentage cell viability

Similar to the leaf extracts, results for the MTT assay (Fig. 30) illustrated that cell viability was dependent on concentration and time. The number of viable cells decreased as the concentration and incubation time increased. The effect of the extracts (ethanol and water) on cell viability was non-significant. At concentrations higher than 250 µg/ml, there was also a significant decrease in the number of viable cells. After 48 h incubation, the ethanol extracts had the overall lowest percentages of viable cells.

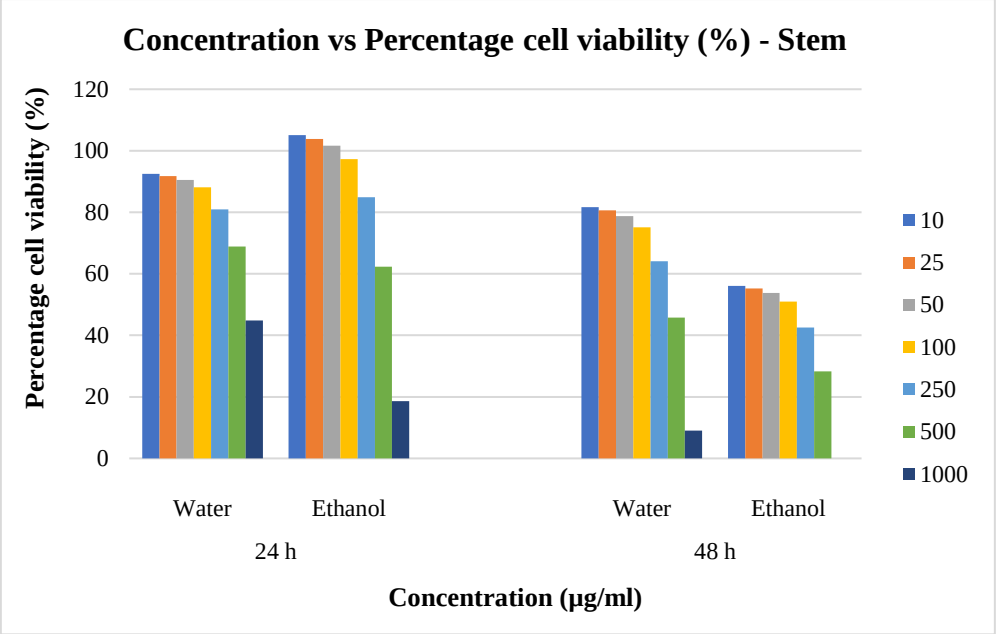

Figure 30: Cell viability (%) of C2C12 cell line after treatment with various concentrations of water and ethanol extracts of the stem extracts of *T. violacea* for both 24 and 48 h

4.4.4.4. Morphological characterization

Similar to the leaf extracts, the C2C12 cells were treated with 10 and 1000 µg/ml of the water and ethanol stem extracts of *T. violacea.* Fresh culture media (untreated cells) (Fig. 31a) and 50% H_2O_2 (Fig. 31f) were used as the negative and positive controls respectively. The C2C12 cells were then incubated for both 24 and 48 h and the morphology of the cells was viewed under a Nikon microscope at 20x magnification (Fig. 31a – j). Compared to the controls, the cells treated with 1000 µg/ml (24 and 48 h) with both water and ethanol extracts, were morphologically abnormal and were roughly rounded and of variable sizes and were aggregated in the form of a dense irregular shaped cellular mass with no recognition of intact cells. This observation was similar to that of the stem extracts. The damaged cells resembled those of the cells treated with 50% H_2O_2 (positive control) (Fig. 31f).

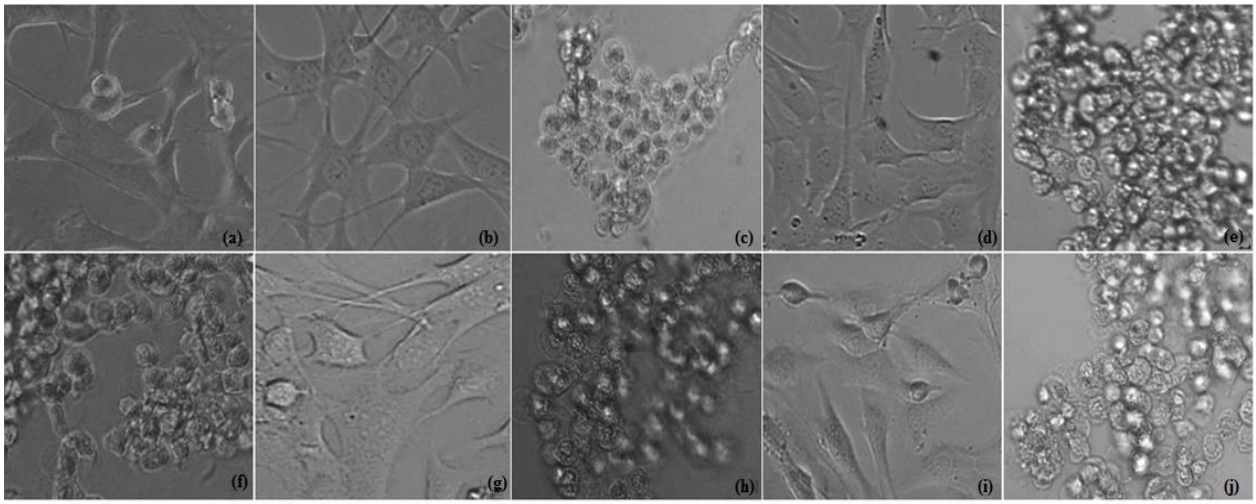

Figure 31: C2C12 were treated with fresh culture media (negative control) (a) and 50% H_2O_2 (positive control) (f). The cells were also treated with 10 and 1000 µg/ml of the water stem extracts incubated for 24 h, (b and c) and 48 h (d and e), respectively. The ethanol extracts results after 24 h incubation for 10 and 1000 µg/ml were (g and h) and after 48 h (i and j) respectively. Magnification 20×.

4.4.5. Crude root extracts

4.4.5.1. Main effects and Interaction plots

Lastly for the root extracts, there were two main effects: concentration (A) and type of extract (C) (Fig. 32a and c). An increase in each of these variables from lower levels (-) to higher levels (+) also resulted in a decrease in the number of viable cells. The main effect of time (B) had no effect on cell viability as indicated by the lower gradient line plotted of the main effect (Fig. 32 (b). In the interaction plots (Fig.e 32d, e, and f), the parallel lines suggests a non-significant interaction between all of the independent variables for the crude extracts of the roots of *T. violacea.*

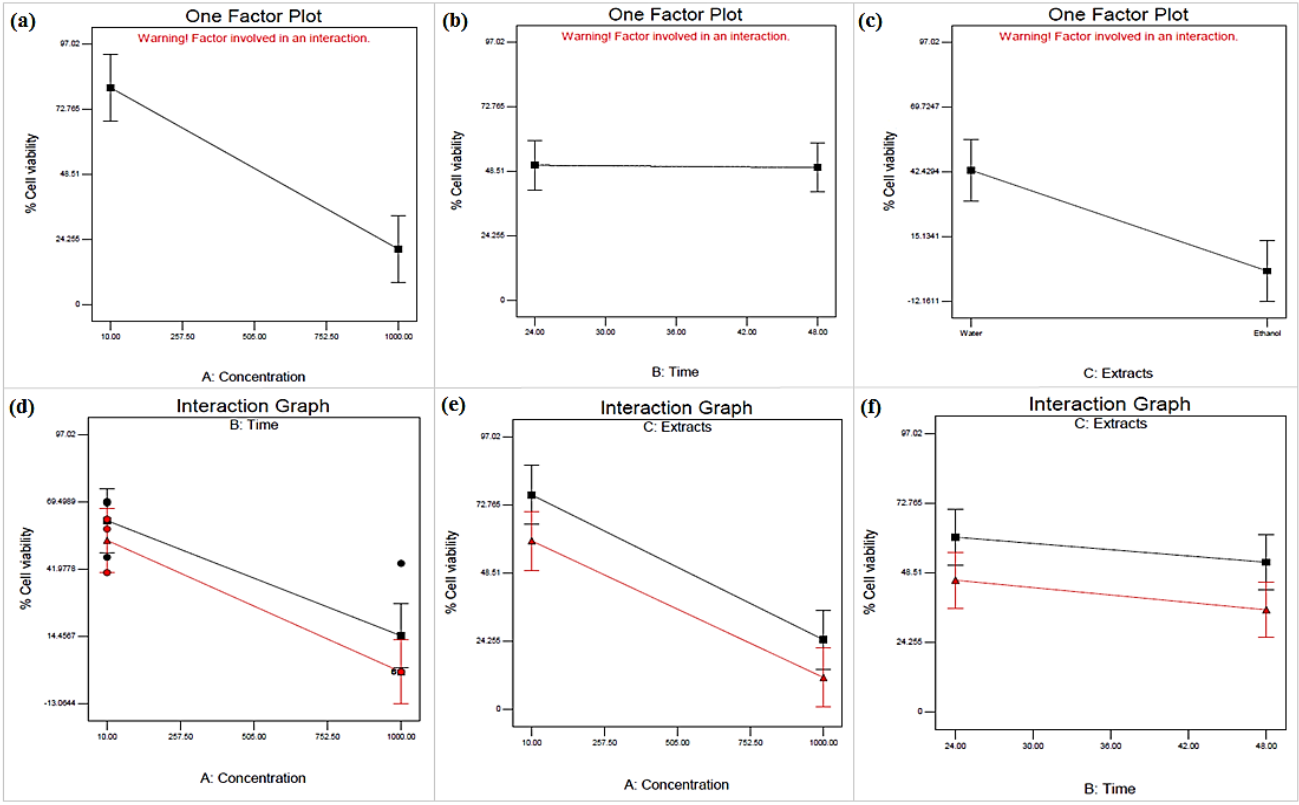

Figure 32: Interaction (a - f) plots on the effect (% cell viability) of the root crude extracts on C2C12 cells.

4.4.5.2. Regression model analysis and ANOVA

The full regression model for the root extracts (Table 14) consisted of both the significant ($p < 0.05$) and non-significant model terms. Similar to the leaf and stem extracts, the main effect of concentration (A) and extracts (C) were significant whereas the effect of time (B) was also non-significant. Similar to the stem extracts, all the two-way interactions concentration x time (AB), concentration x extracts (AC) and time x extracts (BC) were non-significant while the three-way interaction concentration x time x extracts (ABC) was slightly significant.

Table 14: ANOVA report for the full regression model for the root extracts of *T. violacea*.

Source	Sum of squares	Degree of freedom	Mean squares	F-values	P-value
Model	16177.74	7	2311.11	10.14	< 0.0001
A	11850.19	1	11850.19	52.00	< 0.0001
B	225.49	1	225.49	0.99	0.3347
C	1894.00	1	1894.00	8.31	0.0108
AB	663.14	1	663.14	2.91	0.1074
AC	220.37	1	220.37	0.97	0.3401
BC	168.41	1	168.41	0.74	0.4027
ABC	1156.15	1	1156.15	5.07	0.0387
Pure Error	3646.03	16	227.88		
Cor Total	19823.77	23			

SD = 15.10; R^2 = 0.8161; R^2 (adj) = 0.7356; R^2 (pred) = 0.5862; Adeq precision = 9.434; Mean = 41.53

Equations based on the full regression model to predict the percentage cell viability of crude extracts from the leaves were developed (equation 9 and 10).

Extracts -Water

Cell viability = 108.57358−0.11269*Concentration−1.07165*Time + 2.05340E-003* Concentration * Time (9)

Extracts – Ethanol

Cell viability = 70.39805−0.040805*Concentration−0.33300*Time + 2.83530E-004* Concentration * Time (10)

4.4.5.3. Percentage cell viability

Lastly for the root extracts (Figure 33), cell viability was dependent on concentration. The number of viable cells decreased as the concentration increased at both 24 and 48 h. At concentrations higher than 250 µg/ml, there was also a gradual decrease in the number of viable cells. The ethanol extract was lethal (no viable cells) to the C2C12 cells at 1000 µg/ml after 48 h incubation.

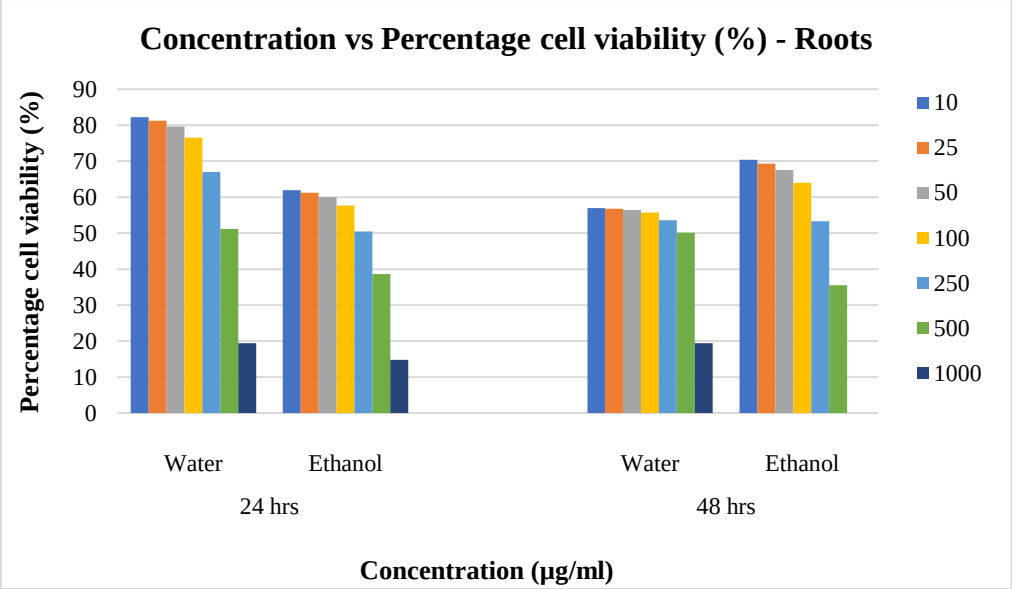

Figure 33: Cell viability (%) of C2C12 cell line after treatment with various concentrations of water and ethanol extracts of the roots of *T. violacea* for both 24 and 48 h.

4.4.5.4. Morphological characterization

Lastly, the C2C12 cells were treated with 10 and 1000 µg/ml of the water and ethanol root extracts of *T. violacea.* Fresh culture media (untreated cells) and 50% H_2O_2 were used as the negative and positive controls, respectively. The C2C12 cells were then incubated for both 24 and 48 h and the morphology of the cells was viewed under a Nikon microscope at 20x magnification (Fig. 34a – j). Compared to the controls, morphological abnormalities resembled those observed in the stem extracts with numerous roughly rounded cells of variable sizes and an aggregation of dense irregular cellular debris with no recognition of intact cells when treated with 1000 µg/ml (24 and 48 h) of the water and ethanol extracts. The damaged cells also resembled those of the cells treated with 50% H_2O_2 (positive control) (Fig. 34f).

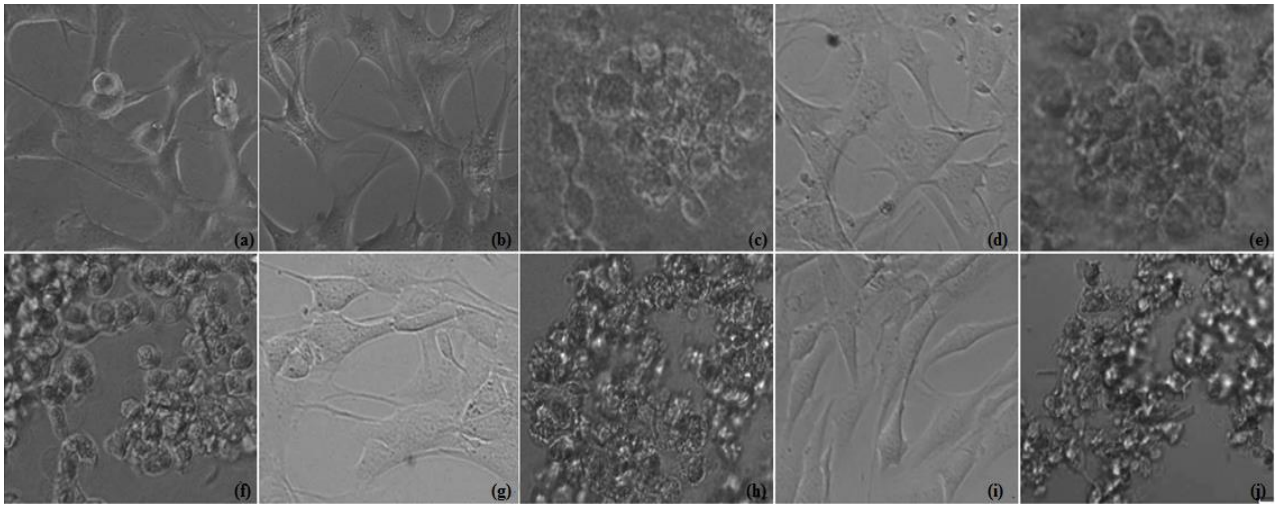

Figure 34: C2C12 were treated with fresh culture media (negative control) (a) and 50% H_2O_2 (positive control) (f). The cells were also treated with 10 and 1000 µg/ml of the water root extracts incubated for 24 h, (b and c) and 48 h (d and e), respectively. The ethanol extracts results after 24 h incubation for 10 and 1000 µg/ml were (g and h) and after 48 h (i and j) respectively. Magnification 20×.

4.4.6. Half maximal inhibitory concentration (IC_{50})

Table 15 shows the IC_{50} values for C2C12 cells treated with crude water and ethanol extracts from the leaves, stems and roots of *T. violacea* after 24 and 48 h incubation. Considering that an IC_{50} value less than 20 µg/ml is highly toxic, the potential toxic activity of *T. violacea* crude extracts on C2C12 cells is negligible. This is due to the fact that the IC_{50}> 30 µg/ml in all three plant parts with both the water and ethanol extracts regardless of the duration of stimulation (both 24 and 48 h).

Table 15: IC_{50} (µg/ml) and 95% CI values of crude extracts from the leaves, stems and roots of *T. violacea* against C2C12 cell line.

	IC_{50} (95% CI)					
Time (hours)	**Leaves**		**Stem**		**Roots**	
	Water	**Ethanol**	**Water**	**Ethanol**	**Water**	**Ethanol**
24	1187 (832.6 to 1692)	535.1 (170.6 to 1678)	881.5 (728.3 to 1067)	580.8 (549.3 to 614.1)	381.0 (275.9 to 526.2)	103.8 (66.51 to 161.8)
48	425.3 (260.4 to 694.6)	97.08 (60.52 to 155.7)	291.1 (209.7 to 404.2)	46.79 (27.12 to 80.72)	107.7 (51.66 to 224.7)	37.82 (21.24 to 67.33)

4.5. *Allium cepa* assay

The aim of the study was to evaluate the genotoxic effect of crude water extracts from the leaves, stems, and roots of *T. violacea*.

4.5.1. Roots growth, Mitotic index (MI) and Chromosomal aberration analysis (CA) with water extracts

The genotoxic inhibitory effects and root growth of various concentrations of the crude water extracts of the leaves, stems and roots of *T. violacea* on *Allium cepa* are shown in Table 16. The results showed a concentration dependent decrease in mitotic index and percentage of aberrant chromosomes as the concentration of the crude extracts increased. There was also a significant ($p < 0.05$) decrease in mean root length as the concentration of the extracts increased. The lowest Mitotic Index (MI) value of 19.69% was obtained with the stem extract at 1000 µg/ml. The mitotic index decreased significantly ($p < 0.05$) with the leaf extracts at 500 µg/ml and 1000 µg/ml with values of 27.78% and 24.54%, respectively. Leaf extracts at 100 µg/ml and 250 µg/ml produced mitotic indices of 43.71% and 58.66%, respectively. For the stem extracts, the higher concentrations (1000, 500 and 250 µg/ml) produced a significant reduction in mitotic indices with values of 19.69%, 32.57% and 33.64%, respectively, when compared to the non-significant 40.56% for 100 µg/ml. Similarly, water extracts of the roots at 1000, 500 and 250 µg/ml produced significant mitotic indices of 22.59%, 31.08% and 37.24%, respectively whereas the 100 µg/ml treatment was non-significant (MI = 58.88). These values were low when compared to the Mitotic index for the negative control (distilled water) which was 61.83%. For the positive control (ethidium bromide), all the tested concentrations produced a significant reduction in the mitotic index and produced the lowest MI value for the highest concentration of 1000 µg/ml of 1.48%.

Table 16: Cytogenetic analysis of *A. cepa* root tips exposed to different concentrations of *T. violacea* leaves, stems and roots extract and ethidium bromide.

Treatment Conc. (µg/ml)	Phenotypic indices			% Chromosomal aberrations ± SEM						% of Aberrant cells ± SEM
	Total no. of cells ± SEM	Mean root length (cm) ± SEM	Mitotic index ± SEM	Bridges	Stickiness	Laggard	C-mitosis	Binucleus	Trinucleus	
Tested parts										
Leaves										
Control	1207 ± 48.53	0.99 ± 0.28	61.83 ± 6.50	1.00 ± 0.19	4.11 ± 0.77	0.33 ± 0.19	4.54 ± 1.35	47.00 ± 4.19	0.33 ± 0.19	4.72 ± 0.49
100	1186 ± 39.94	0.84 ± 0.20	58.66 ± 7.24	1.67 ± 0.58	5.20 ± 1.18	1.11 ± 0.80	5.00 ± 1.07	94.11 ± 9.26*	0.44 ± 0.22	9.07 ± 1.12*
250	1221 ± 39.24	0.58 ± 0.18	43.71 ± 2.93	2.11 ± 1.31	5.33 ± 0.88	1.22 ± 0.73	10.89 ± 5.40	116.44 ± 18.68*	10.56 ± 5.79	12.00 ± 1.68*
500	1118 ± 53.12	0.20 ± 0.19	27.78 ± 6.60*	2.22 ± 1.28	10.00 ± 1.39*	2.33 ± 0.88	11.33 ± 5.84	117.56 ± 24.05*	11.33 ± 4.95*	13.84 ± 1.32*
1000	1263 ± 12.04	0.1 9 ± 0.02*	24.54 ± 5.44*	5.00 ± 0.38*	10.78 ± 2.72*	2.89 ± 0.68*	13.22 ± 4.83*	163.56 ± 11.86*	11.56 ± 5.36*	16.39 ± 1.87*
Stems										
Control	1207 ± 39.43	0.99 ± 0.28	61.83 ± 6.50	1.00 ± 0.19	4.11 ± 0.77	0.33 ± 0.19	4.54 ± 1.35	47.00 ± 4.19	0.33 ± 0.19	4.72 ± 0.49
100	1116 ± 66.67	0.51 ± 0.13	40.86 ± 9.43	1.89 ± 0.22	6.11 ± 0.29	0.56 ± 0.11	6.56 ± 0.78	99.44 ± 9.14*	0.78 ± 0.29	10.20 ± 1.43*
250	1269 ± 24.36	0.31 ± 0.34	33.64 ± 3.86*	4.44 ± 0.22*	7.00 ± 2.17	3.44 ± 0.73*	8.00 ± 0.69*	129.11 ± 10.20*	7.22 ± 5.92	12.56 ± 1.01*
500	1186 ± 2.83	0.27 ± 0.05	32.57 ± 3.14*	5.11 ± 0.87*	13.44 ± 1.25*	5.00 ± 1.58*	8.79 ± 1.97	159.22 ± 30.20*	9.67 ± 8.53	16.97 ± 2.09*
1000	1303 ± 24.45*	0.06 ± 0.05*	19.69 ± 6.67*	6.89 ± 1.98*	15.56 ± 4.15*	7.00 ± 2.27*	13.00 ± 1.71*	209.44 ± 45.57*	20.44 ± 4.80*	20.91 ± 3.04*
Roots										
Control	1207 ± 39.43	0.99 ± 0.28	61.83 ± 6.50	1.00 ± 0.19	4.11 ± 0.77	0.33 ± 0.19	4.54 ± 1.35	47.00 ± 4.19	0.33 ± 0.19	4.72 ± 0.49
100	1263 ± 16.01	0.66 ± 0.67	58.88 ± 5.52	2.56 ± 1.09*	9.22 ± 0.68*	1.78 ± 0.99*	6.56 ± 1.31	94.00 ± 9.26*	0.67 ± 0.33	9.09 ± 1.02*
250	1287 ± 23.93	0.56 ± 0.12	37.24 ± 9.07*	3.33 ± 0.19*	9.78 ± 1.06*	3.78 ± 0.78*	7.44 ± 1.42*	124.11 ± 21.57*	16.66 ± 2.31*	12.83 ± 1.06*
500	1250 ± 22.82	0.37 ± 0.15	31.08 ± 3.14*	3.78 ± 0.29*	10.00 ± 1.61*	4.11 ± 0.78*	8.89 ± 0.99*	137.00 ± 58.28*	20.22 ± 1.16*	14.72 ± 1.82*
1000	1189 ± 24.45	0.09 ± 0.05*	22.59 ± 6.67*	5.44 ± 0.22*	10.33 ± 1.66*	4.33 ± 0.51*	12.67 ± 1.02*	169.89 ± 17.83*	22.44 ± 5.43*	18.93 ± 1.98*
Positive control										
Control	1207 ± 39.43	0.99 ± 0.28	61.83 ± 6.50	1.00 ± 0.19	4.11 ± 0.77	0.33 ± 0.19	4.54 ± 1.35	47.00 ± 4.19	0.33 ± 0.19	4.72 ± 0.49
100	1229 ± 9.75	0.12 ± 0.07*	8.65 ± 6.48*	1.33 ± 0.00	4.22 ± 1.16	0.78 ± 0.48	8.44 ± 1.25*	155.00 ± 8.51*	1.44 ± 1.50	13.93 ± 1.56*
250	1248 ± 19.49	0.04 ± 0.03*	2.20± 0.14*	2.00 ± 1.26	9.56 ± 2.51*	0.89 ± 0.29	9.33 ± 1.33*	190.89 ± 11.28*	2.56 ± 2.17*	17.04 ± 2.35*

500	1167 ± 47.46	0.05 ± 0.04*	1.60 ± 0.80*	3.00 ± 1.20	10.11 ± 1.76*	1.00 ± 0.17	12.33 ± 2.52*	196.11 ± 14.83*	4,11 ± 10.94	19.42 ± 1.56*
1000	1133 ± 68.38	0.00 ± 0.00*	1.48 ± 0.09*	3.44 ± 0.95*	14.00 ± 2.03*	4.89 ± 0.62*	19.89± 5.02*	202.22 ± 11.77*	4.22 ± 10.93*	21.91 ± 1.45

Data represent the mean ± SEM, $n = 3$. (*) represents statistically significant groups compared to the negative control (distilled water) according to the *t*-test: Two-Sample Assuming Equal Variance ($p < 0.05$).

Chromosome aberrations were observed in all stages of mitosis (prophase, metaphase, anaphase and telophase) and showed a mitostatic effect (constant mitotic cell division). Figure 35-39 illustrate the normal cells as well as the six common aberrations observed in the *A. cepa* assay. The six aberrations detected include laggard chromosomes, chromosome bridges, C-mitosis, sticky chromosomes, binucleus and trinucleus. C-mitosis, binucleus and sticky chromosomes were the most common chromosomal aberrations detected (Figure 37B, 38B and 39A). For the negative control group (distilled water), the percentage of aberrant cells was low, 4.72% when compared to highest concentration of 1000 µg/ml of the positive control group (ethidium bromide) which scored 21.91%.

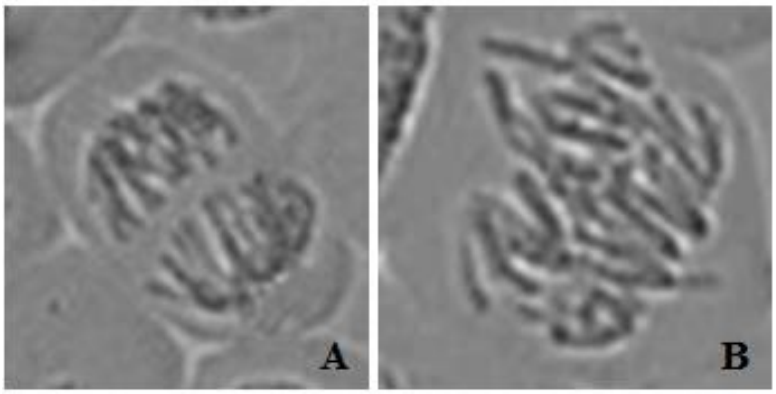

Figure 35: Chromosome aberrations observed in *A. cepa* meristematic cells exposed to water extracts of *T. violacea*. (A), normal anaphase and (B), laggard chromosome. Magnification 400×.

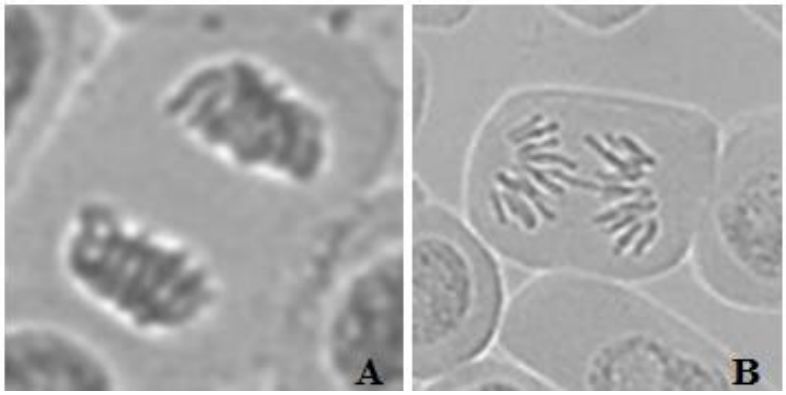

Figure 36: Chromosome aberrations observed in *A. cepa* meristematic cells exposed to water extracts of *T. violacea*. (A), normal telophase and (B), chromosome bridge. Magnification 400×.

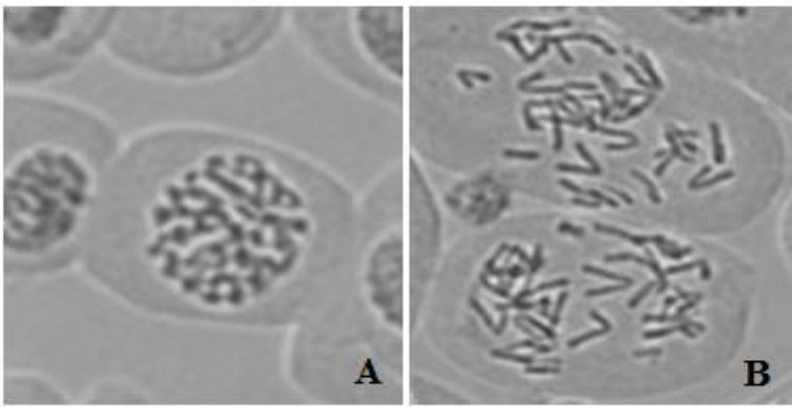

Figure 37: Chromosome aberrations observed in *A. cepa* meristematic cells exposed to water extracts of *T. violacea*. (A), normal prophase and (B), C-mitosis. Magnification 400×.

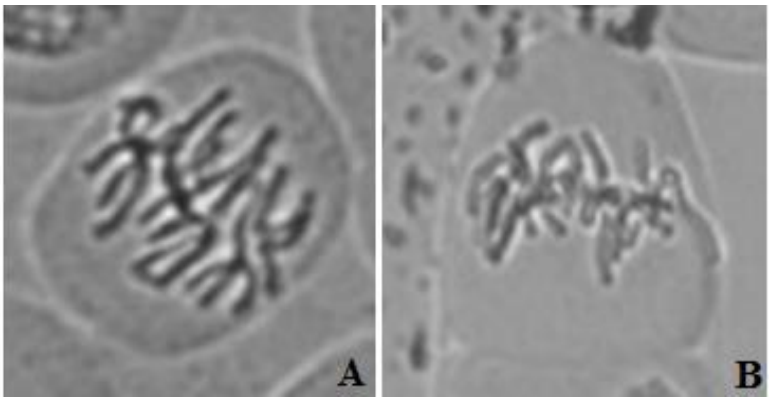

Figure 38: Chromosome aberrations observed in *A. cepa* meristematic cells exposed to water extracts of *T. violacea*. (A), metaphase anaphase and (B), sticky chromosome. Magnification 400×.

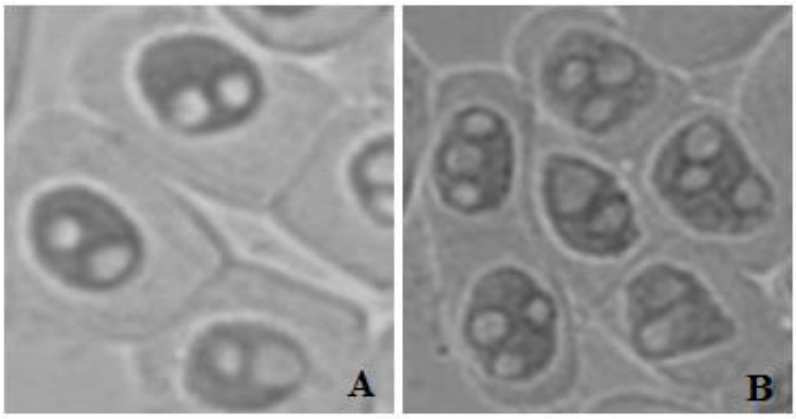

Figure 39: Chromosome aberrations observed in *A. cepa* meristematic cells exposed to water extracts of *T. violacea*. (A), binucleus and (B), trinucleus. Magnification 400×.

4.5.2. Frequency of aberrant cells

Figures (40 - 43) shows graphical representations for the frequency of aberrations and micronucleated cells. The most frequent aberrations detected were binucleated cells, followed by c-mitosis, sticky chromosomes, bridges, trinucleated cells and lastly chromosomal bridges. Extracts from the stem produced the highest frequencies of chromosomal aberrations followed by that from the roots. The lowest number of aberrations were observed in the extracts from the leaves. At the highest concentration of 1000 µg/ml, the total number of aberrations were 207.01, 272.33, and 225.10 for the leaf, stem and root extracts, respectively. The frequency of aberrant cells was concentration and extract dependent. The highest concentration of 1000 µg/ml for the positive control (ethidium bromide) produced a value of 248.66 when compared to the 57.28 of the negative control (distilled water).

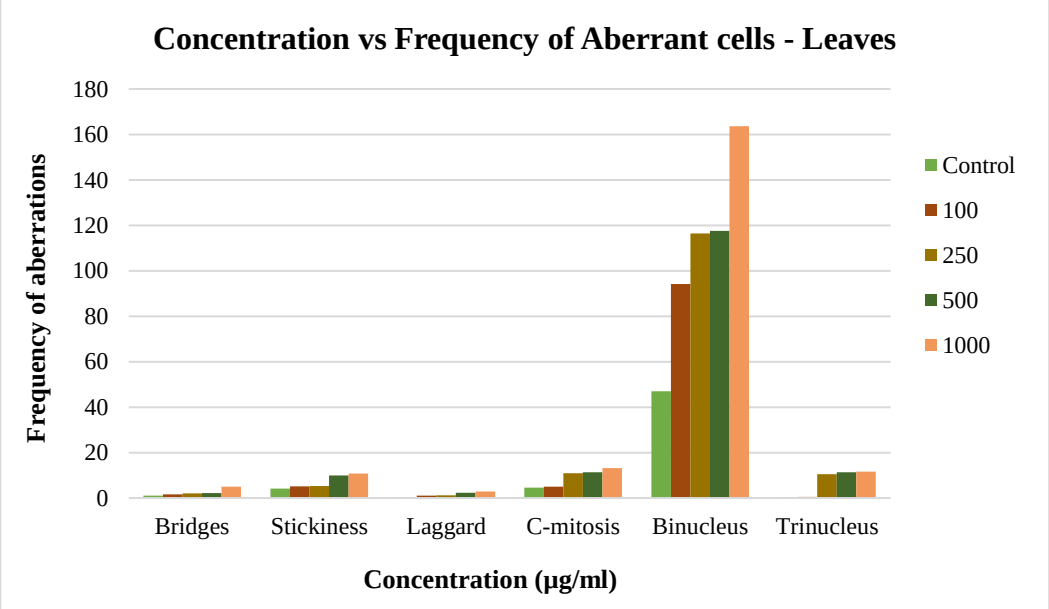

Figure 40: Frequency of aberrations observed in the *A. cepa* roots treated with increasing concentrations of extracts from the leaves. The negative control (distilled water) is also included.

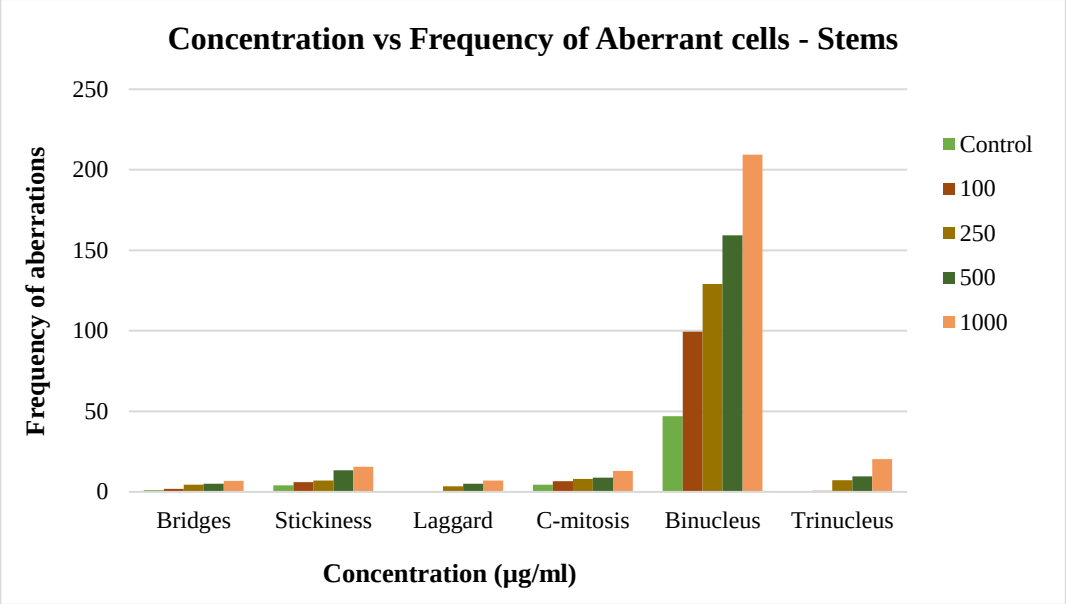

Figure 41: Frequency of aberrations observed in the *A. cepa* roots treated with increasing concentrations of extracts from the stems. The negative control (distilled water) is also included.

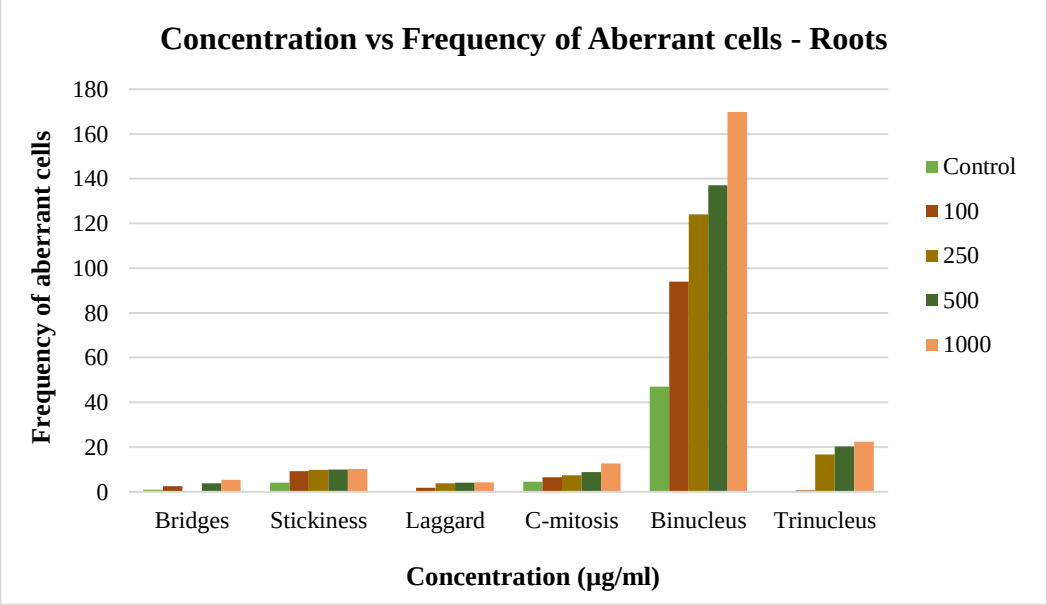

Figure 42: Frequency of aberrations observed in the *A. cepa* roots treated with increasing concentrations of extracts from the roots. The negative control (distilled water) is also included.

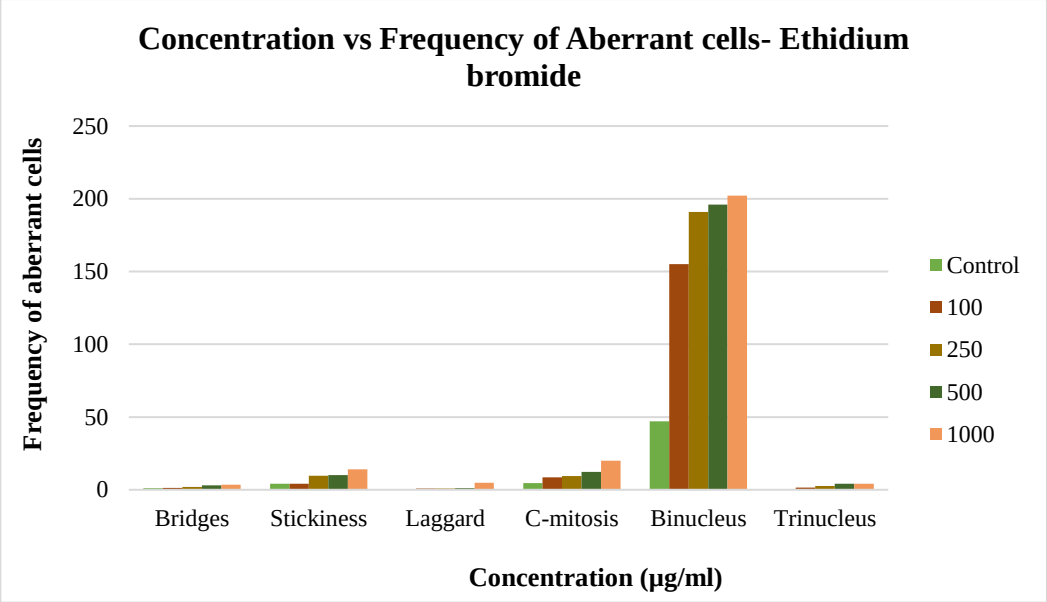

Figure 43: Frequency of aberrations observed in the *A. cepa* roots treated with increasing concentrations of the positive control (ethidium bromide). The negative control (distilled water) is also included.

4.5.3. Micronucleus (MN) assay

The presence of micronuclei (Fig.44) was observed in the roots treated with the different extracts of *T. violacea* and most were significantly different when compared with the negative control ($p < 0.05$) (Table 17). The percentage of micronucleated cells was generally higher at 1000 µg/ml in the stem (3.74%), followed by the root (3.71%) and lastly the leaf (3.68%) crude extracts (Table 17).

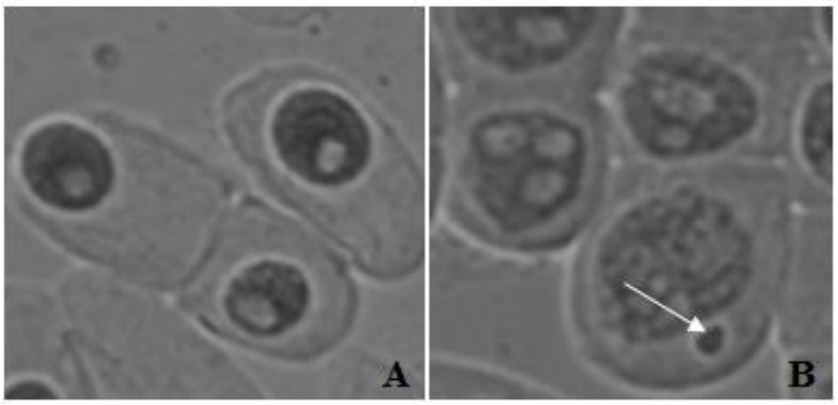

Figure 44: Chromosome aberrations observed in *A. cepa* meristematic cells exposed to water extracts of *T. violacea*. (A), normal interphase (B), micronucleus indicated by arrow. Magnification 400×.

Table 17: Genotoxic effects of *T. violacea* crude extract on cells of *A. cepa* micronucleus assay.

Treatments		
Concentration (µg/ml)	**Mean no. of interphase cells examined ± SEM**	**%Micronucleus ± SEM**
Leaves		
100	876 ± 53.34*	1.20 ± 0.30*
250	653 ± 128.32	1.24 ± 0.60
500	577 ± 9.34	1.41 ± 0.27*
1000	464 ± 57.97	3.68* ± 1.42*
Stem		
100	589 ± 80.99*	2.07 ± 0.51*
250	736 ± 75.81*	2.26 ± 0.80*
500	602 ± 34.26	2.86 ± 0.63*
1000	539 ± 115.33	3.74 ± 0.93*
Roots		
100	713 ± 39.91*	1.57 ± 0.16*
250	674 ± 111.10	1.66 ±0.08*
500	588 ± 79.76	1.98 ± 1.16
1000	370 ± 39.94	3.71 ± 0.64*
Ethidium Bromide		
100	914 ± 29.28*	0.40 ± 0.05
250	914 ± 47.40*	0.41 ± 0.13
500	934 ± 24.64*	0.92 ± 0.68*
1000	939 ± 35.99*	1.11 ± 0.30*
Distilled water	464 ± 57.97	0.31 ± 0.08

Data represent the mean ± SEM, n = 3. (*) represents statistically significant groups compared to the negative control (distilled water) according to the t-test: Two-Sample Assuming Equal Variance ($p < 0.05$).

The frequency of micronucleated cells was concentration dependent with the highest number of frequencies observed at 1000 µg/ml (Fig. 45). The stem water extracts produced the highest number of micronucleated cells with a mean value of 21.11 at 1000 µg/ml when compared to the values of 16.33 and 11.56 for the leaf and root extracts, respectively. The lowest frequency of micronucleated cells was observed in the negative control (distilled water) with a value of 1.00 whereas the positive control had a value of 10.44 at the highest concentration of 1000 µg/ml.

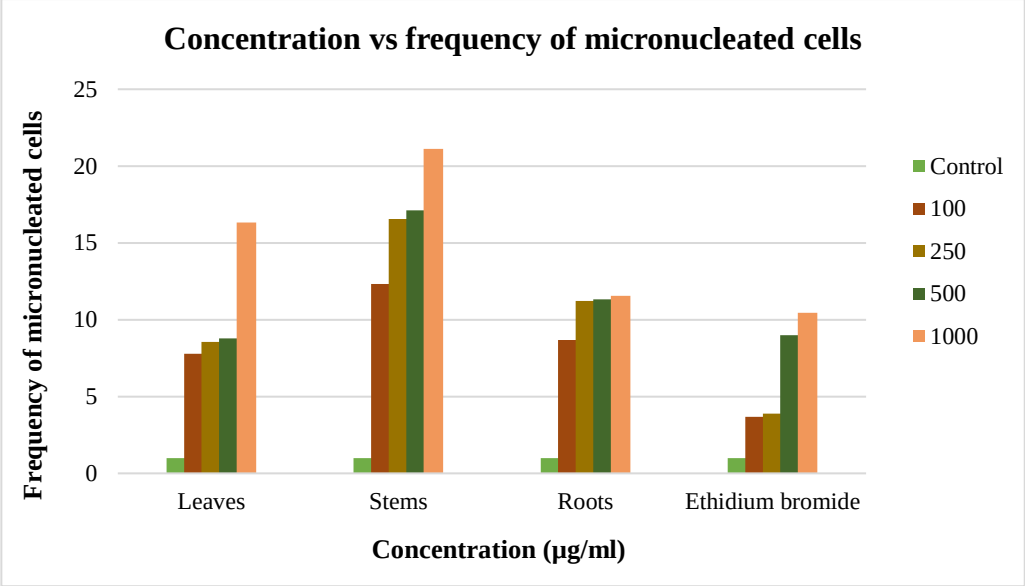

Figure 45: Frequency of micronucleated cells from the leaves, stem and roots water extracts of *T. violacea.* The positive (ethidium bromide) and negative (distilled water) controls are also included.

CHAPTER 5

5. DISCUSSION

5.1. Introduction

This chapter outlines the discussion of the results of the study. The chapter is a summative conclusion for each of the research objectives.

5.2. Discussion

5.2.1. Preliminary phytochemical screening

5.2.1.1. Qualitative phytochemical analysis

The analysis and characterization of bioactive compounds from plants is important to ascertain their medicinal value (Sasidharan *et al.* 2011). This study showed that pharmacologically active compounds such as tannins, terpenoids, flavonoids, saponins, proteins, steroids, cardiac glycosides, phenols and coumarins were present in some organs of *T. violacea* (Table 2). However, phlobatannins, leucoanthocyanins, alkaloids, carbohydrates and anthocyanins were absent in all plant parts (Table 2). An interesting aspect of this study is that the leaves of the plant contained more active compounds than those present in the stem and roots when both water and 70% ethanol were used as extractants. This has importance in conserving the species. Many medicinal plants are being overexploited and are in danger of becoming extinct (Bentley 2010). Since most of the bioactive compounds are present in the leaves of *T. violacea*, it is therefore possible to harvest the leaves while leaving the other parts, especially the underground rhizome of the plant, intact to regenerate itself.

Various chemicals have been used to extract bioactive compounds from plants. In this study, the water and 70% ethanol showed differential extraction of some of the compounds not only within the same organ but also in the different organs of the plant. For example, ethanol extracted less saponins in the leaves while it extracted more flavonoids in the stem when compared to water. The differential extractions may be due to degrading enzymes that may be active or denatured in either of the two extractants. For example, the enzyme polyphenol oxidase degrades polyphenols in water extracts, whereas in ethanol they are inactive (Lapornik *et al.* 2005). The important lesson from this study is that a single solvent may not necessarily extract all the useful bioactive compounds from a plant. Several solvents may have to be used to obtain the best yields of specific compounds. This study also showed that the roots yielded the least number of compounds overall.

With the exception of the 70% ethanol extracts, saponins were present in all parts of the plant with higher quantities observed in the water extracts of the leaves and roots (Table 2). Several studies have outlined the biological importance of saponins that include anti-inflammatory, anti-diabetic, anti-HIV and anti-atherosclerotic properties (Kashiwada *et al.* 1998; Banno *et al.* 2004). Flavonoids were found in all plant extracts, with the highest quantities observed in the water extracts of the leaves and 70% ethanol extracts of the leaves and stem (Table 2). Flavonoids have been reported to possess a wide variety of biological activities among which are antimicrobial, anti-inflammatory, antiangiogenic, analgesic, antiallergic effects, cytostatic and antioxidant, antiviral, anticarcinogenic, anticancer as well as anti-diarrheal properties (Middleton *et al.* 2000; Cushnie *et al.* 2005; Schuier *et al.* 2005; Maikai *et al.* 2009; Cushnie *et al.* 2011). This corresponds to the diverse use of the leaves of *T. violacea* for the treatment of oral fungal infections, gastrointestinal ailments, fever and colds (Burton 1990; Dyson 1998; Kubec *et al.* 2002; Ncube *et al.* 2011a).

Proteins were present in all plant parts, with higher quantities observed in the ethanol extract of the leaves. This means that the nutritional value of these species as a protein supplement cannot be ignored. Studies have reported that the protein hydrolytes from various sources possess antioxidant activity (Shah *et al.* 2006; Luziatelli *et al.* 2010).

Coumarins were also present in all plant parts. Overall, the *T. violacea* plant has high quantities of coumarins with smaller quantities observed only in the 70% ethanol extract of the roots. There are several biological activities that have been reported for coumarins, among which are anti-inflammatory, anticoagulant, antibacterial, antifungal, antiviral, anticancer, antihypertensive, antitubercular, anticonvulsant, antiadipogenic, Cytochrome P450 inhibiting, antihyperglycemic (Venugopala *et al.* 2013), antioxidant, estrogenic, dermal photosensitizing, antihelmentic, hypnotic, analgesic, hypothermic, antiulcer (Monga *et al.* 2012) anticlotting, hypotensive and antitumor activities (Leal *et al.* 2000).

Cardiac glycosides were present in high quantities in all the parts of *T. violacea* except in the water extract of the leaves. Glycosides are natural cardioactive drugs used in the treatment of congestive heart failure and cardiac arrhythmia (Brian *et al.* 1985; Ikeda *et al.* 1995; Denwick 2002). The presence of cardiac glycosides in *T. violacea* may support the usefulness of this plant for the treatment of cardiac diseases (Okwu 2001).

Terpenoids were present in high quantities in most parts of the plant except for the lower quantities observed in the ethanol extracts of the stem and roots. They are known to possess a

wide range of biological activities including antimicrobial, antifungal, antiparasitic, antiviral, antiallergenic, antispasmodic, antihyperglycemic, anti-inflammatory, immunomodulatory properties (Wagner *et al.* 2003; Rabi *et al.* 2009), antimalarial, inhibition of cholesterol synthesis, antibacterial (Mahato *et al.* 1997) and insecticidal properties (Sultana *et al.* 2008). They are also important in the prevention and therapy of several diseases, including cancer (Kappers *et al.* 2005).

Steroids were only observed in the ethanol extracts of the stem with a slightly lower amount in the ethanol extract of the roots. They are responsible for reducing cholesterol levels, for regulating the immune response (Shah *et al.* 2009) and some steroids also have immune-enhancing benefits (Berges *et al.* 1995; Donald *et al.* 1997). Tannins were absent in the 70% ethanol extract of the roots but were present in all the other parts of the plant. The presence of tannins in plants can be affected by the developmental stage of the plant and also by environmental factors (Hatano *et al.* 1986; Salminen *et al.* 2001). Tannins possess a wide variety of biological activities among which are antimicrobial, anti-viral (Lu *et al.* 2004), antibacterial (Akiyama *et al.* 2001) and antiparasitic effects (Kolodziej *et al.* 2005) . Studies have also investigated and reported on the ability of tannins to inhibit HIV replication selectivity and their use as a diuretic (Haslem 1989).

Similar to tannins, phenols were absent in the 70% ethanol extract of the roots but were present in the other parts of the plant (Table 2). It is not clear why the ethanol extract of the roots did not contain phenol (Table 2) since ethanol extracts of the leaves and stems did contain phenol. Several studies have reported that environmental factors, such as soil composition, temperature, rainfall and the incidence of ultraviolet radiation can influence the concentration of phenolic compounds in plants (Kouki *et al.* 2002; Monteiro *et al.* 2006). There are various biological activities have been reported for phenols in plants, among which are antitumor, antiviral, antimicrobial (Robbins 1980) and hypotensive effects (Matsubara *et al.* 1985) as well as antioxidant properties (Robak *et al.* 1988).

The only phytochemical studies on *T. violacea* were conducted by Soyingbe (2012) and Ncube *et al.* (2011b). Soyingbe (2012) examined the essential oils from the roots of *T. violacea* and reported the presence of tannins, alkaloids, and flavonoids while anthraquinones, cardiac glycosides and saponins were absent. Ncube *et al.* (2011b) examined the seeds and the whole plant (leaves and roots) of *T. violacea* and recorded the presence of phenols, flavonoids, gallotannins, condensed tannins and saponins. The results of Soyingbe (2012) and Ncube *et al.*

(2011b) are quite similar to those reported in this research except for the absence of alkaloids in all the plant parts of *T. violacea*. One of the reasons may be that Soyingbe (2012) used essential oils of *T. violacea* instead of plant extracts for the phytochemical analysis. Environmental extremities such as light, temperature, and drought (Ncube *et al.* 2012) may influence the synthesis/ content of certain compounds Penuelas *et al.* (1997) . The class, content and quantity of the compounds may be different depending on the ecological factors present in the area where the plant is cultivated (Liu *et al.* 2015). Whether environmental conditions and genetic variation are factors to be considered for the variation in this research study is a matter of conjecture and needs further studies.

5.2.1.2. Quantitative phytochemical analysis

Table 3 revealed that the total phenolics ranged from 191 to 3.59 mg/g gallic acid equivalent for the water extracts and from 0.98 to 0.15 mg/g gallic equivalent for the ethanol extracts (standard curve for gallic acid Fig. 13). The concentration of total flavonoids ranged from 0.47 to 0.025 mg/g quercetin equivalent for the water extracts and from 0.66 to 0.038 mg/g quercetin equivalent for the ethanol extracts (standard curve for quercetin Fig. 14). This data reveals that the water and 70% ethanol extracts were less effective in extracting flavonoids and phenols from the stems and roots than from the leaves of the plant. However, the data obtained in this study was different from those reported by Olorunnisola *et al.* (2011) who used 100% methanol extracts from fresh and dried *T. violacea* roots and from that of Narendhirakannan *et al.* (2010) who used ethanol extracts from three varieties of *A. sativum* L. The amounts of phenolic compounds from this study was lesser than what was obtained in the fresh and dried root samples as well as in the three varieties of *A. sativum.* These differences may be attributed to the microclimate, processing method as well as the type of solvent employed (Choi *et al.* 2008; Tiwari *et al.* 2011) and genetic variation (Tulay 2012). The high levels of phenolic compounds in the leaves support the medicinal importance of the plant in the management and treatment of oxidative stress induced disorder (Olorunnisola *et al.* 2011). In a study conducted by Ncube *et al.* (2011b), 50% methanol extracts from the whole plant (bulb and leaves) of *T. violacea* were used to determine total phenolic compounds. However, the latter study used an aqueous-methanol solution and different units making it difficult to conduct a comparison of the data obtained with what was reported in this study.

5.2.2. MTT cell proliferation assay

Tulbaghia violacea has been used as a remedy for the treatment of various diseases including pulmonary tuberculosis, HIV/AIDS and cancer (Burton 1990; Dyson 1998; Kubec *et al.* 2002;

Ncube *et al.* 2011a). However, there is contradictory evidence regarding the toxicity and safety of some of the species within the genus *Tulbaghia* (Jäger *et al.* 2012). In this study, the effects of crude water and ethanol extracts from the leaves, stems and roots of *T. violacea* on the viability of a murine macrophage (RAW 264.7) and a skeletal muscle (myoblasts or C2C12) cell line *in vitro* were examined following a 2^3 factorial design as explained in the materials and methods. Water has been used for centuries as an extraction solvent by traditional herbalists (Eloff 1998; Grierson *et al.* 1999; Shale *et al.* 1999; Kelmanson *et al.* 2000; Inngjerdingen *et al.* 2004). Ethanol on the other hand could be used as an alternative to water by traditional herbalists because it is an inexpensive and readily available solvent (Louw *et al.* 2002). Furthermore, the ethanol molecule unlike the water molecule is amphipathic and as a result enables the extraction of both polar and nonpolar compounds.

5.2.2.1. RAW 264.7 cell line

5.2.2.1.1. Experimental observations for the leaf stem and root extracts.

In the experiment the effect of three independent variables of concentration, A (10 and 1000 µg/ml), incubation time B, (24 and 48 h) and type of extracts (water or ethanol) on the crude extracts of the leaves, stem and roots of *T. violaceas* were analyzed using a 2^3 factorial design (Table 4 and 5). The optimum percentage cell viability was predicted for the leaf, stem and root extracts of *T. violacea* with the conditions set in Table 4 using Design expert 6.0.6. The results demonstrated that water extracts at a concentration of 10 µg/ml and incubation time of 24 h should produce the optimum percentage of viable cells. Optimum values of 79.90%, 53.29% and 54.39% (Table 5) should be expected for the leaves, stems and roots extracts, respectively. The predicted values for cell viability were almost similar to the actual values as shown in Table 5.

5.2.2.1.2. Half normal plots for the leaf, stem and root extracts.

According to this study, the main effect of time (B), as well as the interactions: concentration x extract (AC), time x extracts (BC), and concentration x time (AB) with regards to the leaves of *T. violacea* had no significant effect on cell viability (Fig. 15a). The sequence, in decreasing order, of the main effect and interaction terms that significantly influenced cell viability from crude extracts of the leaves of *T. violacea* was C > A and ABC. The main effect of time (B), the interactions: concentration x time (AB), and concentration x time x extract (ABC) with regards to the stems from *T. violacea* also showed no significant effect on cell viability (Fig. 15b). The sequence, in decreasing order, of the main effect and interaction terms that influenced cell viability due to crude extracts from the stems of *T. violacea* was A > C > BC and AC.

Lastly, the main effect of time (B) and interactions: concentration x extract (AC), concentration x time x extract (ABC), and time x extracts (BC) concerning the root extracts had no significant effect on cell viability (Fig. 15c). The sequence, in decreasing order, of the main effect and interaction terms that influenced cell viability when the crude extracts of roots were used was A > C and AB. Overall, the results from the half-normal plots (Fig. 15) revealed that concentration (A) and type of extract (C) had a greater significant effect on cell viability as opposed to the other variables and interactions for all plant parts. The main effect of time was non-significant in all plant parts. The interaction of the three factors: concentration x time x extract (ABC) had an effect of cell viability only with the leaf extracts. The interactions: concentration x extract (AC) and time x extract (BC) had a significant effect on cell viability only with the stem extracts while the interaction: concentration x time (AB) had a significant effect on cell viability only with the root extracts.

5.2.2.1.3. Main effects and interaction plots for the leaf, stem and root extracts.

The main effects and interaction plots for the leaf extracts are shown in Fig. 10. An increase in concentration (A) or type of extract (C) decreased cell growth significantly while incubation time (B) had an almost imperceptible decrease on cell viability. The effects of the interaction between concentration x incubation time (AB), concentration x extract (AC) and time x extract (BC) produced parallel lines indicating that there were no interaction effects on cell viability (Fig 10). The main effects and interaction plots data for the stem extracts are shown in Fig. 19. The main effects (concentration, time, extracts) for the stems on cell viability were similar to that of the leaves while the interactions were different. There was no significant effect when the concentration x time (AB) was increased while the interaction between concentration x extract (AC) and time x extract (BC) were significant. These results would have not been observed in a one-factor-at-a-time (univariate) statistical analysis (Bezerra *et al.* 2008). The main effects and interaction plots for the root extracts are shown in Fig. 22. The main variables: concentration (A), incubation time (B) and type of extract (C) produced an effect similar to that of the leaf and stem extracts. With regards to the interactions, only the concentration x time effect (AB) was significant while the other two interactions AC and BC did not affect cell viability.

5.2.2.1.4. Regression model analysis and ANOVA of leaf, stem and root extracts.

For the leaf extracts (Table 6), the F-value of 19.16 implies the model was significant: there was only a 0.01% chance that an F-value this large could have occurred due to experimental error. The full regression model had a R^2 value = 0.7479. The low p-values ($p < 0.0001$ level)

of the concentration (A) and extraction time (C) suggests that they had the most significant contribution on the percentage viability of the cells (Table 6). The interaction between all three factors (ABC) also had a significant ($p = 0.0390$) effect on cell viability. The regression model developed from the leaf extracts of *T. violacea* indicated that A, C and ABC were significant model terms whereas B, AB, AC and BC were non-significant (Table 6). The p-value less than 0.05 indicated that the model terms are significant whereas values greater than 0.1 indicated non-significance. A comparison of the predicted and adjusted R^2 values was conducted to confirm the adequacy of the regression model. The data is rendered adequate when the predicted R^2 value is high and is less than or nearer to the adjusted R^2 value (Montgomery 2013). The predicted R^2 value of 0.6370 was close to the adjusted R^2 value of 0.7101. This indicated that there was reasonable agreement between both R^2 values, confirming that the model developed for the leaf extracts was desirable.

For the stem extracts (Table 7), the F-value of 21.76 implies the model was significant and there was only a 0.01% chance that an F-value this large could have occurred due to experimental error. The full regression model had a calculated R^2 value of 0.905. The low p-value ($p < 0.0001$) of the concentration (A) suggests that it had the most significant contribution on the percentage cell viability (Table 7). The main effect of the extracts (C), as well as the interactions between the concentration x extract (AC) and the time x extract (BC) also had significant effects on the percentage viability of the cells with p-values of 0.0002, 0.0190 and 0.0177, respectively. The regression model developed from the stem extract of *T. violacea* indicated that A, C, AC and BC were significant model terms whereas B, AB and ABC were non-significant (Table 7). The predicted R^2 value of 0.7861 was close to the adjusted R^2 value of 0.8633. This means that there was reasonable agreement between both R^2 values confirming that the model designed for the stem extracts was desirable.

Lastly for the root extracts (Table 8), the F-value of 8.65 implied that the model was significant, and that there was only a 0.01% chance that a Model F-value this large could have occurred by chance. The full regression model had a calculated R^2 value of 0.7479. The low p-values ($p < 0.0001$) of the concentration (A) and extracts (C) suggests that they had the most significant contribution on the percentage cell viability (Table 8). The interaction between concentration x time (AB) also had significant effects on the percentage viability of the cells with $p = 0.0491$. The regression model developed from the roots of *T. violacea* indicated that A, C and AB were significant model terms whereas B, AC, BC and ABC were non-significant terms (Table 8).

The predicted R^2 value of 0.5421 was close to the R^2 adjusted value of 0.7075 which are in reasonable agreement, confirming that the model for the root extracts was also desirable.

5.2.2.1.5. Percentage cell viability for the leaf, stem and root extracts.

Using numerical optimization, the values for the 25, 50, 100, 250 and 500 µg/ml concentrations were determined and used to calculate the overall percentage of viable cells. This study showed that for RAW 264.7 cells, the concentration and type of plant extract (water or ethanol) (Fig. 17, 20 and 23) had a substantial influence on cell viability whereas the time of growth did not. Alvarez *et al.* (2005) grouped the toxicity of compound into four classes; class 1 (high toxicity) with cell viability values between 0 to 25%, class 2 (moderate toxicity) with cell viability values between 26 to 50%, class 3 (low toxicity) with cell viability values between 51 to 75% and lastly class 4 (non-toxic) with percentage viability values greater than 75%. Overall, treatment of the cells with the ethanol extracts from the leaf, stem and roots of *T. violacea* was toxic (% cell viability < 50%) (Alvarez *et al.* 2005) even at a concentration of 10 µg/ml at both 24 and 48 h incubation periods (Fig. 17, 20 and 23). In another study, Van Huyssteen *et al.* (2011) reported that an ethanol extract from the whole plant of *T. violacea* was toxic to Chang liver cells at the higher levels of 62.5 and 125 µg/ml. The water extract of the leaves was moderately toxic to the RAW 264.7 cells at the highest concentration of 1000 µg/ml (Fig. 17) while that of the stems was moderately toxic at a concentration of 25 µg/ml (Fig. 20). In the case of the roots, concentrations higher than 100 µg/ml were moderately toxic (Fig. 23). In general, there was a significant reduction in the number of viable cells at concentrations higher than 250 µg/ml for all the parts of the plant. This suggests that high concentrations of crude extracts of *T. violacea* are cytotoxic to RAW 264.7 cells. This is the first report that shows that crude extracts of certain concentrations of *T. violacea* can be cytotoxic.

5.2.2.1.6. Morphological effects of plant extracts on RAW 264.7 cells.

The morphological effects of the extracts of *T. violacea* on the RAW 264.7 cells are discussed in this section and shown in Fig. 18, 21 and 24 for the leaves, stems and roots, respectively. There was wide variability in the cell morphologies that was dependent on the concentration and type of extracts, time of incubation as well as the plant parts. The cells treated with DMEM (negative control) appeared smooth and well-rounded with some having a single pointy end that is typical of RAW 264.7 cells (Fig. 18a, 21a, 24a). The cells treated with 50% H_2O_2 (positive control) appeared clumped together, shrunken, with irregular edges (Fig. 18f, 21f, 24f). Compared to the negative and positive controls, the cells treated with the two concentrations of extracts from the plant organs showed different cell configurations at the two

tested times. The most common abnormalities included clumping on the surface of the medium, shrinkage, vacuolisation and detachment from the culture flask (Fig. 18, 21 and 24). These morphological changes are often observed in apoptotic cells (Darzynkiewicz *et al.* 1992; Desagher *et al.* 2000).

For the leaf extracts of *T. violacea* (Fig. 18), incubation of the cells with 10 µg/ml of the leaf water (Fig. 18b and d) extracts did not show any growth abnormalities whereas the 1000 µg/ml (Fig. 18c and e) treatment changed the common smooth shape of the cells to roughly elongated cells after the 48 h incubation. For the leaf ethanol extracts, the 10 µg/ml (Fig. 22g and i) treatments also changed the common shape of the cells to being slightly elongated. However, treatment with 1000 µg/ml (Fig. 18h and j) produced an aggregation of dense irregular cellular debris with no recognition of intact cells that was similar to the positive control group. For the stem extracts (Fig. 21), all the 10 µg/ml treatments for both water (Fig. 21b and d) and ethanol (Fig. 21g and i) extracts appeared to have similar morphological characteristics with the cells appearing roughly elongated. Cell treated with 1000 µg/ml of water (Fig. 21c and e) and ethanol (Fig. 21h and j) extracts produced an aggregation of dense irregular cellular debris with no recognition of intact cells similar to the positive control. Lastly, cells treated with 10 µg/ml root extracts (Fig. 24) produced similar morphologies to that of the stem extracts for both water (Fig. 24b and d) and ethanol (Fig. 24g and i) extracts with cells appearing roughly elongated. Similarly, cells treated with 1000 µg/ml for both water (Fig. 24c and e) and ethanol (Fig. 24h and j) extracts produced an aggregation of dense irregular cellular debris with no recognition of intact cells similar to the positive control group. In general, the ethanol extracts of all the plant parts were cytotoxic. In the case of the water, only the stem and root extracts appeared to have a greater cytotoxic effect on the cells which also appeared to be dependent on time (24 versus 48 h).

5.2.2.1.7. Half maximal inhibitory concentration (IC_{50}).

The half maximal inhibitory concentration (IC_{50}) results indicated a strong cytotoxic effect when the RAW 264.7 cells were treated with the ethanol extracts from leaves, stems and roots of (Table 9). According to Mahavorasirikul *et al.* (2010) compounds with IC_{50} values below 20 µg/ml are considered to be toxic. The IC_{50} values for the ethanol extracts from all three organs of *T. violacea* were below 20 µg/ml. The ethanol leaf extracts were highly toxic to the RAW 264.7 cells with IC_{50} values of 0.010 (0.00077 to 0.1426) µg/ml and 2.177 (0.6176 to 7.671) µg/ml for 24 and 48 h, respectively. However, the water extracts of the leaves and roots were non-toxic except for that of the stem which had a higher IC_{50} value of 22.49 (13.87 to 36.87) at

24 h incubation and showed toxicity after 48 h incubation (IC_{50} = 9.475 (4.061 to 23.39)). This data is interesting as it suggests that the stem extracts of *T. violacea* are toxic to the RAW 264.7 cells compared to that of leaf and root extracts. The study suggests that phytochemicals present in *T. violacea* are cytotoxic to RAW 264.7 cells. This may be attributed to the report of Jordan *et al.* (2010) which associated the toxic activities of the heart, liver, blood, kidney, central nervous system, gastrointestinal disorder such as diarrhoea, and less frequently carcinogenesis and teratogenicity (Teixeira *et al.* 2003) with phytochemicals present in plant material.

The root water extracts showed greater inhibition on the growth of the RAW 264.7 than that of leaf extract at both 24 and 48 h incubation. The findings from this study are different from that of Bungu *et al.* (2006) which showed that the inhibitory effects of *T. violacea* root extracts were higher than those of the leaf extracts. However, Bungu *et al.* (2006) used methanol as an extraction solvent instead of water. It is known that methanol is more polar than ethanol, but it is also cytotoxic in nature and hence may not be an ideal extraction solvent in some situations (Tiwari *et al.* 2011). The findings of this study also appear to be contrary to the report of Saibu *et al.* (2015) which demonstrated that the water extracts of the leaves of *T. violacea* inhibited cell cultures more than extracts from the roots.

5.2.2.2. C2C12 cell line

5.2.2.2.1. Experimental observations for the leaf, stem and root extracts.

This part of the study assessed the effect of three independent variables of concentration A, (10 and 1000 µg/ml), incubation time B, (24 and 48 h) and type of extracts (water or ethanol) on the viability of C2C12 cells with crude extracts from the leaves, stems and roots of *T. violacea.* The experimental design was a 2^3 factorial design (Table 10 and 11) as explained in the materials and methods. The optimum percentage cell viability was predicted for the leaf, stem and root extracts of *T. violacea* with the conditions set in Table 10 using Design expert 6.0.6. The results predicted that water extracts at a concentration of 10 µg/ml and incubation time of 24 h should produce the optimum percentage of viable cells. Optimum values of 118.58%, 92.49% and 82.22% (Table 11) should be expected for the leaves, stems and roots extracts, respectively. The predicted values for cell viability were almost similar to the actual values as shown in Table 11 confirming the adequacy of the designed model.

5.2.2.2.2. Half normal plots for the leaf, stem and root extracts.

This part of the study showed that the interactions between time x extracts (BC) and concentration x time x extract (ABC) with regards to the leaves of *T. violacea* had no effect on

cell viability (Figure 25a). The sequence, in decreasing order, of the main and interaction terms that significantly influenced cell viability with crude extracts from the leaves was A > B > AC > C > and AB. The main effect of the extracts (C) and the interactions: the concentration x time (AB), concentration x extract (AC), time x extracts (BC) for the stems had a non-significant effect on cell viability (Figure 25b). The sequence, in decreasing order, of the main- and interaction terms that significantly influenced cell viability because of crude extracts from the stems of *T. violacea* was A > B and ABC. Lastly, the main effect of time (B) and the interactions: concentration x extract (AC), concentration x time (AB), and time x extracts (BC) for the roots had no significant effect on cell viability (Figure 25c). The sequence, in decreasing order, of the main and interaction terms that significantly influenced cell viability by the crude extracts of the roots was A > C and ABC. Overall, the results from the half-normal plots (Fig. 25) revealed that for all the plant parts of *T. violacea* (leaves, stems and roots), the main effect of concentration (A) had a significant effect on cell viability while the main effect of time (B) was only significant for the leaf and stem extracts. The time x extract (BC) interaction was non-significant in all plant parts whereas the concentration x time (AB) and the concentration x extract (AC) interactions were non-significant only for the stem and root extracts. The main effect of the extract (C) had a significant effect only in the leaf and root extracts while the interaction of the three factors concentration x time x extract (ABC) had an effect of cell viability when the stem and root extracts were evaluated.

5.2.2.2.3. Main effects and interaction plots for the leaf stem and root extracts.

The main effects and interaction plots for the leaf crude extracts are shown in Fig. 26. An increase in the three main effects of concentration (A), incubation time (B) and extracts (C) decreased cell viability. The interaction of concentration x incubation time (AB) and concentration and extract (AC) were significant. However, the interaction of incubation time x extracts (BC) was non-significant. For the stem crude extracts (Fig. 29) the two main effects: concentration (A) and incubation time (B) decreased cell viability as these parameters increased. But the main effect of the extracts (C) did not affect cell viability appreciably. Unlike in the leaves, the interaction of increasing both concentration and incubation time (AB), concentration and extract (AC) as well as the incubation time and extracts (BC) produced a non-significant effect on cell viability. Lastly for the roots (Fig. 32), the two main effects of concentration (A) and type of extract (C) decreased cell viability while time (B) had no effect. Both the stem and root extracts produced non-significant interactions between concentration x

time (AB), concentration x extract (AC) as well as the incubation time and extracts (BC) interactions.

5.2.2.2.4. Regression model analysis and ANOVA for the leaf stem and root extracts.

For the leaf extracts (Table 12), the F-value of 20.81 implies that the model was significant; there was only a 0.01% chance that a Model F-value this large could have occurred due to experimental error. The full regression model had a calculated R^2 value of 0.9010. The low p-value ($p < 0.0001$) with regards to the concentration (A) suggests that it had the most significant contribution on the percentage viability of the cell (Table 12). The study also showed that time (B) ($p = 0.001$) and extracts (C) ($p=0.0072$), as well as the interactions: concentration x extract (AC) ($p=0.0155$) and concentration x time (AB) ($p=0.0023$) affected cell viability significantly. p-values lower than 0.0500 indicated that model terms are significant whereas p values greater than 0.1000 indicated the model terms are non-significant. The regression model developed from the stems of *T. violacea* indicated that A, B, C, AB and AC were significant model terms whereas BC and ABC were non-significant (Table 12). The predicted R^2 value of 0.7773 was closer to the adjusted R^2 value of 0.8577 which are in reasonable agreement, confirming that the designed model for the leaf extracts contained high quality data.

For the stem extracts (Table 13), the F-value of 20.28 implies the model was significant. The full regression model had a calculated R^2 value of 0.8987. Similar to the leaf extracts, the low p-value ($p < 0.0001$) of the concentration (A) suggests that it had the most significant contribution on the percentage cell viability (Table 13). The main effect of time (B) ($p= 0.0003$) and the interaction: concentration x time x extract (ABC) ($p= 0.0415$) also influenced cell viability significantly. The regression model developed from the stems of *T. violacea* indicated that A, B and ABC were significant model terms whereas C, AB, AC and BC were non-significant. A comparison of the predicted and adjusted R^2 values showed that these values were quite close confirming that the model designed for the stem extracts fit the data well. Lastly for the root extracts (Table 14), the F-value of 10.14 implies the model was significant. The full regression model had a calculated R^2 value of 0.8161. Similar to the leaf and stem extracts, the low p-value ($p < 0.0001$) of the concentration (A) suggests that it had the most significant contribution on cell viability (Table 14). The effect of the extracts (C) as well as the interaction between the concentration x time x extract (ABC) had significant effects on the percentage viability of the cells with p-values of 0.0108 and 0.0387, respectively. The regression model developed from the stems of *T. violacea* indicated that A, C and ABC were significant model terms whereas B, AB, AC and BC were non-significant. The predicted and

adjusted R^2 of 0.5862 and 0.7356 are quite close confirming that the designed model for the root extracts fit the data.

5.2.2.2.5. Percentage cell viability for the leaf, stem and root extracts.

Using numerical optimization from the Design expert 6.0.6 software, the values for the 25, 50, 100, 250 and 500 µg/ml concentrations were determined and used to calculate the overall percentage of viable cells. This study showed that for C2C12 cells an increase in the concentration and incubation time (24 or 48 h) (Fig. 27, 30 and 33) decreased cell viability. Lower concentrations of water and ethanol extracts (10 to100 µg/ml) were non-toxic towards the C2C12 cells since cell viability was greater than 50% (Alvarez *et al.* 2005). The water extract of the leaves was only moderately toxic (% cell viability <50%) to the C2C12 cells after a 48 h incubation period and at the highest concentration of 1000 µg/ml (Fig. 27). The effect of time also influenced cytotoxicity of the C2C12 for the ethanol extracts. For example, ethanol extracts of the leaves were moderately toxic at 1000 µg/ml for 24 h, but at 48 h concentrations higher than 250 µg/ml were found to be moderately toxic. Both the water and ethanol extracts of the stems showed moderate and high toxicity, respectively after 24 h at 1000 µg/ml (Fig. 30). But after 48 h of incubation, even lower concentrations of the ethanol and water extracts of the stem showed moderate to high toxicity. For the ethanol extracts, toxicity was observed at concentrations that were greater than 500 µg/ml for 24 h and 100 µg/ml after 48 h while for water it was greater than 250 µg/ml. Lastly, for the water and ethanol extracts of the roots (Fig. 33), moderate and high toxicity were observed at concentrations greater than 500 µg/ml and 250 µg/ml respectively, at 24 and 48 h. In general, the viability of the C2C12 cells was reduced when the concentration of the extracts of all parts of *T. violacea* was greater than 250 µg/ml. Furthermore, the crude extracts from the leaves of *T. violacea* were the least toxic to the C2C12 cells.

5.2.2.2.6. Morphological effects of plant extracts on C2C12 cells.

There was wide variability in the effect of the extracts on the C2C12 cells. These are discussed below. The cells treated with DMEM (negative control) (Fig. 28a, 31a, 34a) appeared thin and elongated with two tapering ends. Treatment with 50% H_2O_2 (positive control) produced slightly rounded with variable sizes that aggregated in the form of dense irregular cellular debris. Widespread cell death occurred since no intact cells were visible (Fig. 28f, 31f, 34f). For the water extracts of the leaves, no cell abnormalities were visible (Fig. 28) when they were incubated with 10 µg/ml (Fig. 28b and d) whereas those cells treated with 1000 µg/ml (Fig. 28c and e) appeared roughly elongated and rounded after the 48h incubation. With the ethanol

extracts of the leaves, the 10 µg/ml (Fig. 28g and i) treatments also produced roughly elongated cells. However, after treatment with 1000 µg/ml (Fig. 28 h and j) the cells appeared more or less like those in the positive control. For the stem extracts (Fig. 31), all the cells treated with 10 µg/ml for both the water (Fig. 31b and d) and ethanol (Fig. 31g and i) showed normal but slightly longer cells. Similar observations were found for the 1000 µg/ml concentrations for both water (Fig. 31c and e) and ethanol (Fig. 31h and j) extracts. The behaviour of the cells for the root extracts (Fig. 34), was similar to that of the stem extracts where all the 10 µg/ml treatments for both water (Fig. 34b and d) and ethanol (Fig. 34g and i) appeared roughly elongated. Likewise, the 1000 µg/ml concentrations for both water (Fig. 34c and e) and ethanol (Fig. 34h and j) extracts also formed numerous roughly rounded cells of variable sizes and a dense aggregated cellular debris similar to that observed in the positive control.

5.2.2.2.7. Half maximal inhibitory concentration (IC_{50}).

The half maximal inhibitory concentration (IC_{50}) results indicated that the potential toxic activity of *T. violacea* crude extracts on C2C12 cells is non-significant (Table 15). This is due to the fact that IC_{50} value less than 20 µg/ml are regarded as highly toxic by Mahavorasirikul *et al.* (2010). For this research study, the IC_{50} value was greater than 30 µg/ml in all three plant parts with both the water and ethanol extracts regardless of the duration of stimulation (both 24 and 48 h). This part of the study showed that the plant extracts from the leaves, stems and roots of *T. violacea* are non-toxic to C2C12 cells. The inhibitory effect of the water and ethanol root extracts on the C2C12 cells was higher than that of similar extracts from the leaves at both 24 and 48 h incubation. This finding is in agreement with that of Bungu *et al.* (2006) who reported that the inhibitory effects of *T. violacea* root extracts were higher than that of the leaves. However, the findings of this study appeared to be contrary to the report of Saibu *et al.* (2015) who found that the inhibitory effects of water extracts of the leaves of *T. violacea* were much higher than water extracts of the roots in cell cultures.

5.2.3. *Allium cepa* assay

Allium cepa (onion) has been used as genetic models for the evaluation of genotoxic effects due to their ability to indicate the presence of mutagenic chemicals (Levan 1938; Fiskesjo 1985) and their kinetic characteristics of proliferation. The *A. cepa* assay allows for the evaluation of different endpoints which are mitotic index (MI), chromosome aberration (CA) assay and the micronucleus assay (MN). The mitotic index has been used as an indicator of cell proliferation biomarkers that effectively measures the proportion of cells in the mitotic phase of the cell cycle. Thus, a reduction in the mitotic index may be concluded as being due to cell death. The

chromosome aberration assay has been reported to be a highly efficient test to investigate potential mutations in a system that has been exposed to putative mutagenic or carcinogenic substances (Rank *et al.* 2002; Leme *et al.* 2008). The MN assay has been evaluated to be one of the most efficient, simple, and fast assays for the analysis of environmental mutagenic/ genotoxic effects (Türkoglu 2007).

5.2.3.1. *Allium cepa* assay: Root growth, Mitotic index (MI) and Chromosomal aberration analysis (CA) with water extracts.

Several morphological abnormalities were observed when the *A. cepa* roots were treated with the water extracts from the leaves, stems and roots of *T. violacea* especially as the concentration of the extracts increased. They included very short, bent, spiral, dark brown/blackish and crochet-like roots especially at 1000 µg/ml. Similar abnormalities were observed in the positive control (ethidium bromide). A decrease of over 45% in root length indicates the presence of toxic substances (Fiskesjo 1985) having sublethal effects on plants (Wierzbicka 1999). In this study, the decrease of over 45% in root length was variable and was dependent on the concentration of the extracts. For example, the leaf and root extracts inhibited root growth at concentrations above 250 µg/ml. However, for the stem extracts concentrations above 100 µg/ml decreased root growth.

According to the literature, a reduction in the mitotic index below 22% of the negative control is considered to cause lethal effects on test organisms while a reduction below 50% has sublethal effects (Sharma *et al.* 2012) and is called cytotoxic limit value. This definition is used in this study. For the leaf extracts, concentrations higher than 100 µg/ml produced a sublethal effect with mitotic index values of 43.71%, 27.78% and 24.54% for the 250, 500 and 1000 µg/ml concentrations, respectively. The 100 µg/ml concentration was non-toxic with a mitotic index value of 58.66%. The stem extracts, produced a sublethal effect on *A. cepa* at 100, 250 and 500 µg/ml with mitotic index values of 40.86%, 33.64% and 32.57%, respectively, whereas the 1000 µg/ml concentration produced a lethal effect with an MI value of 19.69%. The root extracts also produced a sublethal effect on *A. cepa* at concentrations higher than 100 µg/ml with MI values of 37.24%, 31.08%, and 22.59% for the 250, 500 and 1000 µg/ml concentrations, respectively. The 100 µg/ml concentration was also non-toxic with a mitotic index value of 58.88%. Treatment of *A. cepa* with distilled water (negative control) produced a non-toxic effect with an MI value of 61.83. Ethidium bromide has been found to be an extremely effective cytoplasmic mutagen which results in the loss or alteration of DNA and

RNA (Soslau *et al.* 1974). Ethidium bromide was used as a positive control for this study and was observed to be toxic at all tested concentrations with MI values below 22%.

The lower mitotic index values may be an indication of a direct genotoxic effect of *T. violacea* crude extracts. The decrease in mitotic index may be attributed to the obstruction of the onset of prophase, the arrest of one or more mitotic phases, or mainly due to the slowing of the rate of cell progression through mitosis (Christopher *et al.* 1988). Similar reasons may be applicable in this study for the lower mitotic indices. Microscopic analysis revealed a concentration-dependent reduction in mitotic indices (Table 16) with significant differences ($p < 0.05$) between treated groups and the positive control (ethidium bromide) when compared to the mitotic indices of the negative control (distilled water) value of 61.83%.

All the tested concentrations (100, 250, 500 and 1000 µg/ml) of the *T. violacea* plant parts produced different types of chromosomal aberrations in the *A. cepa* assay. Most aberrations in this study were observed in the metaphase and anaphase stages. Armbruster *et al.* (1991) and Kaymak *et al.* (2009) reported a similar observation upon treatment with herbicide dithiopyr and raxil, respectively, and concluded that it may be due to the structural aberration of spindle formation thus resulting in cell division disturbances.

The most common and frequent aberrations included binucleated cells, sticky chromosomes and c-mitosis that were followed by laggards, chromosomal bridges and trinucleated cells (Fig. 35-43. Laggard chromosomes (Fig. 35B) is usually due to failure of the chromosomes to get attached to the spindle fibre and to move to either of the two opposite poles (Tkalec *et al.* 2009). Chromosome bridges were frequently observed in the anaphase and telophase stages (Fig. 36B). Their formation may be attributed to the presence of chromosomal stickiness and subsequent failure of chromosomal separation during anaphase stage (Gömürgen 2005; Türkoglu 2008). Chromosomal bridges signal the clastogenic effect caused by chromosome disruptions or breakages. C-mitosis (Fig. 37B) results when dissociating disulphide bonds prevents spindle microtubules from assembling (Levan 1938). C- mitosis is an indication of a weak toxic effect which may be reversible (Fiskesjo 1985). Sticky chromosomes (Fig. 38B) is usually due to a physiological effect resulting from depolymerisation of DNA, partial dissolution of nucleoproteins, breakage and exchanges of the basic folded fibre units of chromatids and the stripping of the protein covering of DNA in chromosomes (Mercykutty *et al.* 1980). Stickiness may lead to incomplete separation of daughter chromosomes as a result of the cross-linkage of chromoproteins (Kong *et al.* 1999; Tkalec *et al.* 2009). Their presence

is an indication of a highly toxic and irreversible effect, probably leading to cell death (Rencuzogullari *et al.* 2001; Türkoglu 2007). The formation of binucleated/ trinucleated (Fig. 39A and B) cells may be attributed to the inhibition of cytokinesis (Khanna *et al.* 2013).

The percentage of aberrant cells shown in Table 16 was dependent on the concentration of the extracts and was variable for the different plant organs. The highest number of aberrations was observed with the highest concentration of the water extracts of the stem (20.91%) while the lowest was observed with the highest concentration (1000 µg/ml) of the leaf extracts. The aberrations due to the root extracts were almost intermediate between those observed for the stem and leaf extracts. One of the anomalies observed in this study is that the negative control (water) also induced a low number of chromosomal aberrations. There are thousands of cells in the meristematic zone of the roots that are undergoing mitosis at any one time. It is possible that errors in cell division may be expected under these circumstances. The presence of ions in the water may also cause minor chromosomal aberrations (Fiskesjo 1985). The presence of ions in the negative control was overlooked in this research and should be considered in future research. Nonetheless there was a significant difference in the number of chromosomal aberrations when the plant extracts were used compared to those in the negative control (distilled water).

5.2.3.2. Micronucleus assay

The presence of micronuclei bearing cells were also observed at interphase (Fig. 44B). The presence of a micronuclei may be a result of a consequence of clastogenic (chromosome breakage) or aneugenic (chromosome lagging and interference on the spindle behaviour) effect (Meng *et al.* 1992; Yi *et al.* 2003). For this study, a decrease in the percentage of micronucleated cells was variable and concentration dependent. The frequency and percentage of micronucleated cells was higher in the stem extracts than those of the leaves and roots (Fig. 45 and Table 17). The MN assay data confirms the possible genotoxic effect of the stem extracts of *T. violacea.* The positive control (ethidium bromide) had the lowest percentage of micronucleated cells due to the increased number of cells in interphase as opposed to those observed in the *T. violacea* plant extracts. This observation may be attributed to the fact that ethidium bromide prevents subsequent replication of DNA by arresting cell division (Brachet *et al.* 1970).

CHAPTER 6

6. CONCLUSION AND RECOMMENDATIONS

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 obtained data, concluding the thesis.

6.2. Conclusion

The determination of chemical constituents in plants and their potential toxicity are preliminary steps necessary for the discovery and development of novel therapeutic agents with improved efficacy. The phytocompounds present in *T. violacea* suggests that the plant is a potential source of chemotherapeutic compounds. In this study, most of the phytocompounds were found in the leaves of *T. violacea* validating their traditional use in the treatment of various ailments such as fever, colds, asthma, tuberculosis, esophagus cancer, high blood pressure, stomach problems such as gastroenteritis, abdominal pains. The two solvents used in this study were able to extract different bioactive compounds. For example, the water extract of the leaves showed the highest yield of total phenolic content (3.53 ± 0.1 mg of GAE/g of extract) while the highest yields of total flavonoid content (0.66 ± 0.01 mg of QE/g of extract) was obtained with 70% ethanol. This constitutes vital information for those wishing to extract compounds from this plant. This study proved that different factors affect the quantity and composition of the phytocompounds present in an extract such as nature of the solvent, the concentration as well as the polarity of the solvent used.

The findings from the RAW 264.7 study demonstrated that crude water extracts prepared from the leaves and roots of *T. violacea* are not toxic at low concentrations. Extracts prepared from the stem of *T. violacea* were toxic regardless of the extraction solvent. Water was a much safer extraction solvent as opposed to ethanol because the crude water extracts did not exhibit undesirable IC50 values at a low concentration. This study has for the first time showed that crude extracts prepared from the stems of *T. violacea* are toxic to RAW 264.7 cells within the concentration range explored. For all the RAW 264.7 models, the values of the predicted R^2 leaves (0.7101), stems (0.8633) and roots (0.7075) were not greater than the adjusted R^2 values of leaves (0.6370), stems (0.7861) and roots (0.5421) confirming the quality of the data.

Data from the C2C12 cell line demonstrated that crude extracts prepared from the leaves, stems and roots (water or ethanol) of *T. violacea* are not toxic at low concentrations. Similar to the

results obtained with the RAW 264.7 cell line, water was also a much safer extraction solvent as opposed to ethanol because the crude water extracts did not exhibit an undesirable IC_{50} value at a low concentration. For all the C2C12 models, the values of the predicted /adjusted R^2 values for the leaves (0.8577/0.7773) stems (0.8544/ 0.77721) and roots (0.7356/0.5862) were more or less similar confirming the quality of the data.

This research provides preliminary data on the toxicity of crude extracts from *T. violacea* plant parts towards RAW 264.7 and C2C12 cell lines. The results also demonstrated that all extracts were cytotoxic at concentrations higher than 250 μg/ml treatments. This was true for all plant parts for both RAW 264.7 and C2C12 cell lines. According to this study, a concentration of 10 to 100 μg/ml from the water extracts of *T. violacea* plant parts may be considered as the optimal dosage for preparation of traditional remedies. The study also supports the ancient use of only the leaves of *T. violacea* for the preparation of remedies to treat various ailments due to the reported high percentage cell viability as well 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. Consequently, the study showed that low concentrations of *T. violacea* plant extracts have proliferative effects on cells but high concentrations are generally toxic.

The results of the *A. cepa* assay revealed that the water extracts of the stem of *T. violacea* are potentially genotoxic at the concentration range that was evaluated compared to the water extracts of the leaf and root. This finding is similar to that of the MTT assay on RAW 246.7 cells which demonstrated that the water extract of the stems was toxic. It is clear from the study that the crude water extracts from the leaves, stems and roots of *T. violacea* possess chromotoxic and mitodepressive effects at higher concentrations. The data from this research corroborates the use of the *A. cepa* assay to assess the effects of potential genotoxic and cytotoxic substances in plant material.

From this study, *T. violacea* was reported to possesses various secondary metabolites such as tannins, phenols, flavonoids, cardiac glycosides, terpenoids, steroids, coumarins, proteins and saponins. Although this study does not provide any distinct evidence as to which chemical constituents may be responsible for the cytotoxic activity of the *T. violacea* plant extracts, one or more of the above-mentioned compounds may be assumed to be the cause. Ethanol unlike water can efficiently degrade the plant cell wall, thus causing the release of polyphenols

(Lapornik *et al.* 2005). Therefore, increased cytotoxic activity due to the ethanol extracts could have been due to the higher amounts of polyphenols compared to water extracts. Furthermore, the concentration of the cytotoxic compounds could have been higher in the stems of *T. violacea* as opposed to the other parts of the plant since extracts from the stems exhibited high cytotoxicity. The results of this study suggest that the consumption of concoctions and decoctions prepared from *T. violacea* must be done with caution due to their cytotoxic and possible genotoxic effects. However, results of this study should not limit the use of the plant for medicinal purposes because the body system has mechanistic ways in which it can repair DNA damage (Akintonwa *et al.* 2009; Cuyacot *et al.* 2014). Overall, this research study also supports the ancient and frequent use of only the 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 phytocompounds present in the *T. violacea* plant parts will be an interesting area for further study (Sheel *et al.* 2014). Further research is required to exploit the biomedical applications of *T. violacea*. The anti-HIV activity of saponins (Kashiwada *et al.* 1998; Banno *et al.* 2004) is an interesting area for further research as their presence was observed in all the plant parts of *T. violacea. Tulbaghia violacea* plant extracts are rich in cardiac glycosides which have been reported to increase sodium ion levels in the myocytes, thus leading to a rise in the level of calcium ions promoting an increase of calcium ions available for contraction of the heart muscle, which improves cardiac output and reduces distention of the heart (Chukwuebuka *et al.* 2015). They should thus be explored for the treatment of cardiovascular disorders. *In vitro* studies have reported that flavonoids possess antiviral activity against several viruses, among them poliovirus. This raises a need to study *T. violacea* leaf extracts for possible treatment of polio as they are rich in flavonoids (González *et al.* 1990). Studies should also be conducted to isolate, identify, characterize, and elucidate 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. (Handa *et al.* 2008). 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, dichloromethanol, butanol

or methanol may be utilized. However, methanol is cytotoxic, making it unsuitable for extraction in certain kind of studies (Tiwari *et al.* 2011).

Additional studies on RAW 264.7 need to the be done to establish the mechanism by which the crude extracts from *T. violacea* particularly the stem induce cell death. In addition, it would be important to know the effects of the non-toxic concentrations on the cytokine and chemokine profiling of the macrophages. The proliferative action of C2C12 cells when treated with *T. violacea* crude extracts is an interesting area for future research to study aspects of myogenesis, metabolism and muscle biology.

Although the results of this study provide a good initial indication of the toxicity of *T. violacea* plant parts, a direct link to the toxicological effect of the extracts in humans was not established. There is thus a need to conduct *in vivo* cytogenetic studies to ascertain the *in vitro* findings from the *A. cepa* and MTT assays (Akintonwa *et al.* 2009; Cuyacot *et al.* 2014).

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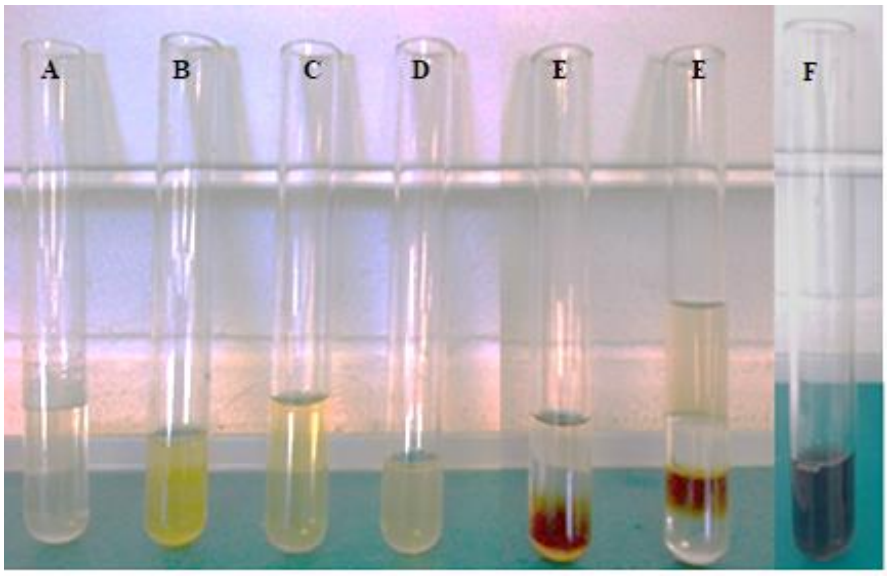
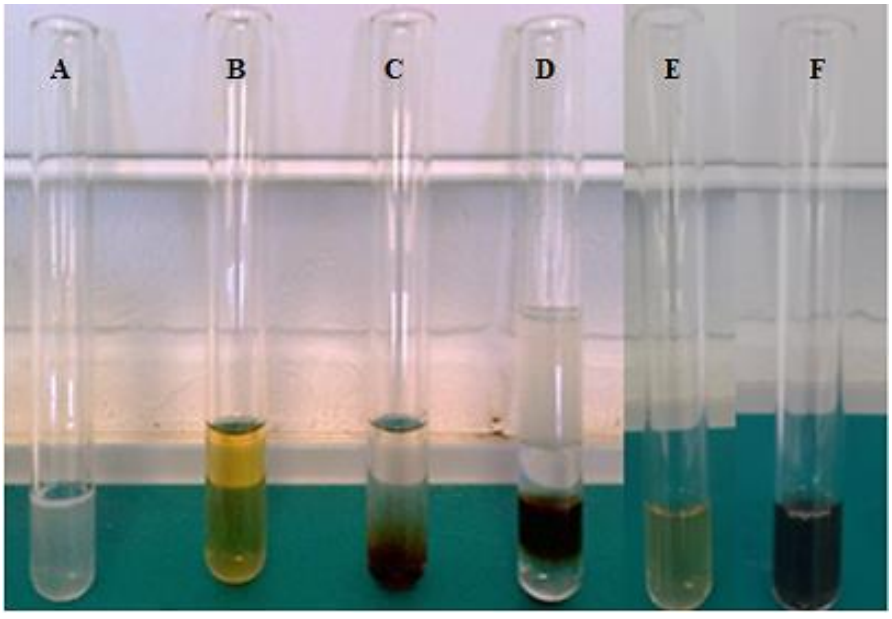
8. APPENDICES

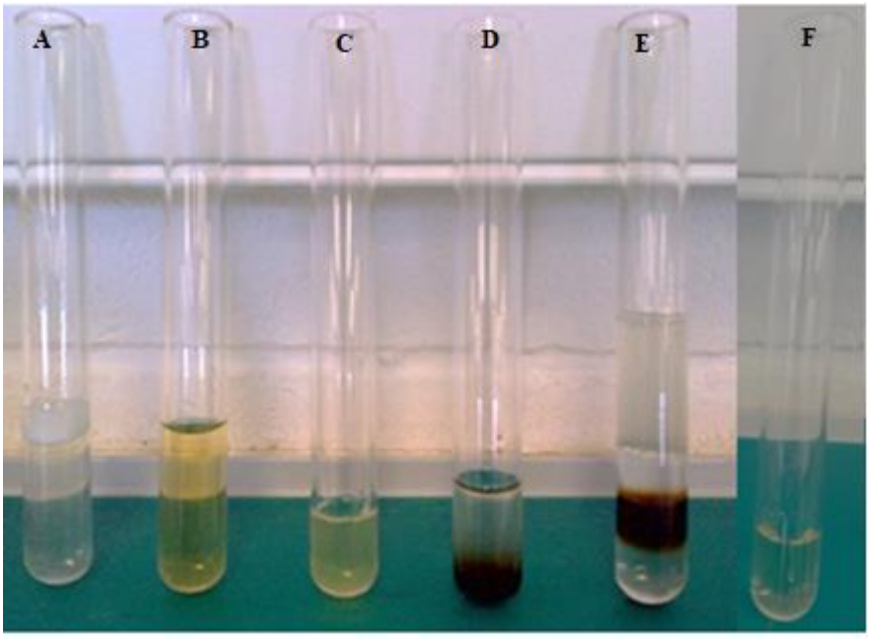
APPENDIX 1: Positive phytochemical results obtained from the water and 70% ethanol extracts of the leaves, stems and roots of *T. violacea*



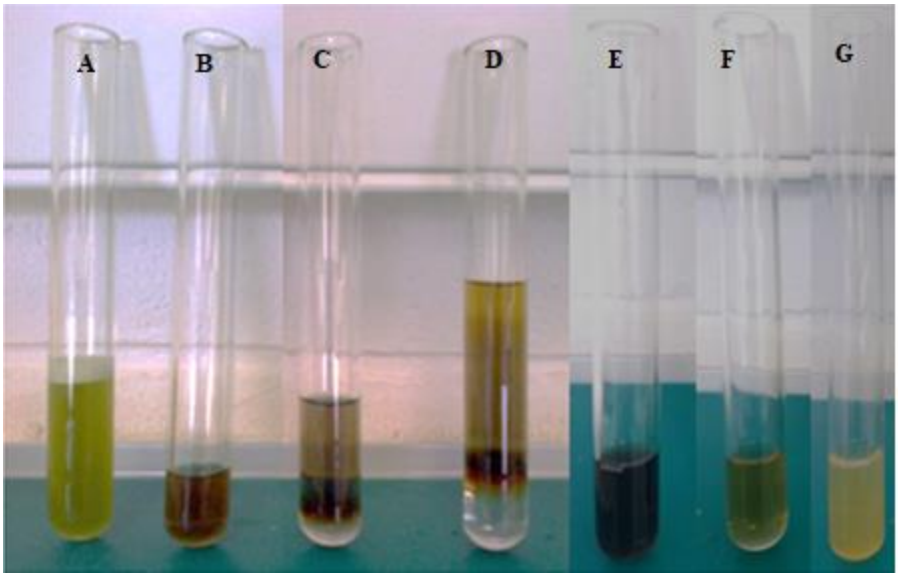
Leaf water extract: A) Saponins, B) Flavonoids, C) Coumarins, D) Protein, E) Cardiac glycosides, F) Terpenoids and G) Tannins/Phenols



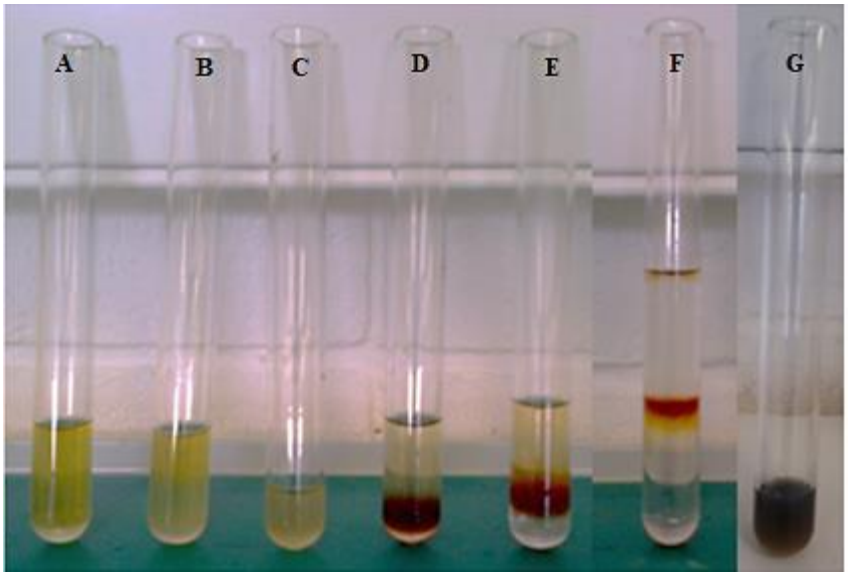
Stem water extract: A) Saponins, B) Coumarins, C) Cardiac glycosides, D) Terpenoids, E) Flavonoids and F) Tannins/Phenols



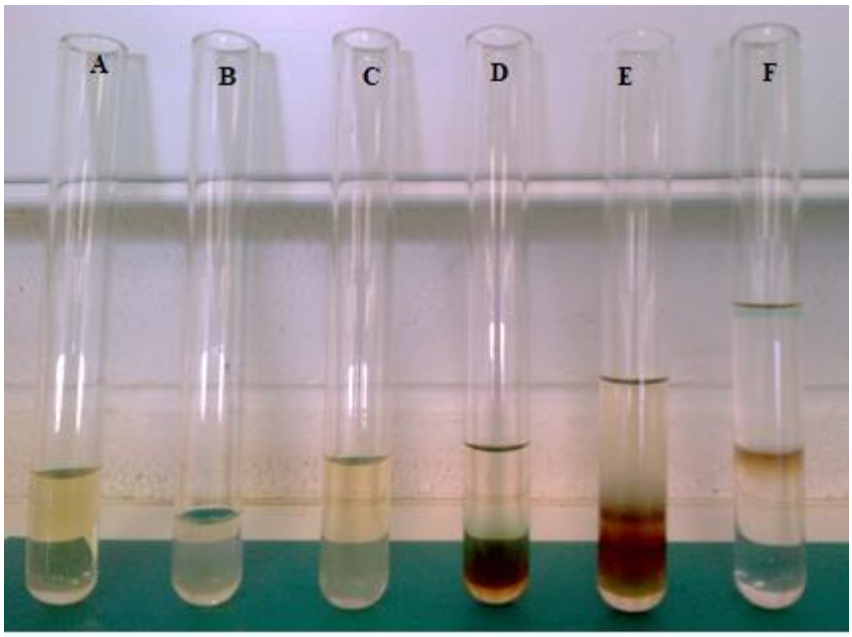
Roots water extract: A) Saponins, B) Coumarins, C) Protein, D) Cardiac glycosides, E) Terpenoids and F) Flavonoids



Leaves 70% ethanol extract: A) Saponins, B) Proteins, C) Cardiac glycosides, D) Terpenoids E) Tannins/Phenols, F) Coumarins and G) Flavonoids

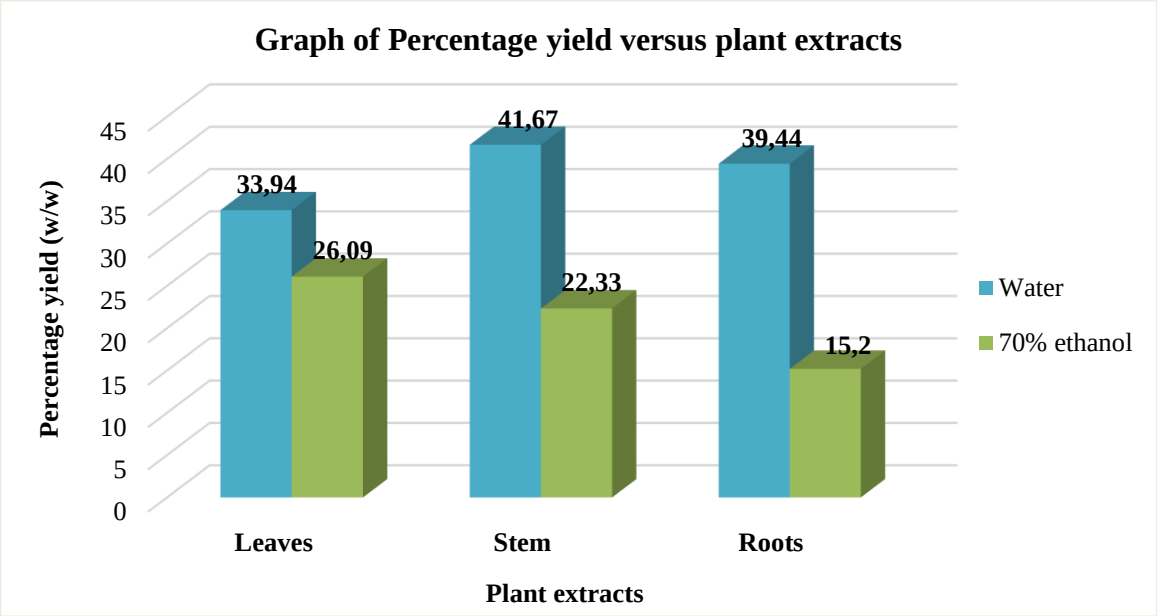


Stems 70% ethanol extract: A) Flavonoids, B) Coumarins, C) Protein, D) Cardiac glycosides, E) Terpenoids, F) Steroids and G) Tannins/ Phenols



Roots 70% ethanol extract: Positive results for 70% ethanol extract of the roots A) Flavonoids, B) Proteins, C) Coumarins, D) Cardiac glycosides, E) Terpenoids and F) Steroids

APPENDIX 2: Percentage yields obtained from the water and 70% ethanol extracts of *T. violacea* leaves, stems and roots.



APPENDIX 3: Absorbance readings for Quantitative phytochemical analysis

Absorbance of standard compound (Quercetin) at λ_{max} = 415 nm

Sample no.	Concentration of Quercetin (µg/ml)	Absorbance at 415 nm
1	0	0.000
2	20	0.374
3	40	0.657
4	60	0.963
5	80	1.508
6	100	1.706

Absorbance of standard compound (Gallic acid) at λ_{max} = 760 nm.

Sample no.	Concentration of Gallic acid (µg/ml)	Absorbance at 760 nm
1	0	0.000
2	50	0.765
3	100	1.196
4	150	1.755
5	200	2.285
6	250	2.834
7	300	3.224

APPENDIX 4: Phytocompounds present in *T. violacea* plant parts with their biological and shared activities

Compounds present	Biological activity	Shared activities
Saponins	Anti-diabetic, anti-HIV and antiatherosclerotic Interfere with cell replication including cancer	anti-inflammatory, antiviral, antifungal, antibacterial, antihyperglycemic, antiparasitic, antimicrobial, anticancer and antiallergenic
Flavonoids	Antiangionic, anticarcinogenic, as well as antidiarrheal. They also help in preventing menopausal symptoms	
Coumarins	anticoagulant, antihypertensive, anti-tubercular, neuroprotective, estrogenic, antihelminthic, sedative, hypnotic, hypothermic, antiulcer and anticlotting activities	
Terpenoids	Antispasmodic, immunomodulatory properties, anti-malarial, inhibition of cholesterol synthesis and insecticidal properties	
Tannins	Hastened healing of wounds and inflamed mucous membranes and treatment of intestinal disorders such as diarrhea and dysentery	
Phenols	Antitumour and hypotensive effects as well as antioxidant properties	
Steroids	Reduce cholesterol levels, regulate the immune response and also have immune-enhancing benefits	
Cardiac glycoside	Used in the treatment of congestive heart failure and cardiac arrhythmia	

Proteins	Potential protein supplements