

**An investigation into the medicinal properties  
of *Tulbaghia alliacea* phytotherapy**

**By**

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**South African Herbal Science and Medicine Institute**

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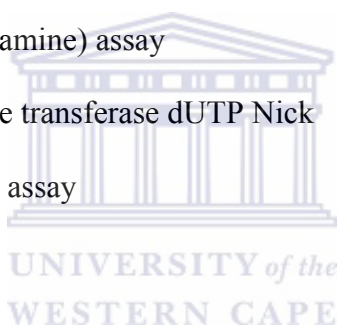



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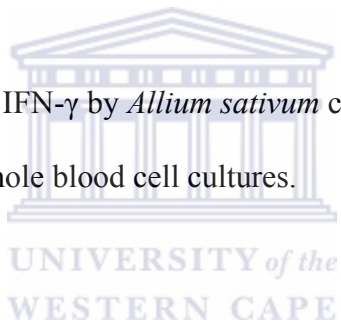


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## DECLARATION

I declare that “**An investigation into the medicinal properties of *Tulbaghia alliacea***” is my own work, only submitted to the University of the Western Cape for examination, and that all research resources I have used in this thesis have been duly acknowledged by means of complete references.

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Samantha Thamburan

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# **CHAPTER 1**

## **GENERAL OVERVIEW**



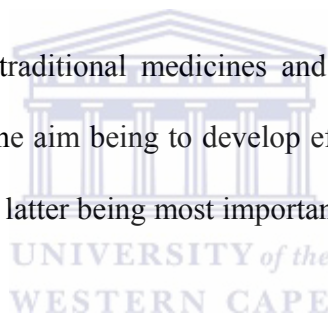
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## 1.1. Medicinal Plants

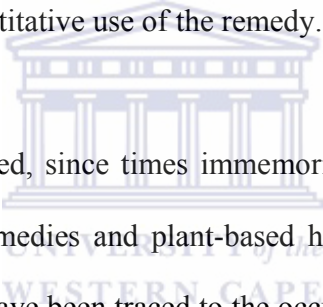
Plants have been used as primary sources of medicine for thousands of years and were our very first medicines. Plants have been selected and used empirically as drugs for centuries, initially as traditional preparations and then as pure active principles (Taylor, Rabe, McGaw, Jager and van Staden, 2001). Out of the 250,000 identified higher plants in the world, about 35,000-70,000 have at one time or another, been used by some people or cultures for medicinal purpose (Hoareau, DaSilva and Edgar, 1999).

The investigation of original traditional medicines and phytotherapies are once again receiving scientific attention; the aim being to develop effective new drugs that are non-toxic and inexpensive, with the latter being most important to developing countries.



A large proportion of the population of developing countries use traditional medicines, either as a result of the high cost of Western pharmaceuticals and health care, or because the traditional medicines are more acceptable from a cultural and spiritual perspective (Cunningham, 1998). The study of African medical plants has not in the past been taken as seriously, or documented as fully, as Indian and Chinese treatments (Taylor *et al.*, 2001), even though over 5000 plants are known to be used for medicinal purposes in Africa; only a few have been described or studied. Documentation of traditional medicinal plants and remedies is becoming increasingly important owing to the rapid loss of natural habitats, with many endemic medicinal plants becoming extinct before they can be investigated (Iwu, 1993).

Ethnopharmacology is defined as the interdisciplinary scientific exploration of biologically active agents traditionally employed or observed by man (Holmstedt and Bruhn, 1995). The aim of this field of study is the validation of traditional preparations either through the isolation of active substances, or through pharmacological findings on indigenous drug preparations. The identification of biologically active principles needs to be interpreted in light of the traditional use and preparation of the plant (Holmstedt and Bruhn, 1995). This should include an analysis and chemical and pharmacological evaluation of original drug preparations (e.g. water infusions) in order to establish dose-effect relationships for the quantitative use of the remedy.



Medicinal plants have been used, since times immemorial in virtually all cultures as a source of medicine. Herbal remedies and plant-based healthcare preparations obtained from traditionally used plants have been traced to the occurrence of natural products with medicinal properties. Moreover, medicinal plants and herbal remedies are re-emerging medical aids whose contribution and significance in the maintenance of good health and well-being is widely accepted (Hoareau *et al.*, 1999). In industrialized societies, the use of traditional medicine and medicinal plants in the treatment of minor ailments is now more acceptable since such use helps lower the increasing costs of personal health maintenance.

The increased interest in herbal medicine is due to the recognition that diseases of the immune system have increased (e.g. AIDS), the number of people suffering from incurable viral conditions is increasing, more and more bacterial infections are becoming resistant to commonly used antibiotics, allergies to foods and other common substances are becoming more prevalent, chronic disability is affecting people more frequently at younger ages, and mental disease is affecting more and more people.

Herbal medicines are an important part of the culture and traditions of African people. Today, most of the population in urban South Africa, as well as smaller rural communities, is reliant on herbal medicines for their health care needs. Apart from their cultural significance, this is because herbal medicines are generally more accessible and affordable (Fennel, Lindsey, McGaw, Sparg, Stafford, Elgorashi, Grace and van Staden, 2004). As a consequence, there is an increasing trend, worldwide, to integrate traditional medicine with primary health care.

It is estimated that more than 80% of the world's population utilise plants as their primary source of medicinal agents (Cordell, 1995) and that moreover, traditional medicine is still the only health resource available to about 60% of the world population (Le Grand and Wondergem, 1989). Estimates by WHO state that between 60 and 90% of Africa's population rely on medicinal plants to totally or partially meet their health care needs. This is true also of South Africa where up to 60% of the population consult one of an estimated 200 000 traditional healers (Van Wyk, Oustshoorn and Gericke, 1997),

especially in rural areas where traditional healers are more numerous and accessible than Western doctors (Cunningham, 1988).

In general, the demand for medicinal plants and herbal remedies and especially its renaissance in the developed countries, is driven by the following factors amongst others (Hoareau *et al.*, 1999; and Leaman, 2002):

- Increasing costs of institutional, pharmaceutical-based healthcare
- Interest of individuals, communities and national governments in greater self-reliance in health care
- Interest of communities and national governments in small and large-scale industrial development based on local/national biodiversity resources
- Increasing success in validating the safety and efficacy of herbal remedies
- Legislation improving the status of the herbal medicine industry
- Renewed interest of companies in isolating useful compounds from plants
- Search for new drugs and treatments of serious and drug-resistant diseases
- Marketing strategies by companies dealing in herbal medicine

### **1.1.1. Plant-based natural products in drug discovery**

Natural products have provided, and continue to provide, essential materials for shelter, furniture, food, clothing, writing, coloring materials, weapons, gifts, and for the treatment of numerous diseases (Balick and Cox, 1996). Bioactive principles were sought in earnest in the very early part of the nineteenth century, when, in a period of fifteen or so years, the investigation of several renowned medicinal plants led to the discovery of a number of the most significant biologically active alkaloids (Cordell, 1981; and Sneader, 1985). Some of these alkaloids; morphine, atropine, papaverine and codeine, subsequently became the cornerstones of many aspects of drug discovery today (Sneader, 1985; and Foye, Lemke and Williams, 1995). Farnsworth and colleagues (1985) indicated that globally there were 119 compounds from 90 plants that were used as single entity medicinal agents. Significantly, 77% of these were obtained as a result of examining the plant based on ethnomedical use, and are employed in a manner that approximates that use (Farnsworth, Akerele, Bingel, Soejarto and Guo, 1985).

Modification of natural products, with a view to enhancing activity or selectivity and reducing side effects or toxicity, developed as organic chemistry grew in the late 19th century (Sneader, 1985). Aspirin was one of the earliest of these chemically modified natural products and recently celebrated 100 years as a commercial entity (Reisch, 1997). Such modifications of natural products are rarely so simple today, and frequently the relationship to a natural product may be barely discernible. However, as O'Neill and Lewis (1993) have pointed out, half of the best-selling pharmaceuticals in 1991 were actually based on a natural product precursor or pharmacophore.

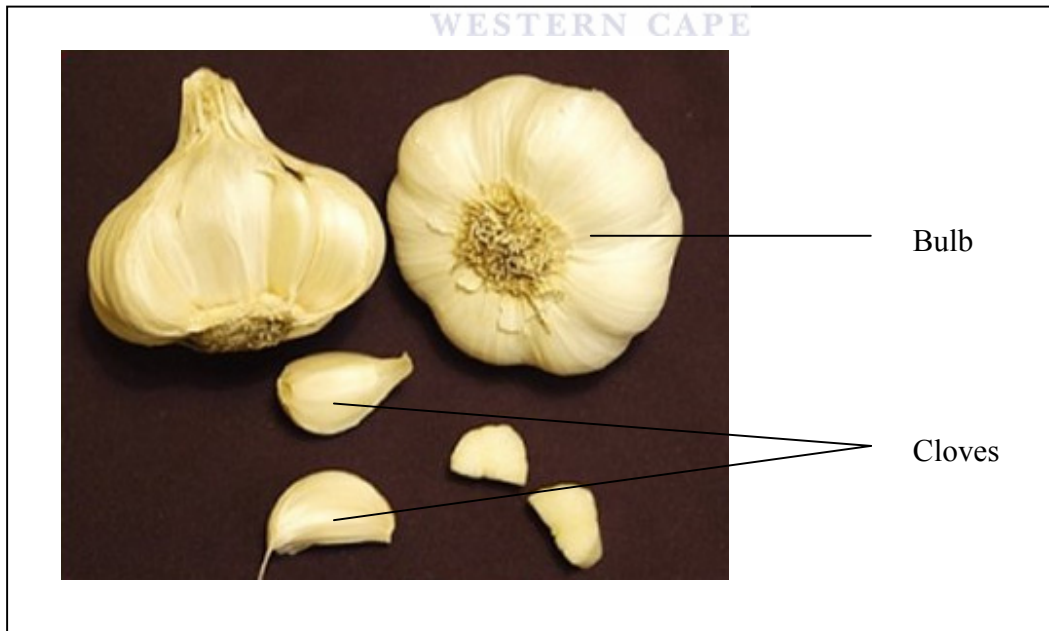
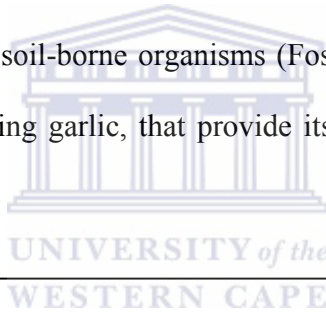
## 1.1.2. Nutraceuticals

An additional important opportunity for natural products, which has emerged in recent years, is their role as nutraceuticals. A nutraceutical is any substance that is a food or a part of a food, and provides medical or health benefits, including the prevention and treatment of disease. Such products may range from isolated nutrients, dietary supplements and specific diets to genetically engineered designer foods, herbal products, and processed foods such as cereals, soups and beverages.

Nutritional status determines normal health and functioning of all systems in the body, including the immune system, which is responsible for host resistance to various infectious diseases. TB co-exists with malnutrition in many parts of the world. The incidence of tuberculosis is unusually high among malnourished people even in developed countries, including the elderly, homeless, alcoholics, drug abusers and HIV-infected individuals (Dolin, Raviglione and Kochi, 1994). The health benefits of garlic include antimicrobial, antineoplastic, anticardiovascular, immuno-stimulatory, and hypoglycemic properties (Ankri and Mirelman, 1999). Such properties make garlic an attractive compound to be included in the daily diet, not only for its unique flavor, but also for its medicinal benefits.

### 1.1.3. Garlic (*Allium sativum*)

Garlic, *Allium sativum* (Fig 1), is a member of the lily family that has been cultivated by humans as a food plant for over 10,000 years. Commercial garlic (*Allium sativum*) is one of the edible plants, which has generated a lot of interest throughout human history as a medicinal panacea. Egyptian records dating to about 1550 BC mention garlic as a remedy for a variety of diseases (Block, 1985). The health benefits of garlic are varied and many. Nature has ingeniously packaged a chemical factory in garlic; it contains a collection of sulfur and selenium aroma compounds that have developed in the plant to protect it from predators such as animals and soil-borne organisms (Foster, 1998). It is these chemicals, released upon cutting or crushing garlic, that provide its health-giving benefits (Foster, 1998).



**Fig.1.** *Allium sativum*

Since the 1980s, scientists have discovered that garlic possesses numerous medicinal properties. A variety of biological activities have been reported for garlic including effects on tumorigenesis, atherosclerosis, microbial growth and blood sugar modulation (Milner, 2001; Siegel, 2004; Tsao, 2001; Iimuro, 2002). The antitumorigenic effects of garlic have been related to induction of apoptosis in tumor cells (Ledezma, 2004; Knowles, 2001; Oommen, 2004; Robert, 2001; Wu, 2001).

A wide range of microorganisms, including bacteria, fungi, protozoa and viruses, have been shown to be sensitive to crushed garlic preparations (Ankri and Mirelman, 1999).

Analysis of steam distillations of crushed garlic cloves (Fig.1) performed over a century ago showed a variety of allyl sulfides. However, it was not until 1944 that researchers isolated and identified the component responsible for the remarkable antibacterial activity of crushed garlic cloves (Cavallito and Bailey, 1994).

The compound turned out to be an oxygenated sulfur compound, which they termed *allicin*, from the Latin name of the garlic plant, *Allium sativum*. Researchers then isolated, identified, and synthesized an oxygenated sulfur amino acid that is present in large quantities in garlic cloves and which they named *alliin* (Stoll and Seebeck, 1951). *Alliin* was found to be the stable precursor that is converted to *allicin* by the action of an enzyme termed *alliinase*, which is also present in the cloves (Ellmore and Feldberg, 1994).



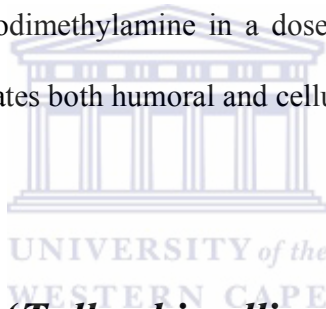
Cross-section studies have indicated that the substrate alliin and the enzyme alliinase are located in different compartments (Koch and Lawson, 1996; and Ellmore and Feldberg, 1994). This unique organization suggests that it is designed as a potential defense mechanism against microbial pathogens of the soil. Invasion of the cloves by fungi and other soil pathogens begins by destroying the membrane which encloses the compartments that contain the enzyme and the substrate. This causes the interaction between alliin and alliinase that rapidly produces allicin and which in turn inactivates the invader.

Garlic has antimicrobial, antineoplastic, anticardiovascular, immuno-stimulatory and hypoglycemic properties. The antimicrobial properties of crushed garlic have been known for a long time and reported frequently (Farbman, Barnett, Bolduc and Klein, 1993; Johnson and Vaughn, 1969; Cavallito and Bailey 1944; Timonin and Thexton, 1950; and Jonkers, van den Broek and van Dooren, 1999). Various garlic preparations have been shown to exhibit a wide spectrum of antibacterial activity against Gram-negative and Gram-positive bacteria including species of *Escherichia*, *Salmonella*, *Staphylococcus*, *Streptococcus*, *Klebsiella*, *Proteus*, *Bacillus*, and *Clostridium*. Even acid-fast bacteria such as *Mycobacterium tuberculosis* are sensitive to garlic (Uchida, Takahashi and Sato, 1975).

In one study, a synergistic effect of allicin against *M. tuberculosis* was found with antibiotics such as streptomycin or chloramphenicol (Gupta and Viswanathan, 1955). A very interesting aspect of the antibacterial activity of allicin is the apparent inability of

most bacteria to develop resistance to it because the mode of action is completely different from that of other antibiotic substances. It has been proposed that the development of resistance to beta-lactam antibiotics is 1000-fold easier than development of resistance to allicin (Gupta and Viswanathan, 1955).

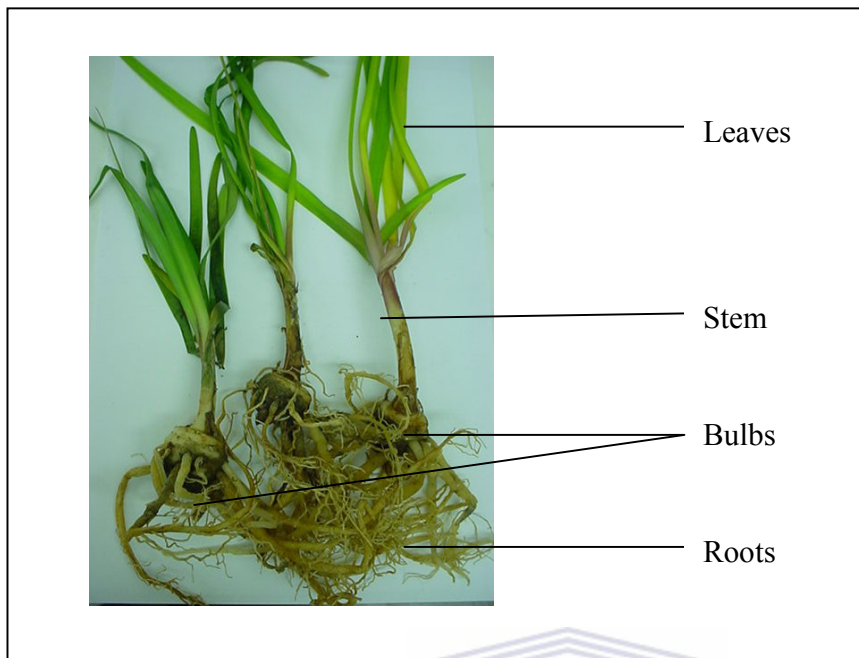
Garlic also has immuno-stimulatory property. Garlic extract or its components, diallyl trisulfide and diallyl sulfide, stimulates T cell proliferation (Lau, Yamasaki and Gridley, 1991; Feng, Zhang and Hao, 1994) and macrophages' cytotoxicities on tumor cell lines (Feng *et al.*, 1994). Pretreatment with diallyl sulfide restores suppression of the antibody response, induced by *N*-nitrosodimethylamine in a dose dependent manner (Jeong and Lee, 1998). Thus, garlic stimulates both humoral and cellular immunity.



#### **1.1.4. African Garlic (*Tulbaghia alliaceae*)**

*Tulbaghia alliaceae* is an indigenous garlic species that occurs mainly in the Eastern Cape and Southern KwaZulu-Natal (Van Wyk, Van Oudtshoorn and Gericke, 2000). Its common names include *wild garlic* (English), *wilde knoffel* (Afrikaans) and *isihaga* (Zulu).

Botanical description: Wild garlic is a bulbous plant with long, narrow, hairless leaves arising from several white, fleshy bases (Fig.2). Pale orange flowers occur in groups of about 10 or more at the tip of a slender stalk (Van Wyk *et al.*, 2000).



**Fig.2.** *Tulbaghia alliacea*

Traditional medicinal uses: *Tulbaghia alliacea* is commonly used for fever, colds, asthma, tuberculosis and stomach problems. In Cape Dutch tradition, it is used as a purgative, and for fits, rheumatism and paralysis (Van Wyk *et al.*, 2000).

Preparation and dosage: The bulbs and leaves are the plant parts used medicinally. The freshly harvested bulbs are boiled in water and the decoctions are either taken orally or as an enema. The leaves are also eaten as vegetables (Van Wyk *et al.*, 2000).

Pharmacological effects: Wild garlic (*Tulbaghia alliacea*) may prove to have the same or similar antimicrobial properties as has been scientifically verified for commercial garlic (*Allium sativum*).

## 1.2. Candidiasis

Candidiasis is the most common oral fungal infection in humans and manifests in a variety of clinical guises ranging from pseudomembranous (thrush), erythematous, and hyperplastic variants to linear gingival erythema associated with HIV infection (Samaranayake and Scully, 1989; Ellepola and Samaranayake, 2000). Oropharyngeal candidiasis (OPC) is the most prevalent oral complication in HIV/AIDS patients (Samaranayake, 1992; Glick, Muzyka, Lurie and Salkin, 1994; Blignaut, Messer, Hollis and Pfaller, 2002), and it is generally accepted that *Candida albicans* (Fig.3) is the most commonly isolated species from clinical specimens (Pfaller, 1994).



**Fig.3.** *Candida albicans*

(Source: <http://naturesvictory.fanspace.com/photo.html>)

### **1.2.1. Predisposing factors for candidal infection**

*Candida albicans* and several related *Candida* species are opportunistic pathogens that live as benign commensal organisms in the oral cavities of healthy individuals. *Candida* are normal inhabitants of the human gastrointestinal tract and may be recovered from up to one third of the mouths of normal individuals and two thirds of those with advanced HIV disease (Fichtenbaum, Koletar, Yiannoutsos, Holland, Pottage, Cohn, Walawander, Frame, Feinberg, Saag, Van der Horst and Powderly, 2000). These microorganisms may change from commensal to pathogenic microorganisms in the mouth in relation to the oral and systemic conditions (Kadir, Uygun and Akyuz, 2005). These are known to include mucosal disorders, intraoral prosthetic devices, diet, smoking, antibacterial drug therapy, and immunocompromising conditions (Arendorf and Walker, 1979; Dreizen, 1984; Arıkan, Kulak and Kadir, 1995; Kadir, Pisiriciler, Akyuz, Yarat, Emekli and Ipbuker, 2002).

Certain patients may be predisposed to the development of oral thrush (white plaque on the buccal mucosa, gums, or tongue), such as patients who are immunocompromised, those with diabetes mellitus, or pernicious anemia. In addition, the use of oral antibiotics, broad-spectrum antibiotics, corticosteroids, or immunosuppressives may predispose individuals to infection (Bruce and Rogers, 2004).

These factors disrupt the balance between the healthy host and the *Candida* organism, which under normal circumstances, may be present in the gastrointestinal flora but is not disease producing (Bruce and Rogers, 2004).

The increasing prevalence of other compromised patient groups in the community, common endocrine disorders such as diabetes mellitus, and nutritional deficiencies have also contributed to the resurgence of oral candidiasis as a relatively common affliction (Ellepola and Samaranayake, 2000). The inter-current administration of antibiotics for the management of infections associated with malignancies may further aggravate candidiasis. For instance, it is well established that broad-spectrum antibiotics such as tetracycline or combination therapy with antibiotics suppress the oral bacterial flora thereby providing the growth of commensal *Candida* species to a very high degree (Samaranayake, Cheung and Samaranayake, 2002).

Oral colonization with inherently drug-resistant organisms is more common in advanced HIV infection (CD4-lymphocyte counts  $<50$  cells/mm<sup>3</sup>) (Fichtenbaum *et al.*, 2000). Although not usually associated with severe morbidity, OPC can be clinically significant as it can interfere with the administration of medications and adequate nutritional intake, and may spread to the esophagus (Tavitian, Raufman and Rosenthal, 1986). Symptoms of OPC may include burning pain, altered taste sensation, and difficulty swallowing liquids and solids.

### 1.2.2. HIV infection and Oral Candidiasis

From the initial description of early cases (Gottlieb, Van der Hedien and Thompson, 1981), candidiasis of the mucosae has been associated with HIV disease. From the beginning of the AIDS epidemic, it was determined that oropharyngeal candidiasis is a marker of dysfunction of the immunological system, and is also a prognostic marker for the development of other resulting infections (Klein, 1984). Candidiasis of the esophagus or of the lungs and bronchi is considered one of the indicator diseases for AIDS.

Oropharyngeal candidiasis (OPC) was among the initial manifestations of HIV-induced immunodeficiency to be recognized (Gottlieb *et al.*, 1981) and typically affects the majority of persons with advanced untreated HIV infection. Presenting months or years before more severe opportunistic disease, oropharyngeal candidiasis may be a sentinel event indicating the presence or progression of HIV disease (Klein, Harris and Small, 1984; Dodd Greenspan, Katz, Westenhouse, Feigal and Greenspan, 1991; Katz, Greenspan, Westenhouse, Heesol, Bichbinder, Lifson, Shiboski, Osmond, Moss and Samuel, 1992).

Candidiasis in HIV patients is strongly related to deficiency of anti-candida defense mechanisms, both topical and systemic, due to the induced immunodeficiency related to HIV infection (Yeh, Fox and Ship, 1988; McCarthy, 1992; Epstein, 1984; McCarthy, Mackie and Koval, 1991). Changes of the standard levels and the efficiency of topical factors, such as secretory IgA globulin, lactoperoxidase, lactoferin, and lysozyme have been proven in HIV patients, and are potentially related to the development of candidiasis (Yeh *et al.*, 1988 and Rigopoulous, Papparizos and Katsambas, 2004).

### 1.2.3. Chemotherapy and Candidiasis

Early studies have shown that *Candida* species are responsible for approximately one half of oral infections that arise during antileukaemic chemotherapy, and for almost two thirds in patients on antineoplastic drugs for solid tumours (Soysa, Samaranayake and Ellepola, 2004). The majority of cancer patients, particularly those with haematological malignancies, are intrinsically colonized by *Candida* species by virtue of their systemic disease, even before they receive chemotherapy or radiotherapy (Soysa *et al.*, 2004).

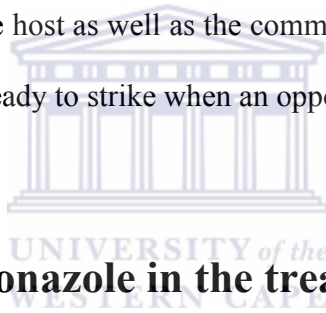
Cytotoxic drugs have a major dampening effect on the cell-mediated arm of the immune component (Soysa *et al.*, 2004) and patients receiving such therapy are particularly vulnerable to fungal and viral infections. In one prospective cohort study on orofacial complications of combination chemotherapy (cyclophosphamide, methotrexate, flurouracil, vincristine and prednisone) in women with breast cancer. McCarthy and Skillings (1992) found intra-oral candidiasis in 12% of their cohort. The increased incidence of oral candidiasis seen in patients with malignancies stems partly from such therapeutic measures and partly from radiotherapy, which are either used singly or in combination depending on factors such as the type, stage, and severity of malignancy (Soysa *et al.*, 2004). In addition, the concurrent administration of broad-spectrum antibiotics may further predispose the patient to oral candidiasis (Bergmann, 1991).

The mechanisms by which therapeutic procedures for oral and other forms of cancer favor the oral yeast carriage or infection are indeed complex. It is highly likely that the



yeast proliferation (infestation) as well as the subsequent infection is related to both the host factors related to the cancer itself and, the side-effects of antineoplastic therapy (Soysa *et al.*, 2004).

From a host perspective, cytotoxic action of chemotherapeutic agents on rapidly dividing oral mucosal cells results in atrophy, thinning and inflammation (mucositis) of the epithelium, which in turn may aggravate the mucosal susceptibility to trauma and infection (Prentice, 1989). The pathogenic mechanisms associated with oral candidiasis consequential to malignant disorders and their therapeutic measures appear to be rather complex and related both to the host as well as the commensal yeasts residing on the oral mucosa as pathogens that are ready to strike when an opportunity arises.

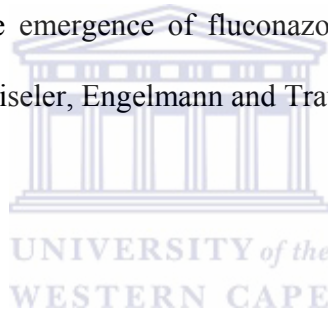


#### **1.2.4. The use of fluconazole in the treatment of Candidiasis**

Since its introduction, fluconazole has been used to prevent and treat *Candida* infections in HIV-1-infected patients because of its efficacy and good tolerability (Koks, Crommentuyn, Mathot, Mulder, Meenhorst and Beignen, 2002). However, after the use of fluconazole for the treatment and prophylaxis of fungal infections became widespread, reports began to appear which suggested reduced effectiveness of the standard fluconazole therapy and also side effects (Ng and Denning, 1993).

The side effects of many azole antifungals such as ketoconazole, itraconazole, fluconazole, posaconazole, and voriconazole are similar; the more common being headache, dyspepsia, diarrhea, nausea, vomiting, hepatitis, and skin rash (Munoz, Moreno, Berenuer, Bernaldo de Quiros and Bouza, 1991).

The clinical response to fluconazole in patients with OPC is usually good but because of incomplete eradication of the fungi due to the fungistatic rather than fungicidal effect of azoles, relapses occur frequently (Rex, Rinaldi and Pfaller, 1995). The prolonged and repeated treatment of OPC in AIDS patients has resulted in an increasing frequency of therapy failures caused by the emergence of fluconazole-resistant *C. albicans* strains (Ruhnke, Eigler, Tennagen, Geiseler, Engelmann and Trautmann, 1994).



### 1.3. Tuberculosis

Tuberculosis (TB) caused by infection with *Mycobacterium tuberculosis* (Fig.4) or *Mycobacterium bovis* remains one of the most important infectious diseases of humans and animals respectively, and continues to inflict a huge cost in both health and financial terms (Hogarth, Jahans, Hecker, Hewinson and Chambers, 2003). Despite the existence of specific antimicrobial agents and the Bacille Calmette-Guérin (BCG) vaccine, it is estimated that a third of the world's population is infected with *M. tuberculosis* (Dye, Scheele, Dolin, Pathania and Raviglione, 1999), with 8 million newly diagnosed cases of TB and up to 2.5 million deaths occurring each year (World Health Organization, 2001).



**Fig.4.** *Mycobacterium tuberculosis* (Scanning electron micrograph)

(Source: <http://medicineworld.org/images/blogs/11-2007/mycobacterium-tuberculosis-299290.jpg>)

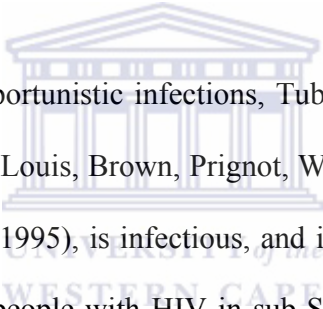
Epidemiological data suggest that up to one-third of the world's population may be latently infected with *M.tuberculosis* and about 5–10 % of these people will become sick or infectious at some time during their life (Olsen and Andersen, 2003). In the Western Cape Province of South Africa, the TB incidence has reached an alarming proportion of 589/100 000 population (Schaaf, Shean and Donald, 2003). Globally, there were an estimated 9.2 million new cases and 1.7 million deaths in 2006 (World Health Organisation, 2008).

Treatment of TB is complex due to the need to use several medications over extended periods of time, and the existence of resistant strains. The current vaccine against TB, Bacille Calmette-Guerin, (BCG) prevents the childhood manifestations of TB but as the efficacy wanes over a period of 10–15 years, it does not prevent the most prevalent disease form, pulmonary TB in adults (World Health Organization, 2004). Considering the overwhelming worldwide problem of TB, it is now clear that the development of a more effective vaccination and therapeutic strategy is urgently needed.

### **1.3.1. The Scourge of HIV and TB**

HIV/AIDS is a global public health emergency demanding effective action. It is fuelling the TB epidemic in sub-Saharan Africa, as individuals infected with Human Immunodeficiency Virus (HIV) are very susceptible to TB and often develop this disease before other manifestations of Acquired Immune Deficiency Syndrome (AIDS) become apparent (Grange and Davey, 1990). TB is therefore, the leading killer of people living

with HIV/AIDS (UNAIDS, 2000). Worldwide, 34.3 million people are living with HIV (Gilks, Katabira and De Cock, 1997). Of these, 24.5 million (71%) live in sub-Saharan Africa (UNAIDS, 2000) and approximately one third are co-infected with TB. Many countries have seen an increase in TB case fatality rates (Dye *et al.*, 1999) and a four-fold rise in their TB caseload (Mukadi, Maher and Harries, 2001). Some countries have documented that up to 70% of TB patients are HIV positive (Raviglione, Harries, Msiska, Wilkinson and Nunn, 1997). Between 1990 and 2003, TB incidence rates tripled in African countries with high HIV prevalence (Corbett, Watt, Walker, Maher, Williams, Raviglione and Dye, 2003).



Unlike other HIV-1-related opportunistic infections, Tuberculosis occurs at all levels of CD-4 count (Mukadi, Perriens, Louis, Brown, Prignot, Williame and Pouthier, 1993; and Ackah, Coulibaly and Digbeu, 1995), is infectious, and its prevention is a major public-health priority. Up to 50% of people with HIV in sub-Saharan Africa develop TB; this rapid increase of TB cases in sub-Saharan African countries has challenged their ability to maintain adequate supplies of TB drugs. Moreover, people infected with HIV and TB are more likely to develop drug-resistant forms of the infection, because compliance is an issue (Organization of African Unity Summit, 2001).

### 1.3.2. Pathogenesis of Tuberculosis

*Mycobacterium tuberculosis* is one of the most successful bacterial parasites of humans, infecting over one-third of the population of the world. This remarkable success is because pathogenic mycobacteria can survive in the hostile habitat of a macrophage, even in the face of a specific T-cell immune response (Silva, Bonato, Lima, Coelho-Castelo, Faccioli, Sartori, De Souza and Leao, 2001).

*M. tuberculosis* infections are acquired through inhalation of infective bacilli. Bacteria are internalized by alveolar macrophages and set up infection foci in the lung tissue. These foci expand through bacterial growth and recruitment of macrophages and lymphocytes that build the granuloma that defines this infection. The granuloma seems to support limited bacterial growth and prevents metastasis of the infection. Nonetheless, the granuloma also protects the bacterium from the immune response and is probably responsible for the persistent or latent nature of the infection. Clinical disease develops when this immune-mediated constriction is abrogated through immune compromise. Even in individuals in whom infection is controlled at the granulomatous state or earlier, any later imbalance of the host's immune system may promote reactivation of the disease (Silva *et al.*, 2001).

TB has an uncanny ability to persist, despite the host immune response to infection and long periods of chemotherapy. TB's slow progression and the need for long periods of treatment make the disease difficult to eradicate. Chemotherapy for TB is notoriously slow compared to other bacterial infections (McKinney, Honer and Munoz-Elias, 2000).

### **1.3.3. The host cell – pathogen relationship**

The etiologic agent *Mycobacterium tuberculosis* is an acid-fast bacillus characterized by a wax rich cell wall. The relevant components comprise long-chain fatty acids, mostly mycolic acids, glycolipids like lipoarabinomannan, phenolic and peptido glycolipids, sulpholipids and others (Brennan and Besra, 1997). The wall is also rich in characteristic polysaccharides, in particular peptidoglycans and arabinogalactans. This highly hydrophobic cell wall is not only responsible for the acid-fastness but also for resistance against complement, acidic or alkaline chemicals, and simple disinfectants, as well as for the high adjuvanticity of the cell wall (Kaufmann and Hess, 2000).

Moreover, the robust cell wall promotes mycobacterial replication within phagosomes of resting macrophages. This is further supported by the capacity of *M. tuberculosis* to arrest phagosome maturation at an early stage (Kaufmann and Hess, 2000). In this way, mycobacterial phagosomes are not acidified and prevented from fusing with lysosomes. Consequently, mycobacteria accommodate an intracellular niche where they can persist for long periods of time within the macrophages – one of the most efficacious host effector cells against other microbial invaders (Schaible, Collins and Kaufmann, 1999).

### 1.3.4. Antimicrobial drug resistance

Antimicrobial resistance is a naturally occurring biological phenomenon amplified many-fold due to human misuse and neglect of antimicrobial drugs (WHO, 2000). The effect of antimicrobial resistance is that it can reduce the curative power of once life-saving medicines to that of a sugar pill.

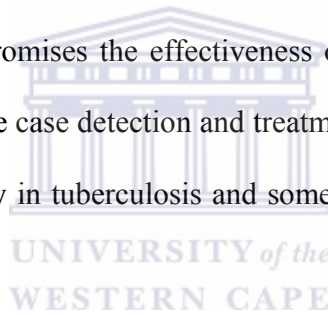
The social causes fuelling the spread of antimicrobial resistance are paradoxical. In some settings – especially in poorer countries – the *under-use* of drugs encourages the development of resistance. For example, where patients are unable to afford the full course of the medicines to be cured of their illnesses, or can only afford to purchase counterfeit drugs on the black market, the weakest microbes in the body may be killed by these insufficient doses while the more resistant microbes are given an opportunity to survive and multiply (WHO, 2000). In wealthy countries, resistance is emerging for the opposite reason – the *overuse* of drugs. Unnecessary demands for drugs by patients are often eagerly met by health services prone to over-prescription (WHO, 2000).

Resistance has been a problem since humans began to use antibiotics, and reflects the Darwinian principle of natural selection, whereby these drugs kill susceptible bacteria but allow resistant ones to survive. Four processes contribute to the accumulation of resistance: firstly, species with inherent resistance are advantaged; secondly, resistant mutants of hitherto susceptible strains are selected; thirdly, transferable resistance genes disseminate among bacterial strains, carried by plasmids, transposons and integrons; and,



last, some resistant strains achieve epidemic spread among patients, hospitals and countries (Livermore and Dudley, 2000). The relative importance of these processes varies with the pathogen and setting.

The rise of antimicrobial resistance in human pathogens poses a growing challenge to medicine and public health. Increasing resistance to preferred therapies has limited the options for treating such diverse infections as HIV and malaria, as well as a variety of hospital- and community-acquired bacterial infections (Bloland, 2000; and Bartlett, 2000). Resistance not only makes treatment of individual patients more complicated and more expensive; it also compromises the effectiveness of disease control programs for those infections where effective case detection and treatment are central to the prevention of disease transmission notably in tuberculosis and some sexually transmitted infections (Farmer and Kim, 1998).



### **1.3.5. Antimicrobial drug resistance and TB**

Strategies to control resistance in TB differ considerably from those contemplated for other community-acquired infections. There are several reasons for these differences: the high case-fatality rate of untreated disease, the importance of treatment in preventing transmission, and the need for sustained multi-drug therapy to prevent emergence of resistance in treated patients (Bloch, 1994).

Highly effective drugs for treating TB were introduced over 30 years ago, yet deaths from the disease continue to increase. New tools are needed, including drugs with activity against multi-drug resistant strains of *Mycobacterium tuberculosis*. Agents that reduce the duration and complexity of the current therapy would have a major impact on compliance and overall cure rate (Duncan, 2003).

Many TB patients fail to comply with the therapy, particularly when their symptoms subside as the drugs exert their effect, or when the side effects become unbearable (Spigelman and Gillespie, 2006). This leads to treatment failure, and worse, to the emergence of drug resistance. Many of the multi-drug resistant TB (MDR-TB) strains encountered today are resistant to the majority of antimycobacterial agents. Treating patients harbouring such organisms is difficult and they are an enormous drain on already strained healthcare resources in many parts of the world (Duncan, 2003). The economic consequences of antimicrobial resistance can be staggering. The cost of treating one person with multidrug-resistant TB is a hundred times greater than the cost of treating non-resistant cases (WHO, 2000).

### 1.3.6. Drug resistant tuberculosis

Drug resistance arises following inadequate chemotherapy which selects for mutated strains with increased survival capabilities. Multi-drug resistant tuberculosis (MDR-TB) has been defined in terms of resistance to at least the two major anti-TB drugs, rifampicin and isoniazid, and requires long and expensive chemotherapy using second-line drugs of higher toxicity (Zager and McNerney, 2008). Extremely drug resistant tuberculosis (XDR-TB) has been reported in all regions of the world and involves resistance to at least Rifampicin, Isoniazid, a second-line injectable drug (capreomycin, kanamycin or amikamycin) and a fluoroquinolone (CDC, 2006).

Insufficient case management of MDR-TB, which allows partially treated and relapsed patients to become sequentially resistant, may play a significant role in the development of XDR-TB (Jones, Hesketh and Yudkin, 2008). Effective treatment of XDR-TB is challenging for various reasons, including an extended period of treatment of up to 2 years, lack of accessibility to and the elevated expense of the relevant drugs, low adherence owing to toxicity of second-line drugs, and the difficulty of co-administration of the medication with antiretroviral therapy in HIV positive patients (Jones *et al.*, 2008).

### 1.3.7. Current TB treatment and problems

By contrast with other infectious diseases, there are relatively few antimicrobial agents clinically effective against *M tuberculosis*. Currently, tuberculosis treatment requires lengthy courses of medication due to the ability of *M tuberculosis* to enter a dormant, persistent, antimicrobial-resistant phase. Furthermore, adherence to treatment of tuberculosis is limited by a multitude of clinical, social, financial and behavioural factors (Ginsburg, Grossett and Bishai, 2003).

Control of the TB epidemic linked with HIV infection will depend largely on the adequate treatment of TB, and possibly on effective chemoprophylaxis, not just for HIV-infected persons but for the community as well (WHO/IUATLD Working Group, 1989). TB therapy has been revolutionized and the present treatment regimes are based on multidrug therapy usually with three or four anti-tuberculosis drugs. However, the problem of multidrug-resistant tubercle bacilli is emerging for various drugs, such as isoniazid, ethambutol, rifampin, streptomycin (Girling, 1989 and Grange and Davey, 1990). Drug-resistant TB is very difficult to treat and requires more and different medications for a longer period of treatment. Sometimes, surgery is needed to remove areas of destroyed lungs that are heavily infected by mycobacteria and inaccessible to drugs (National Jewish Medical and Research Center, 1994).

The need for new anti-tuberculosis agents is urgent because of the increasing resistance of mycobacteria to classic anti-tuberculosis drugs. A recent World Health Organization report states that, globally, 2% of all cases of Tuberculosis are multidrug-resistant - by definition, resistance to rifampicin plus isoniazid (plus/minus other resistances). Such cases can be treated in the USA and other high resource regions but at a great cost (>US\$250000 per case) and using very long courses of rather toxic drugs, thereby raising serious problems of compliance (Lall and Meyer, 1999). South Africa is currently witnessing an explosion in the number of cases of drug-resistant tuberculosis. In some parts of the country, one in ten cases of TB is resistant to treatment (Lall and Meyer, 1999). The recent discovery of extremely drug-resistant (XDR) *Mycobacterium tuberculosis* strains further complicates and exacerbates the problem of drug resistance.



### **1.3.8. Objectives for TB drug development**

Current objectives for TB drug development include shortening the total duration of effective treatment and/or significantly reducing the total number of doses needed to be taken; improving the treatment of MDR-TB; and providing a more effective treatment of latent TB (O' Brien, 2001).

It is essential to have new anti-tuberculosis agents, preferably those that can be readily and simply produced from some local source. The use of antimicrobials from natural vegetation has a great impact on human health care in undeveloped countries. Herbal medicine has been used for centuries in rural areas by local healers and has been

improved in industrialized countries. A number of substances used in modern medicine for the treatment of serious diseases have originated from research on medicinal plants (Irobi, Moo-Young, Anderson and Daramola, 1994).

Natural products continue to play a very significant role in the drug discovery and development process (Newman and Cragg, 2007), and plants are recognized as a useful source of highly active antimycobacterial metabolites (Gibbons, 2005; Pauli, Case, Inui, Wang, Cho, Fsieher and Franzblau, 2005).



### 1.3.9. Immune responses to *M. tuberculosis* infection

The acquired cellular immune response to *M. tuberculosis* infection is complex and involves multiple cellular subsets: CD4 and CD8 T cells, as well as unconventional T cells such as  $\gamma\delta$ T cells and double-negative  $\alpha\beta$ T-cell subsets. Th1 cytokine responses also play an important role in the control of TB in humans, as is demonstrated by the fact that individuals with genetic deficiencies in IFN- $\gamma$  and IL-12 receptors have enhanced susceptibility to disseminated mycobacterial disease (Olsen and Andersen, 2003).

As one-third of the world's population is estimated to be infected with *M. tuberculosis* (World Health Organization, 2001) a vaccine which will control not only initial infection but also disease reactivation is needed. Research on animal models of latency and reactivation of *M. tuberculosis* has demonstrated an important role for CD8 cells as well as IFN- $\gamma$  in maintaining control of the latent stage of *M. tuberculosis* infection (Tascon, Stavropoulos, Lukacs and Colston, 1998 and van Pinxteren, Cassidy, Smedegaard, Agger and Andersen, 2000).

The fact that 5–10% of latently-infected individuals develop clinical disease at some time during their life further suggests that the antigen repertoire primed during the primary infection might not be sufficient to prevent reactivation (Olsen and Andersen, 2003). Antigens expressed in the latent phase of infection could therefore be very relevant components in a future vaccine.

Protection against disease is associated with a Th1 T-cell response involving interferon-gamma-producing CD4+ T-cells. Interferon-gamma (IFN- $\gamma$ ) has a key role in protective immunity through activation of macrophages. In fact, *Mtb*-stimulated whole-blood production of IFN- $\gamma$  is currently the assay most closely associated with protection. T-cell cytokines and, in particular, IFN- $\gamma$  play key roles in determining susceptibility to TB disease, disease severity, and the treatment outcome (Hussain, Talat, Shahid, Dawood, 2007).

Evidence from mouse experiments suggests a major role of CD8 T cells, in conjunction with CD4 T cells, in protection against tuberculosis (Flynn, Goldstein, Triebold, Koller and Bloom, 1992). The T lymphocytes participate in the acquired immune response by means of two major functions. First, they produce cytokines that activate effector functions. Notably, interferon-gamma (IFN- $\gamma$ ) is a major activator of antimicrobial macrophage functions. This cytokine is produced by all T cells stimulated in response to mycobacterial infection, i.e. CD4 T cells, CD8 T cells,  $\gamma\delta$ T cells and CD1-restricted T cells (Schaible *et al.*, 1999).

Secondly, these cells also exhibit cytolytic activities, i.e. they lyse infected target cells (Kaufmann, 1999). Lysis of infected macrophages, themselves insufficiently equipped for mycobacterial killing, could allow release of micro-organisms that are then attacked by more proficient monocytes. (Kaufmann and Hess, 2000). More recently, an additional function of killer cells has been elucidated; CD8 T cells possess granulysin, which can directly attack mycobacteria. The early cytokine response by the innate immune system

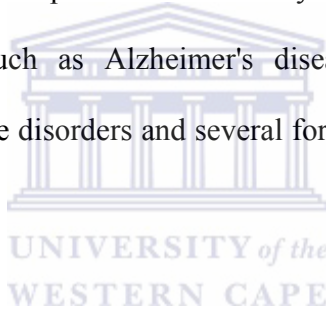


has a decisive influence on the host response to infectious agents (Fearon and Locksley, 1996; and Trinchieri, 1995). Of the early cytokines, IL-12 plays a critical role by generating the Th1 responses required to eliminate intracellular pathogens, such as *M. tuberculosis*.



## 1.4. Apoptosis

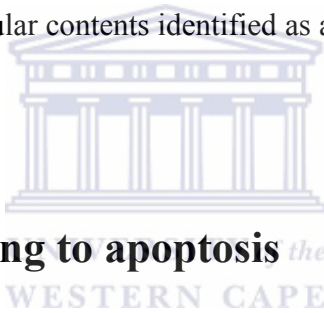
Apoptosis describes the process by which cells are "silently" removed under normal conditions when they reach the end of their life span, are damaged, or superfluous (Israels and Israels, 1999). It is a general tissue phenomenon necessary for development and homeostasis: elimination of redundant cells during embryogenesis, cell atrophy upon endocrine withdrawal or loss of essential growth factors or cytokines, tissue remodelling and repair, and removal of cells that have sustained genotoxic damage (Cohen, 1993). Inappropriate apoptosis is implicated in many human diseases, including neurodegenerative diseases such as Alzheimer's disease and Huntington's disease, ischaemic damage, autoimmune disorders and several forms of cancer (Thompson, 1995; Cohen, 1997).



Apoptotic cell death occurs in two phases: first a commitment to cell death, followed by an execution phase characterized by dramatic stereotypic morphological changes in cell structure (Takashi and Earnshaw, 1996), suggesting the presence in different cells of a common execution machinery (Jacobson, Burne and Raff, 1994).

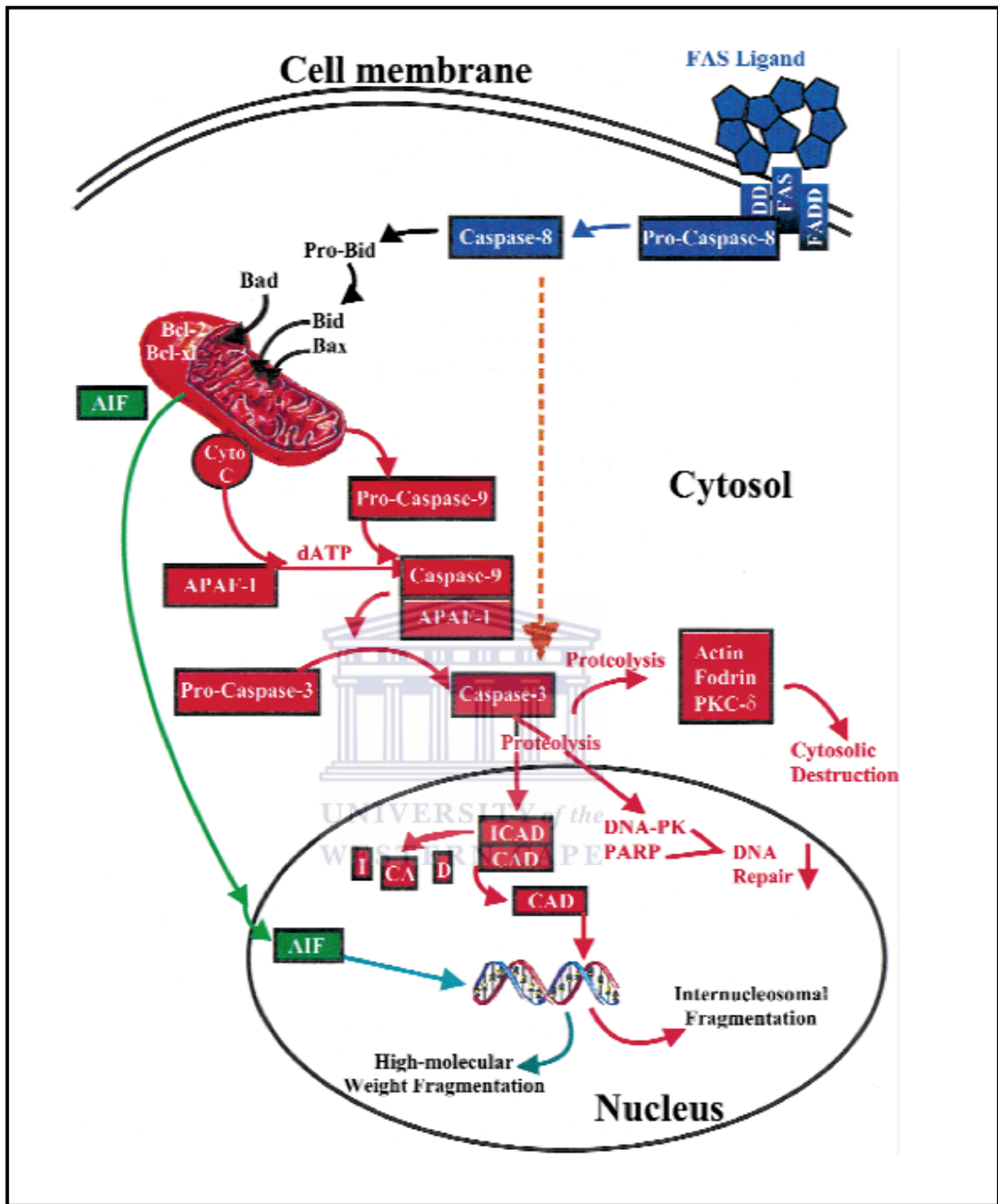
Apoptosis is a physiologically programmed mechanism by which cells die; it leads to cell death through a tightly regulated process resulting in the removal of damaged or unwanted tissue (Lowe, 2000). It is associated with a distinct set of biochemical and physical changes involving the cytoplasm, nucleus and plasma membrane (Fig.5) (Lawen, 2003), and is characterized by chromatin condensation, membrane blebbing, cell

shrinkage, and DNA fragmentation (Wyllie, 1980). Early in apoptosis, the cells round up, losing contact with their neighbors, and shrink. In the cytoplasm, the endoplasmic reticulum dilates and the cisternae swell to form vesicles and vacuoles. In the nucleus, chromatin condenses and aggregates into dense compact masses, and is fragmented internucleosomally by endonucleases (Johnson and Vaughn, 1996). The nucleus becomes convoluted and buds off into several fragments, which are encapsulated within the forming apoptotic bodies. In the plasma membrane, cell junctions are disintegrated, whereby the plasma becomes activate and convoluted, eventually blebbing. The cell breaks up in a florid manner leading to the “falling away” of several membrane spheres containing the “packaged” cellular contents identified as apoptotic bodies of various sizes (Lawen, 2003).



#### **1.4.1. Pathways leading to apoptosis**

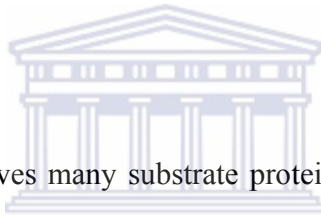
Induction of apoptosis can occur via two general pathways: the receptor or extrinsic pathway, and the mitochondrial or intrinsic pathway. Both apoptotic signaling pathways converge at the level of the specific proteases – the caspases.



**Fig.5.** Schematic diagram illustrating key molecular events in programmed cell death (apoptosis) in mammalian cells. (Source: Graham and Chen, 2001)

## 1.4.2. Caspases: the regulators of apoptosis

The processes that elicit activation of the apoptotic program are diverse. It can be initiated by oxidative stress, which in turn is mediated by the generation of Reactive Oxygen Species (ROS) (Buttke, 1994). Oxidative stress activates caspases, a family of cysteine proteases that are involved in the induction of cell death by apoptosis (Grimm, 1996; Polverino, 1997). Generally, caspases exist in cells as inactive precursor forms (Thornberry, 1998; Enari, 1996). Caspase activation is tightly regulated by an apoptosis activating complex, requiring proteolytic removal of an amino-terminal domain of procaspase to produce an active caspase (Chinnaiuan, 1997; Vaux, 1997; Zou, 1997; Li, 1997).



Once activated, caspase-3 cleaves many substrate proteins including PARP, ICAD, and structural proteins such as actin, fodrin, or lamin to generate the characteristic apoptotic morphology (Lazebnik, 1994; Mashima, 1997; Kothakota, 1997). Caspases are classified as either *initiators* (caspase 2, 8, 9, 10), or *effectors* (caspase 3, 6, 7) of proteolysis - the effector caspases activated downstream of the initiator caspases (Israels, 1999).

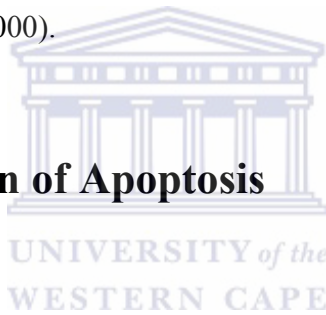
Caspase activity results in: cleavage of cytoskeletal proteins, disruption of the nuclear membrane, disruption of cell-cell contact, and the freeing of the DNA nuclease (CAD, caspase-activated deoxyribonuclease) from its associated protein inhibitor (ICAD) to allow DNA fragmentation (Thornberry and Lazebnik, 1998). The functions of caspases can be summarized as to: (1) arrest the cell cycle and inactivate DNA repair; (2) inactivate the inhibitor of apoptosis; and (3) dismantle the cellular cytoskeleton (Wang, Liu and Cui, 2005).

### 1.4.3. The role of the mitochondria in Apoptosis

The caspase-independent mechanism for commitment to cell death involves the mitochondria. At least three general mechanisms are known, and their effects may be interrelated, including (i) disruption of electron transport, oxidative phosphorylation, and adenosine triphosphate (ATP) production; (ii) release of proteins that trigger activation of caspase family proteases; and (iii) alteration of cellular reduction-oxidation (redox) potential (Green and Reed, 1998).

Mitochondria undergo two major changes during apoptosis induced by neoplastic agents, UV radiation, growth factor withdrawal, and DNA damage (Wang, Liu, Cui., 2005). First, the mitochondrial outer membranes become permeable to proteins, resulting in the release of proteins normally found in the space between the inner and outer membranes (including cytochrome *c*, apoptosis inducing factor, and others). Second, the inner mitochondrial membrane's transmembrane potential is reduced (Chang and Yang, 2000; Green and Kroemer, 1998; Green and Reed, 1998). The release of cytochrome *c* leads to the formation of a heptameric wheel-like caspase-activating complex, which has been termed as apoptosome; this is a high molecular weight complex composed of cytochrome *c*, Apaf-1, deoxyadenosine triphosphate (dATP), and procaspase-9, which forms a platform for the efficient processing and activation of caspase-9 (Wang *et al.*, 2005).

Caspase-9 has a caspase-associated recruitment domain (CARD) in the N-terminus, and this is a key site when it associates with Apaf-1 and cytochrome *c* (Chang and Yang, 2000; Acehan, Jiang, Morgan, Heuser, Wang and Akey, 2002; Zou, Henzel, Liu, Lutschg and Wang, 1999). Activation of caspase-9, in turn, cleaves effector caspases such as caspase-3, -6, and -7 (Nicholson and Thornberry, 1997). Then the effector caspases cleave their target proteins and culminate in the orderly demise of the cell. In this pathway of apoptosis, caspase-3 and -9 may be the most important as their activities influence the process of apoptosis as well as the type of cell death (Martelli, Cappellini, Tazzari, Billi, Tassi, Ricci, Fala and Conte, 2004; Coelho, Holl, Weltin, Lacornerie, Magnenet, Dufour, Bischoff, 2000).



#### **1.4.4. Gene regulation of Apoptosis**

##### **1.4.4.1. The p53 tumor suppressor**

Cell injury resulting in genotoxic events activates *p53*, a transcription regulator gene (Israels and Israels, 1999). The p53 protein product is a regulator of DNA transcription; it binds directly to DNA, recognizes DNA damage (single- or double-strand breaks), and mediates at least two important cellular events: it can induce cell cycle arrest in G<sub>1</sub> or it can promote apoptosis. If cellular damage is "considered" repairable, p53-induced cell cycle arrest allows time for DNA repair (Prives, 1998). With more extensive damage, to prevent the cell with an impaired DNA sequence from proliferating as a defective or malignant clone, p53 moves the cell into the apoptotic pathway (Prives, 1998).

As a sensor of cellular stress, p53 is a critical initiator of the intrinsic apoptotic pathway (Lowe and Lin, 2000). The importance of the p53 gene in suppressing tumor development is illustrated by the fact that it is disrupted in roughly 50% of all human tumors (Hickman, Moroni and Helin, 2002), and loss of p53 function has been shown to both disable apoptosis and accelerate tumor development (Attardi and Jacks, 1999; Ryan, Phillips and Vousden, 2001).

#### **1.4.4.2. Bcl-2/ Bax**

A major downstream regulation of the apoptotic death signal resides with the *bcl-2/bax* gene family (Israels and Israels, 1999). Sixteen members of this family have been recognized: some (including Bcl-2 and Bcl-X<sub>L</sub>) are apoptosis-inhibitory proteins, others (such as Bax, Bad, and Bid) are promoters of apoptosis (Kumar, 1997). Areas of commonality of structure allow these proteins to homo- and hetero- dimerize. A high expression of the Bax group promotes apoptosis; a high expression of the Bcl-2 group inhibits apoptosis — p53 regulates the ratios of the Bax/Bax, Bax/Bcl-2, and Bcl-2/Bcl-2 groups (Johnstone, Ruefli and Lowe, 2002).

The Bcl-2 group of proteins resides on the outer mitochondrial membrane oriented toward the cytosol – they govern ion transport and protect against breaches in the membrane. The Bax proteins reside in the cytosol. Upon receipt of the apoptotic signal, Bax proteins migrate and bind to the mitochondrial membrane "permeability transition pore," inducing loss of selective ion permeability (Green and Reed, 1998). As a result of the membrane changes, there is release into the cytosol of the contents of the intermembrane space,



including cytochrome c and apoptosis-inducing factor (AIF): AIF moves directly to the nucleus, where it produces chromatin condensation and nuclear fragmentation, while cytosolic cytochrome c sets in motion the terminal events of apoptosis (Green and Reed, 1998).

Given the importance of the Bcl-2 family members in regulating the intrinsic apoptotic pathway, it is not surprising that these genes are altered in tumor samples (Johnstone *et al.*, 2002). Bcl-2 is over-expressed in a variety of cancers (Reed, 1999), and its over-expression has been shown to accelerate tumorigenesis in transgenic mice (Adams, Wilkinson, Weiss, Diella, Gale, Deutsch, Risau and Klein, 1999).

#### **1.4.5. Apoptosis and Cancer**



Numerous studies have demonstrated that evasion of apoptosis is one of the most important mechanisms of uncontrolled growth of tumor cells and resistance to the immune system. Hence, apoptosis of initiated and/or neoplastic cells represents a protective mechanism against neoplastic transformation and development of tumors through elimination of genetically damaged cells or cells that may have been inappropriately induced to divide by mitogenic and proliferative stimuli (Owuor 2002).

Considerable attention has been focused on manipulation of apoptosis as a novel and promising strategy for cancer chemoprevention and therapy (Fesus 1995, Holzman 1996, Krzystyniak 2002, Cal 2003, Dong 2003). To achieve this goal, different naturally

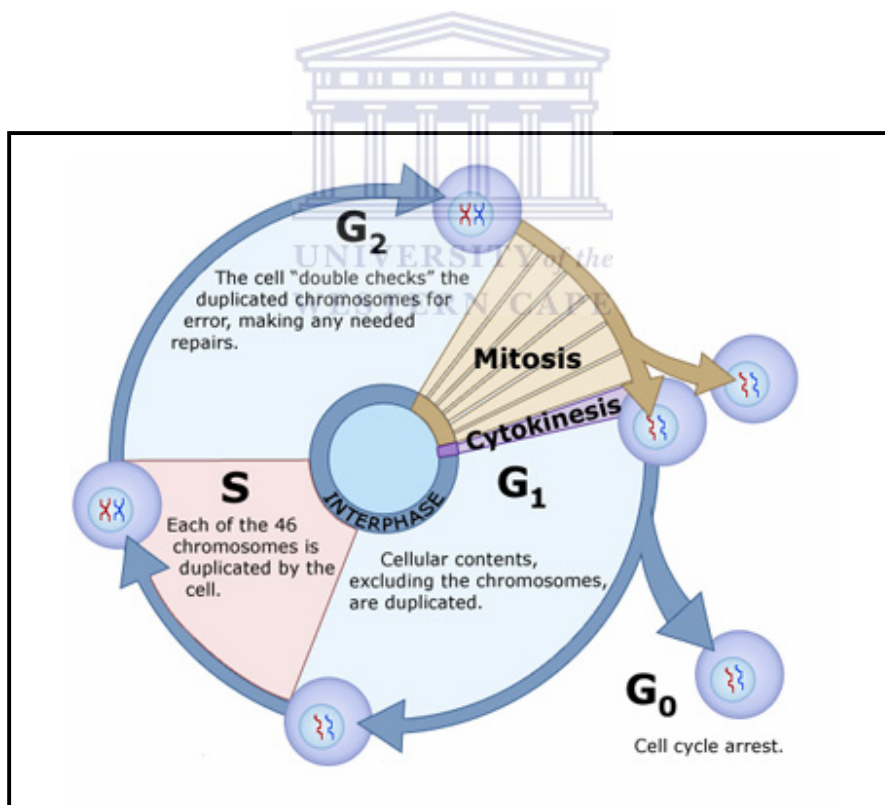
occurring compounds such as resveratrol, curcumin and genistein have been identified and were found to induce apoptosis in malignant cells (Surh, 1999; Kuo, 1996; Lian, 1999). Many such compounds exist in vegetables or fruits that are consumed by humans on a daily basis. Therefore, apoptosis induction by these agents in pre-cancerous and cancerous cells will undoubtedly prevent cancer.

### **1.4.6. The Cell Cycle**

The cell cycle is a conserved mechanism by which eucaryotic cells replicate themselves (MacLachlan, 1995). In metazoans, the process of cell loss and cell gain must be homeostatically balanced in order to generate and maintain the complex architecture of tissues, and also to allow adaptation to changing circumstances (Pucci, Kasten and Giordano, 2000).

Cell cycle deregulation is regarded as an essential step in the multi-step process of tumorigenesis. Many cell cycle regulators controlling the correct entry and progression through the cell cycle are altered in tumors; most, if not all, human cancers show a deregulated control of progression through the cell cycle (Garrett, 2001; Malumbres and Carnero, 2003). The vast majority of tumors have suffered defects that derail the cell cycle machinery, leading to increased cell proliferation. Such defects can target either components of the cell cycle itself or elements of upstream signalling cascades that eventually converge to trigger cell cycle events (Malumbres and Carnero, 2003).

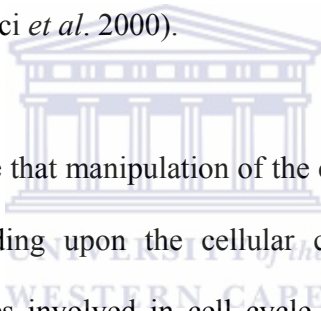
In a normal cell cycle (Fig 6), S-phase is always preceded by M-phase and M-phase does not occur until S-phase is complete. Between the S- and M-phases, there are two preparatory gaps. G<sub>1</sub> separates M from S, and G<sub>2</sub> is between S and M. When the cell undergoes differentiation, it exits from the G<sub>1</sub> phase of the cell cycle to enter into a quiescent state referred to as G<sub>0</sub> (Garrett, 2001). Transmission of genetic information from one cell generation to the next requires genome replication during the S-phase, and its segregation to the two new daughter cells during mitosis or M-phase. S- and M-phases are crucial events rigorously ordered in a cyclic process that allows for the correct duplication of the cell without accumulating genetic abnormalities (Pucci *et al.* 2000).



**Fig.6.** The cell cycle

(Source: <http://images1.clinicaltools.com/images/gene/cellcycle.jpg>)

The timing and order of cell cycle events are monitored during cell cycle checkpoints that occur at the G1/S boundary, in S-phase, and during the G2/M-phases (MacLachlan 1995). The checkpoints are a series of control systems enabling proliferation only in the presence of stimulatory signals such as growth factors, they also contribute to the fidelity with which genetic information is passed from one generation to the next (Pucci *et al.* 2000). The checkpoints are also activated by DNA damage and by mis-aligned chromosomes at the mitotic spindle. In this case, the growth arrest caused by checkpoints allows the cell to repair the damage. After damage repair, progression through the cell cycle resumes. If the damage cannot be repaired, the cell is eliminated through a form of cell death called apoptosis (Pucci *et al.* 2000).



There is accumulating evidence that manipulation of the cell cycle may prevent or induce an apoptotic response depending upon the cellular context (Evan, Brown, Whyte, Harrington, 1995). Many genes involved in cell cycle regulation are also involved in regulation of apoptosis (e.g. *c-myc*, *c-fos*, p53, many kinases and phosphatases) (Jarpe, Widmann, Knall, Schlesinger, Gibson, Yujiri, Fanger, Gelfand, Johnson, 1998). Thus, signals that promote proliferation can also promote apoptosis. If apoptosis is blocked by survival signals, increase in cell numbers occurs, which can manifest in cancer (Salvesen, 2002).

### 1.4.7. Plants as a source of anti-cancer agents

Plants have a long history of use in the treatment of cancer. Drug discovery from medicinal plants has played an important role in the treatment of cancer and most new clinical applications of plant secondary metabolites and their derivatives over the last half century have been applied towards combating cancer (Newman, Cragg, Snader, 2000; 2003; Butler, 2004). Some of the more well-known plant-derived chemotherapeutic agents are vinblastine and vincristine which were isolated from *Catharanthus roseus* and have been used clinically in the treatment of cancer for over 40 years (van Der Heijden, Jacobs, Snoeijer, Hallard, Verpoorte, 2004). Paclitaxel (Taxol<sup>®</sup>) was initially isolated from the bark of the Pacific Yew, *Taxus brevifolia* (Wall and Wani, 1996; Oberlies and Kroll, 2004). Paclitaxel is used in the treatment of breast, ovarian, and non-small cell lung cancer (NSCLC), and has also shown efficacy against Kaposi sarcoma (Cragg and Newman, 2005). Another important addition to the anti-cancer drug armamentarium is the class of clinically active agents derived from *camptothecin*, which is isolated from the Chinese ornamental tree, *Camptotheca acuminata* (Rahier, Thomas, Hecht, 2005). Its derivatives *Topotecan* and *Irinotecan* are used in the treatment of ovarian and small cell lung cancers and colorectal cancers (Cragg and Newman, 2005).

Many experimental investigations have demonstrated that several naturally occurring agents and plant extracts have shown antioxidant and anticancer potential in a variety of bioassay systems and animal models, having relevance to human disease (Azi, Kumae, Ahmad, 2003; Primchanien Nuttavut, Sineenart, Omboon, Narongchai, Neelobol, 2004;

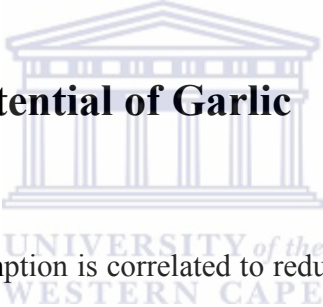
Wang, Zhang, Song, Wang, Ni, Luo, Aung, Xie, Tong, He, Yuan, 2006). In light of the continuing need for effective anticancer agents, and the association of fruit and vegetable consumption with reduced cancer risk, edible plants are increasingly being considered as sources of anticancer drugs (Ferguson, Kurowska, Freeman, Chambers, Koropatnick, 2004). There is significant scientific evidence showing that fruits and vegetables lower the risk of cancer (Chen, Chen, Dou, 2004). Furthermore, cancer chemoprevention, including the use of foods and medicinal herbs have been regarded as one of the most visible fields for cancer control (Jo, Hong, Ahn, Jung, Yang, park, Kim, Lee, Kang, 2004).

#### **1.4.8. Natural products as inducers of apoptosis**

A successful anticancer drug should kill or incapacitate cancer cells without causing excessive damage to normal cells. This ideal situation is achievable by inducing apoptosis in cancer cells. The lifespan of both normal and cancer cells is significantly affected by the rate of apoptosis (Taraphdar, Roy, Bhattacharya, 2001). Thus, modulating apoptosis may be useful in the management and therapy of cancer. Certain products from plants are known to induce apoptosis in neoplastic cells but not in normal cells (Hirano, Abe, Gotoh, Okah, 1995; Jiang, Yang-Yen, Yen, Lin, 1996; Chiao, Carothers, Grunberger, Solomon, Preston, Barrett, 1995).

It has become increasingly evident that apoptosis is an important mode of action for many anti-tumor agents, including ionizing radiation, alkylating agents such as cisplatin and 1,3-bis (2-chloroethyl)-1-nitrosourea (BCNU) (D'Amico and McKenna, 1994), topoisomerase inhibitor etoposide (Kauffman, Desnoyers, Ottaviano, Davidson, Poirier, 1993), cytokine tumour necrosis factor (TNF) (Shih and Stutman, 1996), taxol (Gibb, Taylor, Wan, O' Connor, Doering, Gercel-Taylor, 1997), and N-substituted benzamides such as metoclopramide and 3-chloroprocainamide (Pero, Olsson, Simanaitis, Amiri, Anderson, 1997). Apoptotic induction is thus a target for innovative mechanism-based drug discovery (Fisher, 1996; Workman, 1996; Taraphdar *et al.*, 2001).

#### **1.4.9. Anti-cancer potential of Garlic**



Garlic (*Allium sativum*) consumption is correlated to reduced cancer risk, and its extracts and components have been shown to effectively block experimentally induced tumors in a variety of sites including skin, breast, lung, oesophagus, uterus, cervix and colon, suggesting a general mechanism of action (Hussain, Jannu, Rao, 1990; Milner, 1996; Milner, 2001; Wargovich, 1988; Sumiyoshi, 1990; Liu, 1992; Amagase, 1993; Shukla, 1999; Song, 1999).

A report by Balasenthil, Rao and Nagini (2002) suggested that aqueous garlic extract might exert its chemo-preventive effect by inducing apoptosis. Many organosulfur compounds, the major active principles in garlic, have been shown to inhibit the proliferation of cancer cells, and some of them induced apoptosis in tumor cells of

different tissue origin (Dirsch, Gerbes, Vollmar, 1998; Kwon, Yoo, Ryu, Yang, Rho, Kim, Park, Kim, Park, 2002; Pinto, Lapsia, Shah, Santiago, Kim, 2001; Shirin, Pinto, Kawabata, Soh, Delohery, Moss, Murty, Rivlin, Holt, Weinstein, 2001; and Sigounas, Hooker, Anagnostou, Steiner, 1997). Epidemiological studies have shown that enhanced garlic consumption is closely related with reduced cancer incidence (Buiatti, 1989; Haenszel, 1972; You, 1989; Steinmetz, 1994; Dorant, 1996; Fleischauer, 2001; Hsing, 2002). Several animal experiments have shown that garlic treatment and aged garlic, in particular, counteracts formation and the development of various forms of experimentally induced tumors (Khanum, Anilakumar, Viswanathan, 2004).

Epidemiological investigations in China, Italy, and America have provided evidence that the risks of stomach and colon cancers are inversely related to regular consumption of garlic and garlic products. Experimental carcinogenesis studies indicate that components of garlic (*e.g.*, allyl sulfides) inhibit both the initiation and promotion stages of tumorigenesis for various types of cancer, including colorectal, lung, and skin cancers (Xiao, Pinto, Soh, Deguchi, Gundersen, Palazzo, Yoon, Shirin, Weinstein, 2003).

*Tulbaghia violacea*, commonly known as wild garlic is indigenous to South Africa. This plant is used in traditional medicine for the treatment of fever, asthma, hypertension and oesophageal cancer (Hutchings, Scott, Cunningham, 1996; Van Wyk and Wink, 2004). A study by Bungu, Frost, Brauns, and Van de Venter, (2006) demonstrated that extracts of *T. violacea* exhibited anti-proliferative and pro-apoptotic effects in four human cancer cell lines.



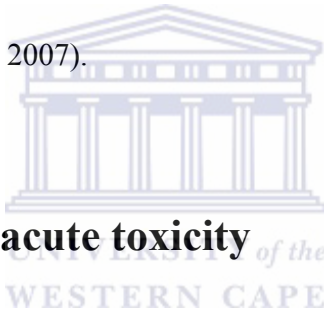
## 1.5. Toxicity

Toxicity is defined as “the potential of a substance to exert a harmful effect on humans or animals, and a description of the effect and the conditions or concentration under which the effect takes place” (Walum, 1998).

In order to support an application for a clinical trial or for the registration of a new drug, it is necessary to satisfy legislation that requires that certain data should be produced from a variety of toxicological investigations that show the safety profile of the compound to which humans may be exposed (Pascoe, 1983; Loomis and Hayes, 1996). Therefore, in the majority of cases of evaluation of the toxicity of most substances, rodents and non-human primates are first used in preclinical animal safety studies before further studies are done in humans. These animals are mainly used because of their biological similarity to humans that allows them to be regarded as suitable metabolic models for humans in a broad range of investigations (Pascoe, 1983; Loomis and Hayes, 1996).

### 1.5.1. Use of the mouse model in toxicological assessments

The mouse is the most commonly used vertebrate species in biomedical research, because it is small and therefore easier to handle and manipulate, can be maintained cost-effectively, and has a short gestation period (~ 19-20 days) (Rosenthal and Brown, 2007; Peters, Robledo, Bult, Churchill, Paigen, Svenson, 2007). In addition, mice are mammals and share a high degree of homology with humans (~99% of their genes) (Waterston, 2002); mice and humans share most physiological and pathological features: similarities in nervous, cardiovascular, endocrine, immune, muscoskeletal and other internal organ systems (Rosenthal and Brown, 2007).



### 1.5.2. Acute and sub-acute toxicity

**Acute toxicity:** Adverse effects are observed within a short time of exposure to the chemical. This exposure may be a single dose, or a short continuous exposure, or multiple doses administered over 24 hours or less.

**Subacute toxicity** Adverse effects are observed following repeated daily exposure to a chemical, or exposure for a significant part of an organism's lifespan (usually not exceeding 10%).

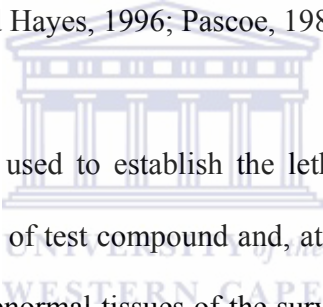
An adverse effect is “any effect that results in functional impairment and/or biochemical lesions that may affect the performance of the whole organism or that reduce the organ’s ability to respond to an additional challenge” (Walum, 1998).

An acute toxicity test is a single test that is conducted in a suitable animal species and may be done for essentially all chemicals that are of any biologic interest. Its purpose is to determine the symptomatology consequent to administration of the compound and to determine the order of lethality of the compound. The test consists of administering the compound to the animals on one occasion (Pascoe, 1983; Loomis and Hayes, 1996; Timbrell, 2002).

Furthermore, acute toxicity tests are those designed to determine the effects, which occur within a short period after dosing. They serve to establish the lethal dose range of the test substance and provide prompt warning if a highly toxic compound is being dealt with (Poole and Leslie, 1989). They also provide information on the limiting toxicity arising from the pharmacological effects of the compound on target organs and, often, on the maximum dose to be used in subsequent sub-acute studies (chronic studies). This latter information is particularly important for predicting the amount of chemical required for future toxicological studies (Poole and Leslie, 1989).

The initial procedure, in an acute toxicity test programme, is to test a series of range-finding single doses of the compound in a single animal species. This necessitates selection of a route of administration, preparation of the compound in a form suitable for administration via the selected route and selection of an appropriate experimental animal species (Poole and Leslie, 1989).

After exposure to single doses of the test compound (or treatment), the animals are monitored for a minimum of 24 hours for any clearly recognized effect (such as changes in locomotor activity; bizarre reactions; sensitivity to pain, sound and touch; changes in social interaction; aggressive behavior; convulsions; paralysis, etc.) seen, as an index of toxicity, shortly or/and consistently after the administration of the chemical (Timbrell, 2002; Loomis and Hayes). However, the most easily recognized and certainly the most significant of effects is that of death and this outcome is usually used as a primary measure of acute toxicity. If the animals appear to be healthy at the end of 24 hours, they are monitored at daily intervals for at least a further one to two weeks for the appearance of delayed toxicity (Loomis and Hayes, 1996; Pascoe, 1983; Timbrell, 2002).



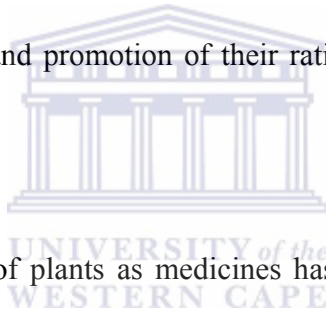
The acute toxicity test is also used to establish the lethal median dose or LD<sub>50</sub>. The animals are given a single dose of test compound and, at the end of 14-days observation period, the major organs and abnormal tissues of the surviving animals are collected and subjected to histopathological investigation. The LD<sub>50</sub> and its confidence limits are calculated from the lethality data using probit analysis (Pascoe, 1983). Since a great range of concentrations or doses of various chemicals may be involved in the production of harmful effects, the LD<sub>50</sub> has been used by some authors to devise categories of toxicity on the basis of the amounts of the chemicals necessary to produce harm. An example of such a categorization, along with respective lethal doses, is given in Table 1.

**Table 1. Classification of toxicity based on LD<sub>50</sub> ranges.**

<b>Category</b>	<b>LD<sub>50</sub> (mg/kg)</b>	<b>LD<sub>50</sub> (mg/kg)</b>	<b>Classification</b>
Extremely toxic	1 or less	< 5	Super-toxic
Highly toxic	1 to 50	5- 50	Extremely toxic
Moderately toxic	50 to 500	50- 500	Very toxic
Slightly toxic	500 to 5000	500- 5000	Moderately toxic
Practically non-toxic	5000 to 15000	5000- 15000	Slightly toxic
Relatively harmless	More than 15000	> 15000	Practically non-toxic
(Loomis and Hayes, 1996)		(Pascoe, 1983)	

### 1.5.3. Toxicity of medicinal plants

The use of traditional medicines and complementary and alternative medicine, in particular herbal medicinal products, continues to grow world-wide. In many parts of the world, policy-makers, health professionals and the public are wrestling with questions about the safety, quality, availability, preservation and further development of this type of health care. Although many medicinal plant therapies have promising potential, and are increasingly used, many of them are untested and their use not monitored. As a result, knowledge of their potential side-effects is limited. This makes identification of the safest and most effective therapies, and promotion of their rational use more difficult (WHO, 2002).



The extensive traditional use of plants as medicines has enabled those medicines with acute and obvious signs of toxicity to be well recognised and their use avoided. However, the premise that traditional use of a plant for perhaps many hundreds of years establishes its safety does not necessarily hold true (De Smet, 1995; De Smet, 1997). There is a mythical yet predominant view that herbal medicines are harmless and free of side effects because they are “natural” (Bateman, Chapman, Simpson, 1998; Stickel, Egerer Seitz, 2000).

The safety of several commercially available herbs, have over recent years, come into question due to reports of adverse reactions and potential interactions with prescription drugs (De Smet, 1997; Fugh-Berman, 2000; Ernst, 1999; Johns-Cupp, 1999; O’Hara,

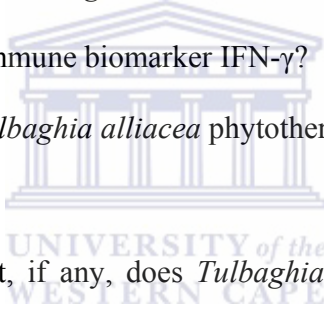
Kiefer, Farrell, Kemper, 1998). The effective and safe use of medicinal herbs has therefore been identified as a top research priority; and the implementation of regulatory procedures and investigations on safety are currently underway in developed countries (Popat, Shear, Malkiewicz, Stewart, Steenkamp, Thomson, Neuman, 2001).



## 1.6. Research Questions

Given the lack of scientific evidence for the indigenous use of *Tulbaghia alliacea* as a traditional garlic medicine to combat bacteria, fungi, and cancer, as well as our limited understanding of its potential toxicity, this investigation aimed at addressing the following questions:

- (i) What effect does *Tulbaghia alliacea* phytotherapy have on *Candidal* fungi?
- (ii) What effect does *Tulbaghia alliacea* have on mycobacteria and the modulation of the immune biomarker IFN- $\gamma$ ?
- (iii) What effect does *Tulbaghia alliacea* phytotherapy have on apoptosis in cancer cells?
- (iv) What toxicity effect, if any, does *Tulbaghia alliacea* phytotherapy have in mice?





## 1.7. References

- Acehan D, Jiang X, Morgan DG, Heuser JE and Wang X (2002). Three-dimensional structure of the apoptosome: implications for assembly, procaspase-9 binding, and activation. *Molecular Cell* 9: 423 – 432.
- Ackah AN, Coulibaly D, and Digbeu H (1995). Response to treatment, mortality, and CD4 lymphocyte counts in HIV-infected persons with tuberculosis in Abidjan, Cote d'Ivoire. *Lancet* 345: 607–610.
- Adams J (2001). Proteasome inhibition in cancer: development of PS-341. *Semin. Oncol.* 28: 613
- Adams RH, Wilkinson GA, Weiss C, Diella F, Gale NW, Deutsch U, Risau W, Klein R (1999). Roles of ephrinB ligands and EphB receptors in cardiovascular development: demarcation of arterial/venous domains, vascular morphogenesis, and sprouting angiogenesis. *Genes Dev* 13: 295 – 306.
- American Lung Association® Fact Sheet (2003). Multidrug-Resistant Tuberculosis. URL: <http://www.lungusa.org/diseases/mdrtbfac.html>
- Ankri S and Mirelman D (1999). Antimicrobial properties of allicin from garlic. *Microbes and Infection* 2: 125-129.
- Arendrof TM and Walker DM (1979). Oral candidal populations in health and disease. *British Dental Journal* 146: 267–272.
- Arikan A, Kulak Y and Kadir T (1995). Comparison of different treatment methods for localized and generalized simple denture stomatitis. *Journal of Oral Rehabilitation* 22: 365–369.

- Attardi LD and Jacks T (1999). The role of p53 in tumour suppression: lessons from mouse models. *Cell. Mol. Life Sci.* 55: 48
- Aziz MH, Kumae R, Ahmad N (2003). Cancer chemoprevention by resveratrol: *in vitro* and *in vivo* studies and the underlying mechanisms. *Int. J. Oncol.* 23: 17–28.
- Balasenthil S, Rao KS, Nagini (2002). Garlic induces apoptosis during 7,12-dimethylbenz[*a*]anthracene-induced hamster buccal pouch carcinogenesis. *Oral Oncol.* 38: 431–436.
- Balick M.J and Cox PA (1996). Plants, People and Culture. The Science of Ethnobotany. New York: Scientific American Library.
- Bartlett JG (2000). Update in infectious diseases. *Annals of Internal Medicine* 133: 285–292.
- Behrend L (2005). Manganese Superoxide Dismutase Induces p53-Dependent Senescence in Colorectal Cancer Cells. *Journal of Molecular and Cellular Biology.* 25: 7758–7769.
- Bergmann OJ (1991). Alteration in oral microflora and pathogenesis of acute oral infections during remission–induction therapy in patients with acute myeloid leukaemia. *Scandinavian Journal of Infectious Diseases* 23: 355–366.
- Biswas SK, Huang J, Persaud S, Basu A (2004). Down-regulation of Bcl-2 is associated with cisplatin resistance in human small cell lung cancer H69 cells. *Journal of Molecular Cancer Therapeutics.* 3: 327–334.
- Blignaut E, Messer S, Hollis RJ, and Pfaller MA (2002). Antifungal susceptibility of South African oral yeast isolates from HIV/AIDS patients and healthy individuals. *Diagnostic Microbiology and Infectious Diseases* 44: 169-174.

- Bloch AB (1994). Nation wide survey of drug-resistant tuberculosis in the United States. *Journal of the American Medical Association* 271: 665–671.
- Block E (1985). The chemistry of garlic and onions. *Sci. Am.* 252: 114–119.
- Bloland PB (2000). Combination therapy for malaria in Africa: hype or hope? *Bull. WHO* 78: 1378–1388.
- Brennan PJ, Besra GS (1997). Structure, function and biogenesis of the mycobacterial cell wall. *Biochem. Soc. Trans.* 25: 188–194.
- Bruce AJ, Rogers RS (2004). Oral manifestations of sexually transmitted diseases. *Clinics in Dermatology* 22: 520-527.
- Buiatti E, Palli D, Decarli A, Amadori D, Avellini C, Bianchi S, Biserni R, Cipriani F, Cocco P, Giacosa A, Mrubini E, Puntoni R, Vindigi C, Frumeni J, Blot W (1989). A case-control study of gastric cancer, and diet in Italy. *Int J Cancer* 44: 611–616.
- Bungu L, Frost CL, Brauns SC, Van de Venter M (2006). *Tulbaghia violacea* inhibits growth and induces apoptosis in cancer cells *in vitro*. *Afr J Biotech* 5: 1936-1943.
- Butler MS (2004). The role of natural product chemistry in drug discovery. *Journal of Natural Products* 67: 2141– 2153.
- Buttke TM, Sandstrom PA (1994). Oxidative stress as a mediator of apoptosis. *Immunol Today* 15: 7–10.
- Cal C, Garban H, Jazirehi A, Yeh C, Mizutani Y, Bonavida B (2003). Resveratrol and cancer: chemoprevention, apoptosis, and chemo-immunosensitizing activities. *Curr. Med. Chem. Anti-cancer Agents* 3: 77–93.

- Calcabrini A (2006). Inhibition of proliferation and induction of apoptosis in human breast cancer cells by lauryl gallate. *Journal of Carcinogenesis*. 27: 1699–1712.
- Camerino DC; Tricarico D, Desaphy, JF (2007). Ion Channel Pharmacology. *The Journal of the American Society for Experimental NeuroTherapeutics* 4: 184–198.
- Cavallito C and Bailey JH (1944). Allicin, the antibacterial principle of *Allium sativum*. Isolation, physical properties and antibacterial action. *Journal of the American Chemical Society* 66: 1944–1952.
- CDC (2006). Revised definition of extensively drug-resistant tuberculosis. *Morbidity and Mortality Weekly Report* 55:1176.
- Chaturvedi R (2004). Induction of Polyamine Oxidase 1 by *Helicobacter pylori* Causes Macrophage Apoptosis by Hydrogen Peroxide Release and Mitochondrial Membrane Depolarization. *The Journal of Biological Chemistry*. 279: 40161–40173.
- Chelli B, Lena A, Vanacore R, Da Pozzo E, Costa B, Rossi L, Salvetti A, Scatena F, Ceruti S, Abbracchio MP, Gremigni V; Martini C (2004). Peripheral benzodiazepine receptor ligands: mitochondrial transmembrane potential depolarization and apoptosis induction in rat C6 glioma cells. *Journal of Biochemical Pharmacology*. 68: 125–134.
- Chen MS, Chen D, Dou QP (2004). Inhibition of proteasome activity by various fruits and vegetables is associated with cancer cell death. *In Vivo* 18: 73.
- Chiao C, Carothers Am, Grunberger D, Solomon G, Preston GA, Barrett JC (1995). *Cancer Res* 55: 3576 – 3583.

- Chien W, Kumagai T, Miller CW, Desmond JC, Frank JM, Said JW, Koeffler HP (2004). Cyr61 Suppresses Growth of Human Endometrial Cancer Cells. *The Journal of Biological Chemistry*. 279: 53087–53096
- Chinnaiyan AM, O'Rourke K, Lane BR, Dixit VM (1997). Interaction of CED-4 with CED-3 and CED-9: a molecular framework for cell death. *Science* 275: 1122–1126.
- Coelho D, Holl V, Weltin D, Lacornerie T, Magnenet P, Dufour P, Bischoff P (2000). Caspase-3-like activity determines the type of cell death following ionizing radiation in MOLT-4 human leukaemia cells. *Br J Cancer* 83: 642 – 649.
- Cohen GM (1997). Caspases: the executioners of apoptosis. *Biochem. J.* 326: 1-16.
- Cohen JJ (1993). Apoptosis. *Immunol Today* 14: 126-130
- Corbett EL, Watt CJ, Walker N, Maher D, Williams BG, Raviglione MC, Dye C (2003). The growing burden of tuberculosis: global trends and interactions with the HIV epidemic. *Archives of Internal Medicine* 163: 1009 – 1021.
- Cordell GA (1995). Changing strategies on natural products chemistry. *Phytochem* 40: 1585-1612.
- Cordell GA (1981). Introduction to Alkaloids – A Biogenetic Approach, Wiley-Interscience. New York.
- Cunningham AB (1988) Overexploitation of medicinal plants in Natal/KwaZulu: Root causes. *Veld & Flora* (September): 85–87
- D'Amico AV, McKenna WG (1994). *Radiother. Oncol.* 33: 3–10.

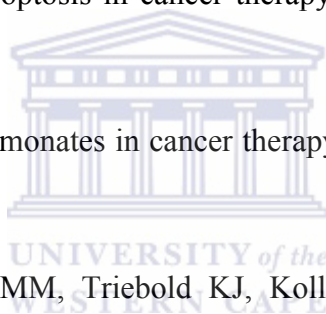
- Deng X, Yin F; Lu X, Cai B, Yin W (2006). The Apoptotic Effect of Brucine from the Seed of *Strychnos nux-vomica* on Human Hepatoma Cells is Mediated via Bcl-2 and Ca<sup>2+</sup> Involved Mitochondrial Pathway. *Journal of Toxicological Sciences*. 91: 59–69
- De Smet PAGM (1995). Health risks of herbal remedies. *Drug Safety* 13: 81-93.
- De Smet PAGM (1997). Adverse effects of herbal remedies. *Adverse drug reaction bulletin* 183: 695-698.
- DeSmet PAGM, Keller K, Hansel R, Chandler RF (1997). *Adverse effects of herbal drugs*, volumes 1, 2 and 3. Springer, New York.
- Dietze EC (2001). Tamoxifen but Not 4-Hydroxytamoxifen Initiates Apoptosis in p53 – normal Human Mammary Epithelial Cells by Inducing Mitochondrial Depolarization. *The Journal of Biological Chemistry*. 276: 5384–5394.
- Dirsch VM, Gerbes AL, Vollmar AM (1998). Ajoene, a compound of garlic, induces apoptosis in human promyeloleukemic cells, accompanied by generation of reactive oxygen species and activation of nuclear factor kappa B. *Mol. Pharmacol.* 53: 402–407.
- Dodd CL, Greenspan D, Katz MH, Westenhause JL, Feigal DW and Greenspan JS (1991). Oral candidiasis in HIV infection: pseudomembraneous and erythematous candidiasis show similar rates of progression to AIDS. *Journal of Acquired Immunodeficiency Syndrome* 5: 1339-1343.
- Dolin PJ, Raviglione MC and Kochi A (1994). Global tuberculosis incidence and mortality during 1990-2000. *Bulletin of the World Health Organization* 72: 213-220.

- Dong Z (2003). Molecular mechanism of the chemopreventive effect of resveratrol. *Mutat. Res.* 523–524: 145–150.
- Dreizen S (1984). Oral candidiasis. *American Journal of Medicine* 77: 28–33.
- DüBmann H (2003). Outer mitochondrial membrane permeabilization during apoptosis triggers caspase-independent mitochondrial and caspase-dependent plasma membrane potential depolarization: a single-cell analysis. *Journal of Cell Science.* 116: 525–536.
- Duncan K (2003) Progress in TB drug development and what is still needed. *Tuberculosis* 83: 210-207.
- Dye C, Scheele S, Dolin P, Pathania V and Raviglione MC (1999). Global burden of tuberculosis: estimated incidence, prevalence, and mortality by country. WHO global surveillance and monitoring project. *Journal of American Medical Association* 28: 677–686.
- Ellepola ANB and Samaranayake LP (2000). Antimycotic agents in oral candidosis: an overview. 1. Clinical variants. *Dent. Update* 27: 111–116.
- Ellmore GS and Feldberg RS (1994). Alliin lyase localization in bundle sheaths of garlic clove (*Allium sativum*). *American. Journal of Botany* 81:89–94.
- Enari M, Talanian RV, Wong WW, Nagata S (1996). Sequential activation of ICE-like and CPP32-like proteases during Fas-mediated apoptosis. *Nature* 380: 723–726.
- Epstein JB, Truelove EL and Izutzu KT (1984). Oral candidiasis: pathogenesis and host defense. *Review of Infectious Diseases* 6: 96–106.

- Ernst E (1999). Second thoughts about safety of St. John's wort. *The Lancet* 354: 2014-2015.
- Evan GI, Brown L, Whyte M, Harrington E (1995). Apoptosis and cell cycle. *Current Opinion in Cell Biology* 7: 825 – 834.
- Evan G, Littlewood T (1998). A matter of life, and cell death. *Science* 281: 317–322.
- Farbman KS, Barnett ED, Bolduc GR, Klein JO (1993). Antibacterial activity of garlic and onions: a historical perspective. *Journal of Pediatric Infectious Diseases* 12: 613.
- Farmer P, Kim JY (1998). Community based approaches to the control of multi-drug resistant tuberculosis: introducing 'DOTS-plus'. *British Medical Journal* 317: 671–674.
- Farnsworth NR, Akerele O, Bingel AS, Soejarto DD, Guo Z (1985). *Bulletin of the World Health Organization* 63: 965.
- Fearon DT, Locksley RM (1996). The instructive role of innate immunity in the acquired immune response. *Science* 272: 50–53.
- Feng ZH, Zhang GM, Hao TL (1994). Effect of diallyl trisulfide on the activation of T cell and macrophage-mediated cytotoxicity. *Journal of Tongji Medical University* 14: 142.
- Fennell CW, Lindsey KL, McGaw LJ, Sparg SG, Stafford GI, Elgorashi EE, Grace OM, van Staden J (2004). Assessing African medicinal plants for efficacy and safety: pharmacological screening and toxicology. *Journal of Ethnopharmacology* 94: 205-217.



- Ferguson PJ, Kurowska E, Freeman DJ, Chambers AF, Koropatnick DJ (2004). A flavonoid fraction from cranberry extract inhibits proliferation of human tumor cell lines. *J. Nutr.* 134: 1529.
- Fesus L, Szondy Z, Uray I (1995). Probing the molecular program of apoptosis by cancer chemopreventive agents. *J. Cell Biochem. Suppl.* 22: 151–161.
- Fichtenbaum CJ, Koletar S, Yiannoutsos C, Holland F, Pottage J, Cohn SE, Walawander A, Frame P, Feinberg J, Saag M, Van der Horst C, Powderly WG (2000). Refractory mucosal candidiasis in advanced human immunodeficiency virus infection. *Clinical Infectious Diseases* 30: 749-756.
- Fisher DE (1994). Apoptosis in cancer therapy: crossing the threshold. *Cell* 78: 539–542.
- Flescher E (2007). Jasmonates in cancer therapy. *Journal of Cancer Letters.* 245: 1–10
- Flynn JL, Goldstein MM, Triebold KJ, Koller B, Bloom BR (1992). Major histocompatibility complex class I-restricted T cells are required for resistance to *Mycobacterium tuberculosis* infection. *National Academy of Science, USA*, 89: 12013–12017.
- Foster S (1998). *Garlic*. Available from URL:  
<http://www.herbphoto.com/education/monograph/garlic.html>
- Foye WO, Lemke TL, Williams DA (1995). Principles of Medicinal Chemistry. 4th ed. Baltimore: Williams & Wilkins.
- Fugh-Berman A (2000). Herb-drug interactions. *The Lancet* 355: 134-138.



- Fukumori T, Kanayama H, Raz A (2007). The role of galectin-3 in cancer drug resistance. *Journal of Drug Resistance Update*. 10: 101–108.
- Fukumori T (2006). Galectin-3 Regulates Mitochondrial Stability and Antiapoptotic Function in Response to Anticancer Drug in Prostate Cancer. *Journal of Cancer Research*. 66: 3114–3119.
- Gao X (2007). Synthetic triterpenoids inhibit growth and induce apoptosis in human glioblastoma and neuroblastoma cells through inhibition of prosurvival Akt, NF- $\kappa$ B and Notch1 signaling. *Journal of Neurooncology*. 84:147–157.
- Garrett MD (2001). Cell cycle control and cancer. *Current Science*. 81: 515 – 522.
- Gavrieli Y, Sherman Y, Ben-Sasson SA (1992). Identification of programmed cell death *in situ* via specific labeling of nuclear DNA fragmentation. *J Cell Biol* 119: 493-501.
- Gescher A (1998). Analogs of staurosporine: potential anticancer drugs. *Gen Pharmacol*. 31: 721 – 728.
- Gibb RK, Taylor DD, Wan T, O'Connor DM, Doering, DL, Gercel-Taylor, C (1997). *Gynaecol. Oncol*. 65: 13–22.
- Gibbons S (2005). Plants as a source of bacterial resistance modulators and anti-infective agents. *Phytochem Reviews* 4: 63 – 78.
- Gilks C, Katabira E, De Cock KM (1997). The challenge of providing effective care for HIV/AIDS in Africa. *AIDS* 11: 99-106.
- Ginsburg AS, Grosset JH, Bishai WR (2003). Fluoroquinolones, tuberculosis, and resistance. *The Lancet Infectious Diseases* 3: 432-442.

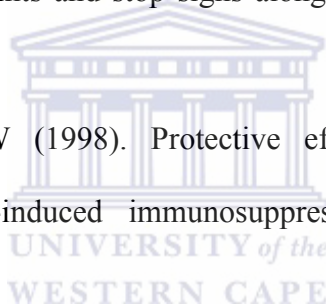


- Girling DJ (1989). The chemotherapy of tuberculosis. In: Ratledge C, Stanford JL and Grange JM. *The Biology of the Mycobacteria*. London: Academic Press. p. 43–47.
- Glick M, Muzyka BC, Lurie D, Salkin LM (1994). Oral manifestations associated with HIV-related disease as markers for immune suppression and AIDS. *Oral Surgery, Oral Medicine, and Oral Pathology* 77: 344–345.
- Goto H, Yanagimachi M, Kajiwara R, Kuroki F, Yokota S (2007). Lack of mitochondrial depolarization by oxidative stress is associated with resistance to buthionine sulfoximine in acute lymphoblastic leukemia cells. *Journal of Leukemia Research*. 31: 1293–1301.
- Gottlieb E, Van Der Heiden MG, Thompson CB (2000). Bcl-xL Prevents the Initial Decrease in Mitochondrial Membrane Potential and Subsequent Reactive Oxygen Species Production during Tumor Necrosis Factor Alpha-Induced Apoptosis. *Journal of Molecular and Cellular Biology* 20: 5680–5689.
- Gottlieb MS, Schroff R, Weisman JD, Fan PT, Wolf RA, Saxon A, Schanker HM (1981). *Pneumocystis carinii* pneumonia and mucosal candidiasis in previously healthy homosexual men: evidence of a new acquired cellular immunodeficiency. *New England Journal of Medicine* 305: 1425–1431.
- Graham SH, Chen J (2001). Programmed cell death in cerebral ischemia. *J Cereb Blood Flow Metab* 21: 99-109.
- Grange JM, Davey RW (1990). Detection of anti-tuberculosis activity in plant extracts. *Journal of Applied Bacteriology* 68: 587–591.
- Green DR, Reed JC (1998). Mitochondria and apoptosis. *Science* 281: 1309.

- Grimm S, Bauer MKA, Baeuerle PA, Schulze-Osthoff K (1996). Bcl-2 down-regulates the activity of transcription factor NF- $\kappa$ B induced upon apoptosis. *J Cell Biol* 134: 13–23.
- Gupta KC, Viswanathan R (1955). Combined action of streptomycin and chloramphenicol with plant antibiotics against tubercle bacilli. I. Streptomycin and chloramphenicol with cepharanthine. II. Streptomycin and allicin. *Antibiotic Chemotherapy (Wash. DC)* 5:24–27.
- Haenszel W, Kurihara M, Segi M, Lee RK (1972). Stomach cancer among Japanese in Hawaii. *J Natl Cancer Inst* 49: 969–988.
- Ham YM (2006). Ginsenoside-Rh2-Induced Mitochondrial Depolarization and Apoptosis Are Associated with Reactive Oxygen Species and Ca<sup>2+</sup>-Mediated c-Jun NH2-Terminal Kinase 1 Activation in HeLa Cells. *The Journal of Pharmacology and Experimental Therapeutics*. 319: 1276–1285.
- Hartwell JL (1982). Plants used against cancer. Quarterman, Lawrence, MA.
- Hickman ES, Moroni MC, Helin K (2002). The role of p53 and pRB in apoptosis and cancer. *Current Opinion in Genetics & Development* 12: 60.
- Hirano T, Abe K, Gotoh M, Oka K (1995). *Br. J. Cancer* 72: 1380 – 1388.
- Hoareau L, DaSilva D, Edgar J (1999). Medicinal Plants: A Re-emerging Health Aid. *EJB Electronic Journal of Biotechnology* 2: 2.
- Hogarth PJ, Jahans JK, Hecker R, Hewinson RG, Chambers RA (2003). Evaluation of adjuvants for protein vaccines against tuberculosis in guinea pigs. *Vaccine* 21: 977-982.

- Holmstedt BR, Bruhn JG (1995). Ethnopharmacology – a challenge. In: Schultes RE and von Reis S (eds) *Ethnobotany. Evolution of a Discipline*. Portland: Dioscorides Press, 332 – 336.
- Holzman D (1996). Apoptosis provides new targets for chemotherapy. *J. Natl. Cancer Inst.* 88: 1098–1100.
- Hussain R, Talat N, Shahid F, Dawood G (2007). Longitudinal Tracking of Cytokines after Acute Exposure to Tuberculosis: Association of Distinct Cytokine Patterns with Protection and Disease Development. *Clinical and Vaccine Immunology* 14: 1578 – 1586
- Hussain SP, Jannu LN, Rao AR (1990). Chemopreventive action of garlic on methylcholanthrene-induced carcinogenesis in the uterine cervix of mice. *Cancer Lett.* 49: 175–180.
- Hutchings AH, Scott GL, Cunningham A (1996). *Zulu Medicinal Plants: An Inventory*. University of Natal Press, Scottsville, South Africa.
- Ibrado AM, Huang Y, Fang G, Liu L, Bhalla K (1996). Overexpression of Bcl-2 or Bcl-xL inhibits Ara-C-induced CPP32/Yama protease activity and apoptosis of human acute myelogenous leukemia HL-60 cells. *Cancer Res* 56: 4743–4748.
- Iimuro M, Shibata H, Kawamori T, Matsumoto T, Arakawa M, Sugimura T (2002). Suppressive effects of garlic extract on *Helicobacter pylori*-induced gastritis in Mongolian gerbils, *Cancer Lett* 187: 61–68.
- Irobi ON, Moo-Young M, Anderson WA, Daramola SO (1994). Antimicrobial activity of bark extracts of *Bridelia ferruginea*. *Journal of Ethnopharmacology* 43: 185–190.

- Israels LG, Israels ED (1999). Apoptosis. *Stem Cells* 17: 306-313.
- Ivanova D, Gerova D, Chervenkov T, Yankov T (2005). Polyphenols and antioxidant capacity of Bulgarian medicinal plants. *J. Ethnopharmacol.* 96: 145
- Iwu MM (1993). Handbook of African Medicinal Plants. Florida: CRC Press. ISBN 0-8493-4266-X
- Jacobson MD, Burne JF, Raff MC (1994). Programmed cell death and Bcl-2 protection in the absence of a nucleus. *EMBO J.* 13: 1899.
- Jarpe MB, Widmann C, Knall C, Schlesinger TK, Gibson S, Yujiri T, Fanger GR, Gelfand EW, Johnson GL (1998). Anti-apoptotic versus pro-apoptotic signal transduction: checkpoints and stop signs along the road to death. *Oncogene* 17: 1475 – 1482.
- Jeong HG, Lee YW (1998). Protective effects of diallyl sulfide on *N*-nitrosodimethylamine-induced immunosuppression in mice. *Cancer Letters* 134:73.
- Jiang MC, Yang-Yen HF, Yen JJ, Lin JK (1996). *Nutr. Cancer* 26: 111 - 120
- Jin YH, Yim H, Park JH, Lee SK (2003). Cdk2 activity is associated with depolarization of mitochondrial membrane potential during apoptosis. *Journal of Biochemical and Biophysical Research Communications* 305: 974–980
- Jo EH, Hong HD, Ahn NC, Jung JW, Yang SR, Park JS, Kim SH, Lee YS, Kang KS (2004). Modulations of the Bcl-2/Bax family were involved in the chemopreventive effects of licorice root (*Glycyrrhiza uralensis* Fisch) in MCF-7 human breast cancer cell. *J. Agric. Food Chem.* 52: 1715.



- Johnson MG, Vaughn RH (1969). Death of *Salmonella typhimurium* and *Escherichia coli* in the presence of freshly reconstituted dehydrated garlic and onion. *Applied Microbiology* 17:903.
- Johns-Cupp M (1999). Herbal remedies: adverse effects, and drug interactions. *Am Family Physician* 59: 239–1244.
- Johnstone RW, Ruefli AA, Lowe SW (2002). Apoptosis: a link between cancer genetics and chemotherapy. *Cell* 108: 153.
- Jones KDJ, Hesketh T, Yudkin J (2008). Extensively drug-resistant tuberculosis in sub-Saharan Africa: an emerging public-health concern. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 102: 19-224.
- Jonkers D, Van den Broek E, Van Dooren I (1999). Antibacterial effect of garlic and omeprazole on *Helicobacter pylori*. *Journal of Antimicrobial Chemotherapy* 43:837
- Kadir T, Pisiriciler R, Akyüz S, Yarat A, Emekli N, Ipbüker A (2002). Mycological and cytological examination of oral candidal carriage in diabetic patients and non-diabetic control subjects: thorough analysis of local aetiologic and systemic factors. *Journal of Oral Rehabilitation*. 29: 452–457.
- Kadir T, Uygun B, Akyuz S (2005). Prevalence of *Candida* species in Turkish Children: relationship between dietary intake and carriage. *Archives of Oral Biology* 50: 33-37.
- Katz MH, Greenspan D, Westenhouse J, Heesol NA, Bichbinder SP, Lifson AR, Shiboski S, Osmond D, Moss A, Samuel M (1992). Progression to AIDS in HIV-

infected homosexual and bisexual men with hairy leukoplakia and oral candidiasis. *Journal of Acquired Immune Deficiency Syndrome* 6: 95-100.

- Kaufmann SH, Desnoyers S, Ottaviano Y, Davidson NE, Poirier GG (1993). Specific Proteolytic Cleavage of Poly (ADP-ribose) Polymerase: An Early Marker of Chemotherapy-induced Apoptosis. *Cancer Res.* 3: 3976–3985.
- Kaufmann SHE and Hess J (2000). Immune response against *Mycobacterium tuberculosis*: implications for vaccine development. *Journal of Biotechnology* 83: 13-17.
- Kaufmann SHE (1999). Cell-mediated immunity: Dealing a direct blow to pathogens. *Current Trends in Biology* 9: R97–R99.
- Kerr JFR, Winterford CM, Harmon BV (1994). Apoptosis – its significance in cancer and cancer therapy. *Cancer* 73: 2013 – 2026.
- Khanum F, Anilakumar KR, Viswanathan KR (2004). Anticarcinogenic properties of garlic: a review. *Critical Reviews in Food Science and Nutrition* 44: 479 – 488.
- Klein RS, Harris CA and Small CB (1984). Oral candidiasis in high-risk patients as the initial manifestation of the acquired immunodeficiency syndrome. *New England Journal of Medicine* 311: 354–358.
- Knowles LM, Milner JA (2001). Possible mechanism by which allyl sulfides suppress neoplastic cell proliferation. *J Nutr* 131: 1061S–1066S.
- Koch HP and Lawson LD (1996). Garlic, the science and therapeutic application of *Allium sativum L.* and related species. Baltimore: Williams & Wilkins. p.33.



- Koks CHW, Crommentuyn KML, Mathot RAA, Mulder JW, Meenhorst PL and Beijnen JH (2002). Prognostic factors for the clinical effectiveness of fluconazole in the treatment of oral candidiasis in HIV-1-infected individuals. *Pharmacological Research* 46: 89-94.
- Kothakota S, Azuma T, Reinhard C, Klippel A, Tang J, Chu K, McGarry TJ, Kirschner MW, Koths K, Kwiatkowski DJ, Williams LT (1997). Caspase-3-generated fragment of gelsolin: effector of morphological change in apoptosis. *Science* 278: 294–298.
- Kroemer G, Zamzami N, Susin SA (1997). Mitochondrial control of apoptosis. *Immun Today* 18: 44-51.
- Krohn AJ, Wahlbrink T, Prehn JHM (1999). Mitochondrial Depolarization Is Not Required for Neuronal Apoptosis. *The Journal of Neuroscience* 19: 7394–7404
- Krzystyniak KL (2002). Current strategies for anticancer chemoprevention and chemoprotection. *Acta Pol. Pharm.* 59: 473–478.
- Kuo ML, Huang TS, Lin JK (1996). Curcumin, an antioxidant and anti-tumor promoter, induces apoptosis in human leukemia cells. *Biochim. Biophys. Acta* 1317: 95–100.
- Kwon KB, Yoo SJ, Ryu DG, Yang JY, Rho HW, Kim JS, Park JW, Kim HR and Park BH (2002). Induction of apoptosis by diallyl disulfide through activation of caspase-3 in human leukemia HL-60 cells. *Biochem. Pharmacol.* 63: 41–47.
- Lall N and Meyer JJM (1999). In vitro inhibition of drug-resistant and drug-sensitive strains of *Mycobacterium tuberculosis* by ethnobotanically selected South African plants. *Journal of Ethnopharmacology* 66: 347-354.

- Lau BHS, Yamasaki T and Gridley DS (1991). Garlic compounds modulate macrophage and T-lymphocyte functions. *Molecular Biotherapy* 3:103.
- Lazebnik YA, Kaufmann SH, Desnoyers S, Poirier GG, Earnshaw WC (1994). Cleavage of poly-(ADP-ribose) polymerase by a proteinase with properties like ICE. *Nature* 371: 346–347.
- Le Grand A and Wondergem P (1989). Herbal medicine and health promotion. A comparative study of herbal drugs in primary health care. Royal Tropical Institute Amsterdam, pp 7–20. ISBN 90-6832-033-5.
- Le HV, Minn AJ, Massague J (2005). Cyclin-dependent Kinase Inhibitors Uncouple Cell Cycle Progression from Mitochondrial Apoptotic Functions in DNA-damaged Cancer Cells. *The Journal of Biological Chemistry* 280: 32018–32025.
- Leaman D (2002). *Medicinal plants. Briefing notes on the impacts of domestication/cultivation on conservation.* Paper for the "Commercial captive propagation and wild species conservation" workshop. Jacksonville.
- Ledezma E, Apitz-Castro R, Cardier J (2004). Apoptotic and antiadhesion effect of ajoene, a garlic derived compound, on the murine melanoma B16F10 cells: possible role of caspase-3 and the alpha (4) beta (1) integrin. *Cancer Lett* 206: 35–41.
- Lee HH (2003). Differential effects of natural polyphenols on neuronal survival in primary cultured central neurons against glutamate- and glucose deprivation-induced neuronal death. *Journal of Brain Research* 986: 103–113.

- Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES, Wang X (1997). Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* 91: 479–489.
- Lian F, Li Y, Bhuiyan M, Sarkar FH (1999). p53-independent apoptosis induced by genistein in lung cancer cells. *Nutr. Cancer* 33: 125–131.
- Lin HL, Yang JS, Yang JH, Fan SS, Chang WC, Li YC, Chung JG (2006). The Role of Ca<sup>2+</sup> on the DADS-induced Apoptosis in Mouse–Rat Hybrid Retina Ganglion Cells (N18). *Journal of Neurochemical Research* 31:383–393.
- Livermore DM and Dudley MN (2000). Antimicrobials: better use, better drugs, or both? *Current Opinion in Microbiology* 3: 487-488.
- Loomis TA, Hayes AW (1996) Loomis's essentials of toxicology . 4<sup>th</sup> edition, *Academic Press*, California 208-245.
- Lowe SW, Lin AW (2000). Apoptosis in cancer. *Carcinogenesis* 21: 485 – 495.
- Lu D (2007). Protein kinase C- $\epsilon$  protects MCF-7 cells from TNF-mediated cell death by inhibiting Bax translocation. *Journal of Apoptosis*. 12: 1893–1900.
- Malekova L, Kominkova V, Ferko M, Stefanik P, Krizanova P, Ziegelhoffer A, Szewczyk A, Ondrias K (2007). Bongkrekie acid and atractyloside inhibits chloride channels from mitochondrial membranes of rat heart. *Journal of Biochimica et Biophysica Acta*. 1767: 31–44.
- Martelli AM, Cappellini A, Tazzari P, Billi AM, Tassi C, Ricci, Falà F, Conte R (2004). Caspase-9 is the upstream caspase activated by 8-methoxypsoralen and ultraviolet-A radiation treatment of Jurkat T-leucemia cells and normal T-lymphocytes. *Haematologica* 89: 471–479.

- Mashima T, Naito M, Noguchi K, Miller DK, Nicholson DW, Tsuruo T (1997). Actin cleavage by CPP-32/apopain during the development of apoptosis. *Oncogene* 14: 1007–1012.
- McCarthy GM (1992). Host factors associated with HIV-related oral candidiasis. A review. *Oral Surgery, Oral Medicine and Oral Pathology* 73: 181–186.
- McCarthy GM and Skillings JR (1992). Orofacial complications of chemotherapy for breast cancer. *Oral Surgery, Oral Medicine and Oral Pathology* 74: 172–178.
- McCarthy GM, Mackie ID and Koval J (1991). Factors associated with increased frequency of HIV-related oral candidiasis. *Journal of Oral Pathology and Medicine* 20: 332-336.
- McKinney JD, Höner zu Bentrup K and Muñoz-Elías EJ (2000). Persistence of *Mycobacterium tuberculosis* in macrophages and mice requires the glyoxylate shunt enzyme isocitrate lyase. *Nature* 406: 735-738.
- Milner JA (1996). Garlic: its anticarcinogenic and antitumorigenic properties. *Nutr. Rev.* 54: S82–S86.
- Milner JA (2001). A historical perspective on garlic and cancer. *J. Nutr.* 131: 1027S–1031S.
- Milner JA (2001). Mechanisms by which garlic and allyl sulphur compounds suppress carcinogen bioactivation. Garlic and carcinogenesis, *Adv Exp Med Biol* 492: 69–81.
- Miyamoto S, Howes AL, Adams JW, Dorn GW, Brown JH (2005). Ca<sup>2+</sup> Dysregulation Induces Mitochondrial Depolarization and Apoptosis: Role of

Na<sup>+</sup>/Ca<sup>2+</sup> Exchanger and AKT. *The Journal of Biological Chemistry*. 280: 38505–38512.

- Modrak DE (2004). Synergistic Interaction between Sphingomyelin and Gemcitabine Potentiates Ceramide-Mediated Apoptosis in Pancreatic Cancer. *Journal of Cancer Research*. 64: 8405–8410.
- Mukadi Y, Perriens JH, Louis ME, Brown C, Prignot J, Willame JC and Pouthier F (1993). Spectrum of Immunodeficiency in HIV-1-Infected Patients with Pulmonary Tuberculosis in Zaire. *Lancet* 342: 143–46.
- Mukadi YD, Maher D and Harries A (2001). Tuberculosis case fatality rates in high HIV prevalence populations in sub-Saharan Africa. *AIDS* 15:143-52.
- Munoz P, Moreno S, Berenuer J, Bernaldo de Quiros JC, and Bouza E (1991). Fluconazole-related hepatotoxicity in patients with acquired immunodeficiency syndrome. *Archives of Internal Medicine* 5:1020-1021.
- Murphy AN, Fiskum G, Flint Beal M (1999). Mitochondria in neurodegeneration: bio-energetic function in cell life and death. *J Cereb Bood Flow Metab* 19: 231-245.
- National Jewish Medical and Research Center (1994). Medfacts from the National Jewish Centre for Immunology and Respiratory Medicine. National Jewish Medical and Research Center. Colorado.
- Natoni F (2005). Sodium butyrate sensitises human pancreatic cancer cells to both the intrinsic and the extrinsic apoptotic pathways. *Journal of Biochimica et Biophysica Acta*. 1745: 318–329.

- Newman DJ, Cragg GM, Snader KM (2000). The influence of natural products upon drug discovery. *Natural Product Reports* 17: 215– 234.
- Newman DJ, Cragg GM, Snader KM (2003). Natural products as sources of new drugs over the period 1981–2002. *Journal of Natural Products* 66: 1022–1037.
- Newman DJ, Cragg GM (2007). Natural products as sources of new drugs over the last 25 years. *J. Nat. Prod.* 70: 461–477.
- Ng TT and Denning DW (1993). Fluconazole resistance in *Candida* in patients with AIDS - a therapeutic approach. *Journal of Infection* 26: 117–125.
- Nicotera P, Leist M (1997). Energy supply and the shape of cell death in neurons and lymphoid cells. *Cell Death Differ* 4: 435-442.
- Nigro P, Bloise E, Turco MC, Skhirtladze A, Montoro P, Pizza C, Piacente S, Belisario MA (2007). Antiproliferative and pro-apoptotic activity of novel phenolic derivatives of resveratrol. *Journal of Life Sciences*. 81: 873–883.
- O’Neill MJ and Lewis JA (1993). In: Kinghorn AD and Balandrin MF. *Human Medicinal Agents from Plants, ACS Symposium* 534. American Chemical Society. Washington DC.
- Oberlies NH, Kroll DJ (2004). Camptothecin and taxol: historic achievements in natural products research. *Journal of Natural Products* 67: 129– 135.
- O’Brien RJ (2001). Scientific blueprint for TB drug development *Tuberculosis* 81: 1-52.
- O’Hara M, Kiefer D, Farrell K, Kemper K (1998). A review of 12 commonly used medicinal herbs. *Arch of Fam Med* 7: 523–536.

- Ohta T, Kinoshita T, Naito M, Nozaki T, Masutani M, Tsuruo T, Miyajima A (1997). Requirement of the caspase-3/CPP32 protease cascade for apoptotic death following cytokine deprivation in hematopoietic cells. *J Biol Chem* 272: 23111–23116.
- Olsen AW and Andersen P (2003). A novel TB vaccine; strategies to combat a complex pathogen. *Immunology Letters* 85: 207-211.
- Oommen S, Anto RJ, Srinivas G, Karunagaran D (2004). Allicin (from garlic) induces caspase-mediated apoptosis in cancer cells, *Eur J Pharmacol* 485: 97–103.
- Organization of African Unity Summit on HIV/AIDS, TB and Other Infectious Diseases (2001). Nigeria. Available from: URL: <http://www.infectiousdiseaseneews.com/default.asp?article=idnhome.asp>
- Owuor ED, Kong AN (2002). Antioxidants and oxidants regulated signal transduction pathways, *Biochem. Pharmacol.* 64: 765–770.
- Papucci L, Schiavone N, Witort E, Donnini M, Lapucci A, Tempestini A, Formigli L, Zecchi-Orlandini S, Orlandini G, Carella G, Brancato R, Capaccioli S (2003). Coenzyme Q10 Prevents Apoptosis by Inhibiting Mitochondrial Depolarization Independently of Its Free Radical Scavenging Property. *The Journal of Biological Chemistry.* 278: 28220–28228
- Pascoe D (1983) Toxicology. *Edward Arnold Limited*, London, England 1-60.
- Pauli GF, Case RJ, Inui T, Wang Y, Cho S, Fischer NH, Franzblau SG (2005). New perspectives on natural products in TB drug research. *Life Sciences* 78: 485-494.

- Pero RW, Olsson A, Simanaitis M, Amiri A Anderson I (1997). *Pharm. Toxicol.* 80: 231 –239.
- Peters LL, Robledo RF, Bult CJ, Churchill GA, Paigen BJ, Svenson KL (2007) The mouse as a model for human biology: a resource guide for complex trait analysis. *Nature Reviews Genetics* 8: 58-69.
- Pfaller MA (1994). Epidemiology and control of fungal infections. *Clinical Infectious Diseases* 19: S8–13.
- Piedrafita FJ, Pfahl M (1997). Retinoid-induced apoptosis and Sp1 cleavage occur independently of transcription and require caspase activation. *Mol Cell Biol* 17: 6348–6358.
- Pinto JT, Lapsia S, Shah S, Santiago H, Kim G (2001). Antiproliferative effects of garlic-derived and other allium related compounds. *Adv. Exp. Med. Biol.* 492: 83–106.
- Polverino AJ, Patterson SD (1997). Selective activation of caspases during apoptotic induction in HL-60 cells. Effects of a tetrapeptide inhibitor. *J Biol Chem* 272: 7013–7021.
- Poole A, Leslie GB (1989). A practical approach to toxicological investigations. 1<sup>st</sup> edition. Great Britain. *Cambridge University Press* 30-117.
- Popat A, Shear NH, Malkiewicz I, Stewart MJ, Steenkamp V, Thomson S, Neuman MG (2001). The toxicity of *Callilepis laureola*, a South African traditional herbal medicine. *Clinical Biochemistry* 34: 229-236.
- Prentice AG (1989). Oral and gastrointestinal candidosis: prophylaxis during immunosuppressive therapy. *Mycoses* 32: 42–46.



- Primchanien M, Nuttavut K, Sineenart K, Omboon L, Narongchai P, Neelobol N. (2004). Antiproliferation, antioxidation and induction of apoptosis by *Garcinia mangostana* (mangosteen) on SKBR3 human breast cancer cell line, *J. Ethnopharmacol.* 92: 161–166.
- Prives C (1998). Signalling by p53: breaking the MDM2-p53 circuit. *Cell*: 95: 5.
- Pucci B, Kasten M, Giordano A (2000). Cell cycle and apoptosis. *Neoplasia* 291 – 299.
- Qanungo S, Das M, Haldar S, Basu A (2005). Epigallocatechin-3-gallate induces mitochondrial membrane depolarization and caspase-dependent apoptosis in pancreatic cancer cells. *Journal of Carcinogenesis* 26: 958–967
- Quadriatero J, Hoffman-Goetz L (2004). N-Acetyl-L-cysteine prevents exercise-induced intestinal lymphocyte apoptosis by maintaining intracellular glutathione levels and reducing mitochondrial membrane depolarization. *Journal of Biochemical and Biophysical Research Communications*. 319: 894–901
- Rahier NJ, Thomas CJ, Ehcht SM (2005). Camptothecin and its analogs. In: Cragg GM, Kingston DGI, Newman DJ, Editors, *Anticancer Agents from Natural Products*, Brunner-Routledge Psychology Press, Taylor & Francis Group, Boca Raton, FL pp. 5–22 (Chapter 2).
- Ramljak D (2005). Pentameric procyanidin from *Theobroma cacao* selectively inhibits growth of human breast cancer cells. *Journal of Molecular Cancer Therapy*. 4: 537–546.
- Raviglione MC, Harries AD, Msiska R, Wilkinson D and Nunn P (1997) Tuberculosis and HIV: current status in Africa. *AIDS* 11:115-123.

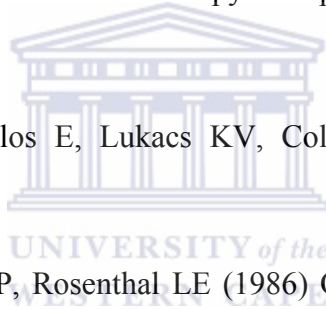
- Reed JC (1997). Cytochrome c: can't live with it - can't live without it. *Cell* 91: 559-562.
- Reed JC (1999). Dysregulation of apoptosis in cancer. *J. Clin. Oncol.* 17: 2941.
- Reisch M. 1997. Aspirin is 100 years old. *Chemical and Engineering News*. p.12.
- Reungpatthanaphong P (2003). Rhodamine B as a mitochondrial probe for measurement and monitoring of mitochondrial membrane potential in drug-sensitive and -resistant cells. *Journal of Biochemical Biophysical Methods*. 57: 1–16.
- Rex JH, Rinaldi MG, Pfaller MA (1995). *Antimicrobial Agents and Chemotherapeutics* 39: 1–8.
- Rigopoulos D, Pappas V, Katsambas A (2004). Cutaneous markers of HIV infection. *Clinics in Dermatology* 22: 487-498.
- Robert V, Mouille B, Mayeur C, Michaud M, Blachier F (2001). Effects of the garlic compound diallyl disulfide on the metabolism, adherence and cell cycle of HT-29 colon carcinoma cells: evidence of sensitive and resistant subpopulations. *Carcinogenesis* 22: 1155–1161.
- Rosenthal N, Brown S (2007) The mouse ascending: perspectives for human disease models. *Nature Cell Biology* 9: 993-999.
- Rotem R (2005). Jasmonates: Novel Anticancer Agents Acting Directly and Selectively on Human Cancer Cell Mitochondria. *Journal of Cancer Research*. 65: 1984–1993.
- Ruhnke M, Eigler A, Tennagen I, Geiseler B, Engelmann E and Trautmann M (1994). *Journal of Clinical Microbiology* 32: 2092–2098.

- Ryan KM, Phillips AC, Vousden KH (2001). Regulation and function of p53 tumor suppressor protein. *Curr. Opin. Cell Biol.* 13: 332.
- Sakahira H, Enari M, Nagata S (1998). Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis. *Nature* 391: 96–99.
- Salvesen GS, Renatus M (2002). Apoptosome: the seven-spoked death machine. *Dev Cell* 2: 256 – 257.
- Samaranayake LP and Scully C (1989). Oral candidiasis in HIV infection *Lancet* ii: 1491–1492.
- Samaranayake LP, Cheung LK and Samaranayake YH (2002). Candidiasis and other fungal diseases of the mouth. *Dermatological Therapeutics* 15: 252–270.
- Samaranayake LP (1992). Oral mycoses in human immunodeficiency virus infection: a review. *Oral Surgery, Oral Medicine and Oral Pathology* 73: 171–180.
- Schaaf HS, Shean K, Donald PR (2003). Culture confirmed multidrug resistant tuberculosis: diagnostic dealy, clinical features, and outcome. *Archibves of Disease in Childhood* 88: 1106-1111.
- Schaible UE, Collins H, Kaufmann SHE (1999). Confrontation between intracellular bacteria and the immune system. *Advances in Immunology* 71: 267–377.
- Schlegel J, Peters I, Orrenius S, Miller DK, Thornberry DA, Yamin TT, Nicholson DW (1996). CPP32/apopain is a key interleukin 1 $\beta$  converting enzyme-like protease involved in Fas-mediated apoptosis. *J Biol Chem* 271: 1841–1844.

- Sharif-Askari E, Nakhaei P, Oliere S, Tumilasci V, Hernandez E, Wilkinson P, Lin R, Bell J, Hiscott J (2007). Bax-dependent mitochondrial membrane permeabilization enhances IRF3-mediated innate immune response during VSV infection. *Journal of Virology* 365: 20–33.
- Shibayama-Imazu T (2006). Production of superoxide and dissipation of mitochondrial transmembrane potential by vitamin K2 trigger apoptosis in human ovarian cancer TYK-nu cells. *Journal of Apoptosis* 11: 1535–1543
- Shih SC, Stutman O (1996). Cell cycle-dependent tumor necrosis factor apoptosis. *Cancer Res.* 56: 1591–1598.
- Shirin H, Pinto JT, Kawabata Y, Soh JW, Delohery T, Moss SF, Murty S, Rivlin RS, Holt PR, Weinstein IB (2001). Antiproliferative effects of S-allylmercaptocysteine on colon cancer cells when tested alone or in combination with sulindac sulfide. *Cancer Res.* 61: 725–731.
- Siegel G, Malmsten M, Pietzsch J, Schmidt A, Buddecke E, Michel F (2001). The effect of garlic on arteriosclerotic nanoplaque formation and size, *Phytomedicine* 11: 24–35.
- Sigounas G, Hooker J, Anagnostou A, Steiner M (1997). S-allylmercaptocysteine inhibits cell proliferation and reduces the viability of erythroleukemia, breast, and prostate cancer cell lines. *Nutr. Cancer* 27: 186–191.
- Silva CL, Bonato VLD, Lima KM, Coelho-Castelo AAM, Faccioli LH, Sartori A, De Souza AO and Leão SC (2001). Cytotoxic T cells and mycobacteria. *FEMS Microbiology Letters* 197: 11-18.

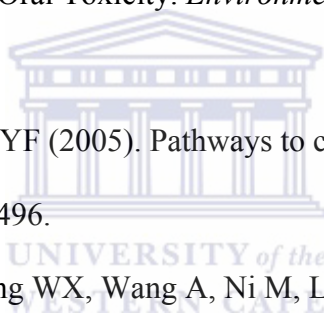
- Sneader W (1985). *Drug Discovery: The Evolution of Modern Medicines*. Wiley: Chichester.
- South African Tuberculosis Association (1998). *Tuberculosis*. Southern Africa: Sunsite.
- Souza-Fagundes EM (2003). Myriadenolide, a labdane diterpene isolated from *Alomia myriadenia* (asteraceae) induces depolarization of mitochondrial membranes and apoptosis associated with activation of caspases-8, -9, and -3 in Jurkat and THP-1 cells. *Journal of Experimental Cell Research*. 290: 420–426.
- Soysa NS, Samaranayake LP and Ellepola ANB (2004). Cytotoxic drugs, radiotherapy and oral candidiasis. *Oral Oncology* 40: 970-978.
- Spigelman M, Gillespie S (2006). Tuberculosis drug development pipeline: progress and hope. *The Lancet* 367: 945 – 947.
- Steller H (1995). Mechanisms and genes of cellular suicide. *Science* 267: 1445–1449.
- Stepczynska A, Lauber K, Engels IH, Janssen O, Kabelitz D, Wesselborg S (2001). Staurosporine and conventional anticancer drugs induce overlapping, yet distinct pathways of apoptosis and caspase activation. *Oncogene* 20: 1193 – 202.
- Stoll A, Seebeck E (1951). Chemical investigations of alliin, the specific principle of garlic. *Advances in Enzymology* 11: 377–400.
- Sun XM, MacFarlane M, Zhuang J, Wolf BB, Green DR, Cohen GM (1999). Distinct caspase cascades are initiated in receptor-mediated and chemical-induced apoptosis. *J. Biol. Chem.* 274: 5053–5060.

- Surh YJ, Hurh YJ, Kang JY, Lee E, Kong G, Lee SJ (1999). Resveratrol, an antioxidant present in red wine, induces apoptosis in human promyelocytic leukemia (HL-60) cells. *Cancer Lett.* 140: 1–10.
- Susin SA, Zamzami N, Castedo M, Hirsch T, Marchetti P, Macho A, Daugas E, Geuskens M, Kroemer G (1996). Bcl-2 inhibits the mitochondrial release of an apoptogenic protease. *J Exp Med* 184: 1331-1342.
- Takahashi A. and Earnshaw WC (1996). ICE-related proteases in apoptosis. *Curr. Opin. Genet. Dev.* 6: 50
- Taraphdar AK, Roy M, Bhattacharya RK (2001). Natural products as inducers of apoptosis: Implication for cancer therapy and prevention. *Current Science* 80: 1387 – 1396.
- Tascon RE, Stavropoulos E, Lukacs KV, Colston MJ (1998). *Infection and Immunity* 66: 830–834.
- Tavitian A, Raufman JP, Rosenthal LE (1986) Oral candidiasis as a marker for esophageal candidiasis in the acquired immunodeficiency syndrome. *Ann Intern Med* 104: 54 – 55.
- Taylor JLS, Rabe T, McGaw LJ, Jager AK, van Staden J (2001). Towards the scientific validation of traditional medicinal plants. *Plant Growth Regulation* 34: 23–37.
- Thompson CB (1995). Apoptosis in the pathogenesis and treatment of disease. *Science* 267: 1456 – 1462.
- Thornberry NA, Lazebnik Y (1998). Caspases: enemies within. *Science* 281: 1312–1316.



- Tiano L, Fedeli D, Ballarini P, Santoni G, Falcioni G (2001). Mitochondrial membrane potential in density-separated trout erythrocytes exposed to oxidative stress in vitro. *Journal of Biochimica et Biophysica Acta*. 1505: 226–237.
- Timbrell J (2002) Introduction to toxicology. 3<sup>rd</sup> edition *Taylor and Francis* 163-179.
- Timonin MI and Thexton RH (1950). The rhizosphere effect of onion and garlic on soil microflora. *Soil Sci Soc Am Proc* 15: 186.
- Trinchieri G (1995). Interleukin-12 a proinflammatory cytokine with immunoregulatory functions that bridges innate resistance and antigen-specific adaptive immunity. *Annual Review of Immunology* 13: 251–276.
- Tsao M, Yin M (2001). In vitro activity of garlic oil and four diallyl sulphides against antibiotic-resistant *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*, *J Antimicrob Chemother* 47: 665–670.
- Tsujimoto Y (1997). Apoptosis and necrosis: intracellular ATP level as a determinant for cell death modes. *Cell Death Differ* 4: 429-434.
- Uchida Y, Takahashi T, Sato N (1975). The characteristics of the antibacterial activity of garlic. *Japanese Journal of Antibiotics* 28: 638–642.
- UNAIDS Report on the global HIV/AIDS epidemic (2000) *UNAIDS*, New York.
- Van der Heijden R, Jacobs DI, Snoeijer W, Hallard D, Verpoorte R (2004). The *Catharanthus* alkaloids: pharmacognosy and biotechnology. *Current Medicinal Chemistry* 11: 607–628.
- Van Pinxteren L, Cassidy JP, Smedegaard JHC, Agger EM and Andersen P (2000). *European Journal of Immunology* 30: 3689–3698.

- VanWyk B-E, van Oudtshoorn B, Gericke N (1997). Medicinal plants of South Africa. Pretoria: Briza Publications. ISBN 1 875093 09 5.
- Van Wyk B, Van Oudtshoorn B, Gericke N (2000). Medicinal Plants of South Africa. Briza Publications, South Africa.
- Van Wyk B-E, Wink M (2004). Medicinal Plants of the World. Briza Publications, South Africa.
- Vaux DL (1997). CED-4 - the third horseman of apoptosis. *Cell* 90: 389–390.
- Wall ME, Wani MC (1996). Camptothecin and taxol: from discovery to clinic. *Journal of Ethnopharmacology* 51: 239– 253.
- Walum E (1998) Acute Oral Toxicity. *Environmental Health Perspectives* 106: 497-503.
- Wang ZB, Liu YQ, Cui YF (2005). Pathways to caspase activation. *Cell Biology International* 29: 489 – 496.
- Wang CZ, Zhang B, Song WX, Wang A, Ni M, Luo X, Aung HH, Xie JT, Tong R, He TC, Yuan CS (2006). Steamed American Ginseng Berry: Ginsenoside analyses and anticancer activities. *J. Agric. Food Chem.* 54: 9936 – 9942.
- Waterston RH (2002) Initial sequencing and comparative analysis of the mouse genome. *Nature* 420: 520-562.
- WHO/IUATLD Working Group (1989). Tuberculosis and AIDS. *Bulletin of the International Union Against Tuberculosis and Lung Disease* 64: 8–11.
- Workman P (1996). *Apoptosis and Cell Cycle Control in Cancer*. BIOS Scientific Publishers Ltd., Oxford, pp 205–232.





- World Health Organization (2008). Global tuberculosis control: surveillance, planning and financing. Geneva. URL:  
[http://www.who.int/tb/publications/global\\_report/2008/download\\_centre/en/index.html](http://www.who.int/tb/publications/global_report/2008/download_centre/en/index.html)
- World Health Organization (2000). Press Release 41. Available from URL:  
<http://www.who.int/inf-pr-2000/en/pr2000-41.html>
- World Health Organization (2001). Global tuberculosis control. WHO Report, Geneva pp.181.
- World Health Organization (2002). WHO Traditional Medicine Strategy 2002 – 2005.
- World Health Organization (2004). BCG Vaccine. *Weekly Epidemiological Record*, No 4.
- Wu CC, Sheen LY, Chen HW, Tsai SJ, Lii CK (2001). Effects of organosulfur compounds from garlic oil on the antioxidation system in rat liver and red blood cells, *Food Chem Toxicol* 39: 563–569.
- Wyllie AH, Kerr JF, Currie AR (1980). Cell death: the significance of apoptosis. *Int Rev Cytol* 68: 251–306.
- Wyllie AH (1980). Glucocorticoid induced thymocyte apoptosis is associated with endogenous endonuclease activation. *Nature* 284: 555–556.
- Xiao D, Pinto JT, Soh JW, Deguchi A, Gundersen GG, Palazzo AF, Yoon JT, Shirin H, Weinstein IB (2003). Induction of Apoptosis by the Garlic-Derived Compound *S*-Allylmercaptocysteine (SAMC) Is Associated with Microtubule

Depolymerization and c-Jun NH<sub>2</sub>-Terminal Kinase Activation. *Cancer Research* 63: 6825 – 6837.

- Yeh CK, Fox PC, Ship JA (1988). Oral defense mechanisms are impaired early in HIV-1 infected patients. *Journal of Acquired Immune Deficiency Syndrome* 1: 361–366.
- Zager EM, McNerney R (2008). Multidrug-resistant tuberculosis. *BioMedCentral Infectious Diseases* 8: 10.
- Zamzami N, Susin SA, Marchetti P, Hirsch T, Gomez-Monterrey I, Castedo M, Kroemer G (1996). Mitochondrial control of nuclear apoptosis. *J Exp Med* 183:1533-1544.
- Zhang XD, Gillespie SK, Hersey P (2004). Staurosporine induces apoptosis of melanoma by both caspase-dependent and - independent apoptotic pathways. *Mol Cancer Ther* 3: 187 – 97.
- Zhao K (2005). Mitochondria-targeted peptide prevents mitochondrial depolarization and apoptosis induced by tert-butyl hydroperoxide in neuronal cell lines. *Journal of Biochemical Pharmacology*. 70: 1796–1806.
- Zhivotovsky B, Burgess DH, Vanags DM, Orrenius S (1997). Involvement of cellular proteolytic machinery in apoptosis. *Biochem Biophys Res Commun* 230: 481–488.
- Zou H, Henzel WJ, Liu X, Lutschg A, Wang X (1997). Apaf-1, a human protein homologous to *C. elegans* CED-4, participates in cytochrome c-dependent activation of caspase-3. *Cell* 90: 405–413.

- Zunino SJ, Storms DH (2006). Resveratrol-induced apoptosis is enhanced in acute lymphoblastic leukemia cells by modulation of the mitochondrial permeability transition pore. *Journal of Cancer Letters*. 240: 123–134.



## **CHAPTER 2**

***Tulbaghia alliacea* phytotherapy:  
A potential anti-infective remedy for Candidiasis**



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## 2.1. Abstract

The reproductive health of individuals is severely compromised by HIV infection, with candidiasis being the most prevalent oral complication in patients. Although not usually associated with severe morbidity, oropharyngeal candidiasis can be clinically significant, as it can interfere with the administration of medications and adequate nutritional intake, and may spread to the esophagus. Azole antifungal agents are commonly prescribed for the treatment and prophylaxis of candidal infections. However, the emergence of drug resistant strains and dose limiting toxic effects have complicated the treatment of candidiasis. Consequently, safe and effective and affordable medicine is required to combat this fungus. Commercial garlic (*Allium sativum*) has been used time since immemorial as a natural antibiotic, however very little is known about the antifungal properties of two indigenous South African species of garlic, namely *Tulbaghia alliacea* and *Tulbaghia violacea*, that are used as folk medicines for a variety of infections. This study compares the *in vitro* anti-candidal activity of *Tulbaghia alliacea*, *Tulbaghia violacea* and *Allium sativum* extracts. It was found that the greatest concentrations of inhibitory components were extracted by chloroform or water. The IC<sub>50</sub> concentrations of *Tulbaghia alliacea* were between 0.007 – 0.038% (w/v). Assays using *S. cerevisiae* revealed that the *T. alliacea* extract was fungicidal, with a killing half-life of approximately 2 hours. This inhibitory effect of the *T. alliacea* extracts was observed via TLC, and may be due to an active compound called Marasmicin, that was identified using NMR. This investigation confirms that extracts of *T.alliacea* exhibit anti-infective activity against candida species *in vitro*.

**Key words:** Reproductive health, candidiasis, HIV, *Tulbaghia alliacea*, *Tulbaghia violacea*, *Allium sativum*, indigenous, anti-fungal and anti-infective.

## 2.2. Introduction

The reproductive health of individuals is severely compromised by HIV infection, with candidiasis being the most prevalent oral complication in patients. HIV/AIDS is a public health emergency in Africa and according to the UN, South Africa has the fastest growing epidemic in the world, with more than 4.5 million HIV-positive people, thousands of AIDS deaths, 2500 new infections daily and 100,000–150,000 AIDS orphans annually (Stegmann, 2001). Studies of AIDS all over the world show that 58–81% of all patients contract a fungal infection at some time during the primordial stage or after developing AIDS, and 10–20% have died as a direct consequence of fungal infections (Drouhent and Dupont, 1989).

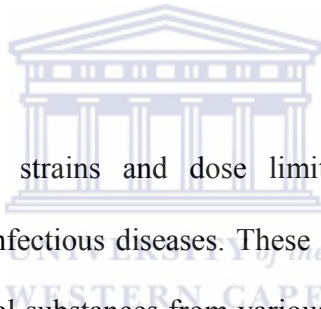
Candidiasis is the most common oral fungal infection in humans and manifests in a variety of clinical guises ranging from pseudomembraneous (thrush), erythematous, and hyperplastic variants to linear gingival erythema associated with HIV infection (Samaranayake and Scully, 1989; Ellepola and Samaranayake, 2000). Oropharyngeal candidiasis (OPC) is the most prevalent oral complication in HIV/AIDS patients (Samaranayake, 1992; Glick, Muzyka, Lurie, Salkin, 1994; Blignaut, Messer, Hollis,

Pfaller, 2002), and it is generally accepted that *Candida albicans* is the most commonly isolated species from clinical specimens (Pfaller, 1994). *Candida* species are normal inhabitants of the human gastrointestinal tract and may be recovered from up to one third of the mouths of normal individuals and two thirds of those with advanced HIV disease (Fichtenbaum, Koletar, Yiannoutsos, Holland, Pottage, Cohn, Walawander, Frame, Feinberg, Saag, Van Der Horst, Powderly, 2000). Oral colonization with inherently drug-resistant organisms is more common in advanced HIV infection (CD4-lymphocyte counts  $<50$  cells/mm<sup>3</sup>) (Fichtenbaum *et al.*, 2000).

Although not usually associated with severe morbidity, OPC can be clinically significant as it can interfere with the administration of medications and adequate nutritional intake, and may spread to the esophagus (Tavitian, Raufman, Rosenthal, 1986).

The increasing prevalence of other compromised patient groups in the community, together with common endocrine disorders such as diabetes mellitus, and nutritional deficiencies have also contributed to the resurgence of oral candidiasis as a relatively common affliction (Ellepola and Samaranayake, 2000). In addition, the use of oral antibiotics, broad-spectrum antibiotics (Samaranayake, Cheung, Samaranayake, 2002), corticosteroids, or immunosuppressives may predispose individuals to infection (Soysa, Samaranayake, Ellepola, 2004). These factors disrupt the balance between the healthy host and the *Candida*, which under normal circumstances, may be present in the gastrointestinal flora but is not disease producing (Bruce and Rogers, 2004)

Since its introduction, fluconazole has been used to prevent and treat *Candida* infections in HIV-1-infected patients because of its efficacy and good tolerability (Koks, Crommentuyn, Mathot, Mulder, Meenhorst, Beijnen, 2002). However, after the use of fluconazole for the treatment and prophylaxis of fungal infections became widespread, reports began to appear which suggested reduced effectiveness of the standard fluconazole therapy and also side effects (Ng and Denning, 1993). The side effects of many azole antifungals such as ketoconazole, itraconazole, fluconazole, posaconazole, and voriconazole are similar; the more common being headache, dyspepsia, diarrhea, nausea, vomiting, hepatitis, and skin rash (Munoz, Moreno, Berenuer, Bernaldo de Quiros, Bouza, 1991).



Emergence of drug resistant strains and dose limiting toxic effects has further complicated the treatment of infectious diseases. These complications have necessitated the search for new antimicrobial substances from various sources. Extracts of plants and phytochemicals have been shown to possess activity against many infectious diseases, and may thus be a good source of new active agents (Mekaway, Meselhy, Kusumoto, Kadota, Hattori, Namba, 1995; Vlietinck, Bruyne, Apers, Pieters, 1998; Matsuse, Lim, Hattori, Correa, Gupta, 1999). One of the most widely used medicinal plants is garlic (*Allium sativum*), which is reported to be effective against a wide range of microorganisms, including bacteria, fungi, protozoa and viruses (Ankri and Mirrelman, 1999). *Tulbaghia alliacea* and *Tulbaghia violacea* are two indigenous South African garlic species, which are traditionally used as remedies for a variety of infections and ailments (Van Wyk *et al.*, 2000).



This investigation therefore aimed to assess the antifungal properties of *T.alliacea*, with a view to using this indigenous phytotherapy as a potential anti-candidal remedy.

## **2.3. Materials and Methods**

### **2.3.1. Plant material**

Bulbs of *Tulbaghia alliacea* were obtained from Rastafarian herbal traders in Cape Town, South Africa; *Tulbaghia violacea* was obtained from the Educational and Environmental Resources Unit (EERU) at the University of the Western Cape, while a commercial garlic brand of *Allium sativum* was purchased from a retail store. All three species were verified by a botanist at the University of the Western Cape Herbarium where voucher specimens (Thamburan001 for *T.alliacea*, Thamburan002 for *T.violacea* and Thamburan003 for *A.sativum*) were deposited.

### **2.3.2. Preparation of plant extracts**

Bulbs of *T. alliacea* and *T.violacea* and cloves of *A. sativum* were washed with distilled water, peeled and chopped into tiny pieces (Eloff, 1998). Solvent extractions of different concentrations (0.06, 0.15 and 0.30 %) of the bulbs and cloves were prepared using solvents of differing polarity (water, methanol and chloroform). Extracts were prepared by soaking the chopped plant material in the solvents overnight, and then vacuum filtering it.

### 2.3.3. Microorganisms and growth media

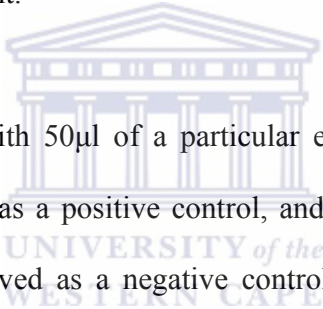
For the preliminary antifungal screening, a culture of *Candida albicans* was obtained from the Department of Medical Biosciences at the University of the Western Cape, South Africa. *C.albicans* was maintained on Sabouraud dextrose agar. Subcultures of *C.albicans* were inoculated in nutrient broth and spread onto prepared agar plates using sterile swab sticks under aseptic conditions. Plated cultures were incubated at 37 °C for 24 hours. The agar plate method for plant extract susceptibility testing is well standardized and widely used (Vanden Berghe and Vlietinck, 1991).

For more detailed antifungal analyses, the broth dilution antifungal susceptibility testing method (Agarwal, Rogers, Baerson, Jacob, Barker, Cleary, Walker, Nagle, Clark, 2003) was used to assess the growth inhibitory activity of *T.alliacea* extracts on a variety of pathogenic fungi. The yeast strains: *Saccharomyces cerevisiae* (JC746-9D), *Candida albicans* (ATCC 90028), *Candida glabrata* (ATCC 90030), *Candida krusei* (ATCC 6258) and *Cryptococcus neoformans* (ATCC 90113) were obtained from the National Center for Natural Products Research, University of Mississippi, Oxford. YPD medium contained 10g yeast extract, 20g peptone and 20g glucose per liter. Minimal medium contained 7g yeast nitrogen base, 20g glucose, 10g succinic acid, 4.5g NaOH per liter and was supplemented with amino acids (Guthrie and Fink, 1991). Solid media contained 20g agar per liter.

## 2.3.4. Antifungal screening

### 2.3.4.1. Disk diffusion assays

All solvent (aqueous, methanol and chloroform) extracts of *T. alliacea*, *T. violacea* and *A. sativum* were tested for *in vitro* antifungal activity against *C. albicans* using the agar plate disk diffusion method. The pharmaceutical drug, fluconazole (40 µg/disk) was used as a positive control, as it is a broad-spectrum antibiotic. Filter paper disks (9mm) (Schleider and Schuell) were autoclaved at 120°C in a Speedy Autoclave (Lasec) for 20 min, and then incubated at 37°C overnight.



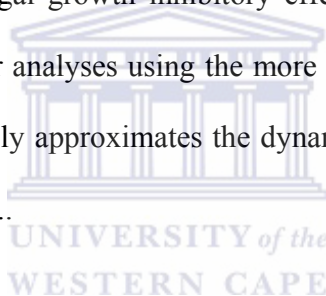
Each disk was impregnated with 50µl of a particular extract and was allowed to dry overnight. Fluconazole served as a positive control, and the extraction solvents (water, methanol, and chloroform) served as a negative control. The dried impregnated disks were placed on the surface of agar plates inoculated with the candidal culture. Each plate consisted of the following disks: a positive control (Fluconazole), a negative control (particular solvent) and a different extraction of each solvent (e.g. 0.30 % water). Each extract was tested in triplicate. After being accurately labeled, the agar plates were inverted and incubated at 37°C for 24 hours. After incubation, inhibition zones were recorded as the diameter of the growth-free zones around the disk.

#### **2.3.4.2. Minimum Inhibitory Concentrations (MIC)**

The MIC's for each extraction were calculated by preparing serial dilutions of the original extractions. The extract concentration exhibiting a 1mm zone of inhibition was taken to be the MIC value for that solvent extraction, as a 1mm zone is the smallest zone that can be detected as growth inhibition of microorganisms *in vitro* using the agar plate disk diffusion method.

#### **2.3.4.3. Broth dilution antifungal susceptibility testing**

After establishing the anti-fungal growth inhibitory effect of *T.alliacea* using the disk diffusion assay method, further analyses using the more sensitive optical density growth inhibition method, which closely approximates the dynamics of the fungus in biological fluid, was used for assessments..



The fungal strains [*Saccharomyces cerevisiae* (JC746-9D), *Candida albicans* (ATCC 90028), *Candida glabrata* (ATCC 90030), *Candida krusei* (ATCC 6258), and *Cryptococcus neoformans* (ATCC 90113)] were grown overnight in YPD medium. The YPD medium was diluted to  $3 \times 10^6$  cells/ml in yeast minimal medium and 175 $\mu$ l of diluted cells was placed in each of the wells of a 96-well plate (Nunc) with the extract dilution.

Absorbance at 630 nm was measured at zero time and after 24 hours of growth using a Dynatech MR5000 microplate reader. The plate wells were sealed with Parafilm and the

plate was shaken at 250 rpm at 30°C. Dilutions of chloroform extracts were made in a final volume of 50µl chloroform in 1.5ml polypropylene tubes (Sarstedt).

Chloroform was evaporated from the uncapped tubes in a chemical hood for three hours at 22°C. Tubes were filled with 400µl of diluted cells from above, capped, and shaken at 250 rpm at 30°C. Cell densities were measured from 200µl aliquots placed into a 96-well plate as above. All assays were done in triplicate and the average data were plotted.

#### **2.3.4.4. Viability assay**

A viability assay to determine the mechanism of action (fungistatic or fungicidal) of *T.alliacea* extracts was carried out. An *S. cerevisiae* culture was diluted to  $3 \times 10^6$  cells / ml in 400µl yeast minimal medium in a 1.5ml polypropylene tube. Aqueous *T. alliacea* extract was added to a final 0.075% (w/v) (four-fold dilution of aqueous extract stock). The capped tube was incubated at 30°C with 250 rpm shaking. Samples of cells were removed before extract addition and after 5 and 24 hours of growth were diluted and plated on YPD plates. Viable cell numbers were calculated from the colony forming units after three days of growth.

### 2.3.5. Thin-Layer Chromatography–Bioautographic Assay

For direct bioassay on TLC plates, a 30µl aliquot of each extract was applied to 5 x 20 cm silica F254 (Merck) glass backed plates in duplicate for fingerprinting. All plates were developed in hexane: ethyl acetate (1:2 v/v) (Wagner and Blade, 1996). The developed TLC plates were dried overnight and then viewed under ultraviolet (UV) light (366 nm).

Thereafter, one set of plates (of each extract) was sprayed with vanillin-sulphuric acid reagent, made up by dissolving 3g vanillin in 30ml ethanol, to which 5ml concentrated sulphuric acid was added. Some compounds that are not visible under UV light become visible upon spraying and heating (for approximately 20 min) of the developed plates.

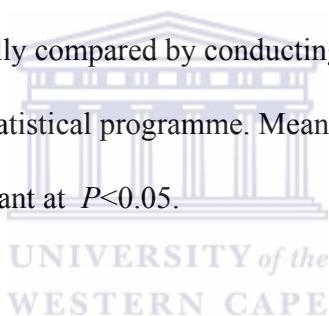
The duplicate plates were used for the bioautographic assay (inoculated TLC), whereby Sabouraud dextrose agar inoculated with *C.albicans* was poured over the plates under aseptic conditions. These plates were placed on damp paper towels in a plastic tray, covered with cling wrap and incubated at 37 °C for 24 hours. To detect the antimicrobial activity on the plates, they were sprayed with a 0.2mg/ml solution of 2,5-diphenyltetrazolium bromide (MTT) (Sigma Chemicals Co.). Clear zones on the chromatogram indicated inhibition of growth (Beugue and Kline, 1972).

### **2.3.6. Nuclear Magnetic Resonance (NMR) analyses**

The active compound from the chloroform extract was isolated by preparative TLC using a mixture of hexane and ethyl acetate (4:1) as mobile phase. The  $^1\text{H}$  NMR spectrum was recorded on a Gemini Varian 200 instrument at 200MHz, in deuteriochloroform as solvent and tetramethylsilane as internal standard; the chemical shifts were reported in ( $\delta$ ) ppm.

### **2.3.7. Statistical analyses**

Inhibition zones were statistically compared by conducting an unpaired  $t$ -test using the MedCalc (version 7.1, 2000) statistical programme. Means  $\pm$  SEM (standard error of the mean) were considered significant at  $P < 0.05$ .



## 2.4. Results

### 2.4.1. Disk diffusion assays

Fluconazole (2 %) served as a positive control and inhibited the growth of *C.albicans* with a zone of 7mm. The negative controls (extraction solvents) did not have any effect on the fungus and thus no zones of inhibition were observed around these disks. Inhibition zones caused by extracts of *A.sativum*, *T.alliacea* and *T.violacea* were compared using an unpaired *t*-test (Table 1).

At 0.06% concentration, aqueous extracts of *T. violacea* had no effect on *C.albicans*, whereas *A. sativum* and *T. alliacea* had similar inhibitory effects ( $P = 0.1012$ ), however at both 0.15 and 0.30% *T. alliacea* exhibited a statistically bigger zone of inhibition than *A. sativum* ( $P < 0.05$ ) and *T. violacea* ( $P < 0.0001$ ). *T. alliacea* methanol extracts at all concentrations exhibited statistically bigger zones of inhibition than *A. sativum* and *T. violacea* ( $P < 0.05$ ). Chloroform extracts of *T. violacea* only became active at the highest concentration (0.30%), whereas *A. sativum* and *T. alliacea* inhibited *Candida* at all concentrations, with *T. alliacea* exhibiting the biggest zone of inhibition (10.67mm) at 0.30%.

In general, extracts of *T. violacea* were poorest in inhibiting the growth of the fungus; they were inactive at the lowest concentration of 0.06% and only exhibited small zones of



inhibition at the higher concentrations. In contrast, all extracts of *T. alliacea* at all concentrations exhibited antifungal activity.

**Table 1.** The anti-infective activity of *Tulbaghia alliacea*, *Tulbaghia violacea* and *Allium sativum* extracts against *Candida albicans*

Extract	Concentration (%)	Zone of Inhibition (mm ± SEM)			Statistical significance*		
		<i>A. sativum</i> (a)	<i>T. alliacea</i> (b)	<i>T. violacea</i> (c)	ab	ac	bc
Aqueous (H <sub>2</sub> O)	0.06	3.33 ± 0.33	4.33 ± 0.33	0	0.1012	0.0006	0.0002
	0.15	4.00 ± 0.00	6.33 ± 0.33	0	0.0022	0.0002	0.0001
	0.30	5.33 ± 0.33	8.33 ± 0.33	1.00 ± 0.00	0.0031	0.0002	0.001
Methanol (MeOH)	0.06	1.67 ± 0.33	4.00 ± 0.00	0	0.0023	0.0075	0.0002
	0.15	3.67 ± 0.33	5.33 ± 0.33	0.66 ± 0.33	0.0241	0.0031	0.0006
	0.30	5.33 ± 0.33	7.33 ± 0.33	3.33 ± 0.33	0.0132	0.0132	0.0011
Chloroform (CHCl <sub>3</sub> )	0.06	4.00 ± 0.00	5.33 ± 0.33	0	0.1012	0.0006	0.0002
	0.15	4.33 ± 0.33	7.33 ± 0.33	0	0.0031	0.0002	0.0001
	0.30	5.33 ± 0.33	10.67 ± 0.33	2.33 ± 0.33	0.0004	0.0031	0.0001

\* *P* values for comparing main effects: ab = *A. sativum* and *T. alliacea*, ac = *A. sativum* and *T. violacea*, bc = *T. alliacea* and *T. violacea*.

# Fluconazole (2%) inhibited *C. albicans* with a 7mm zone of inhibition.

Negative controls (water, methanol and chloroform) did not inhibit *C. albicans*, hence no zone of inhibition was observed.

An MIC value is defined as the lowest concentration of an extract, which visibly inhibits the growth of the micro-organism after incubation. *T.alliacea* extracts were shown to have lower MIC values than both *A.sativum* and *T.violacea* (Table 2). The lowest MIC value against *C.albicans* was obtained with the 0.024 % chloroform extract of *T.alliacea*.

**Table 2:** The minimum inhibitory concentrations (MIC) for *Tulbaghia alliacea*, *Tulbaghia violacea* and *Allium sativum* extracts against *Candida albicans*\*

Solvent	Extract Concentration (%)			Zone of Inhibition (mm)
	<i>A. sativum</i>	<i>T. alliacea</i>	<i>T. violacea</i>	
Aqueous (H <sub>2</sub> O)	0.132	0.036	0.300	1
Methanol (MeOH)	0.048	0.048	0.150	1
Chloroform (CHCl <sub>3</sub> )	0.048	0.024	0.060	1

\* The MIC for Fluconazole was 0.25 % (1mm zone of inhibition).

## 2.4.2. Broth dilution antifungal susceptibility testing

All five yeast species grew well in YPD and minimal medium normally used for *S. cerevisiae* growth. Therefore, these media were used for growth inhibition assays following a described method (Agarwal *et al.*, 2003). Chloroform extracts required a significant alteration in protocol because chloroform dissolved the 96-well plates. Growth inhibition by chloroform extracts was assayed by growth in polypropylene tubes after chloroform was evaporated. The methanol extraction solvent and evaporated chloroform residue did not inhibit yeast growth.

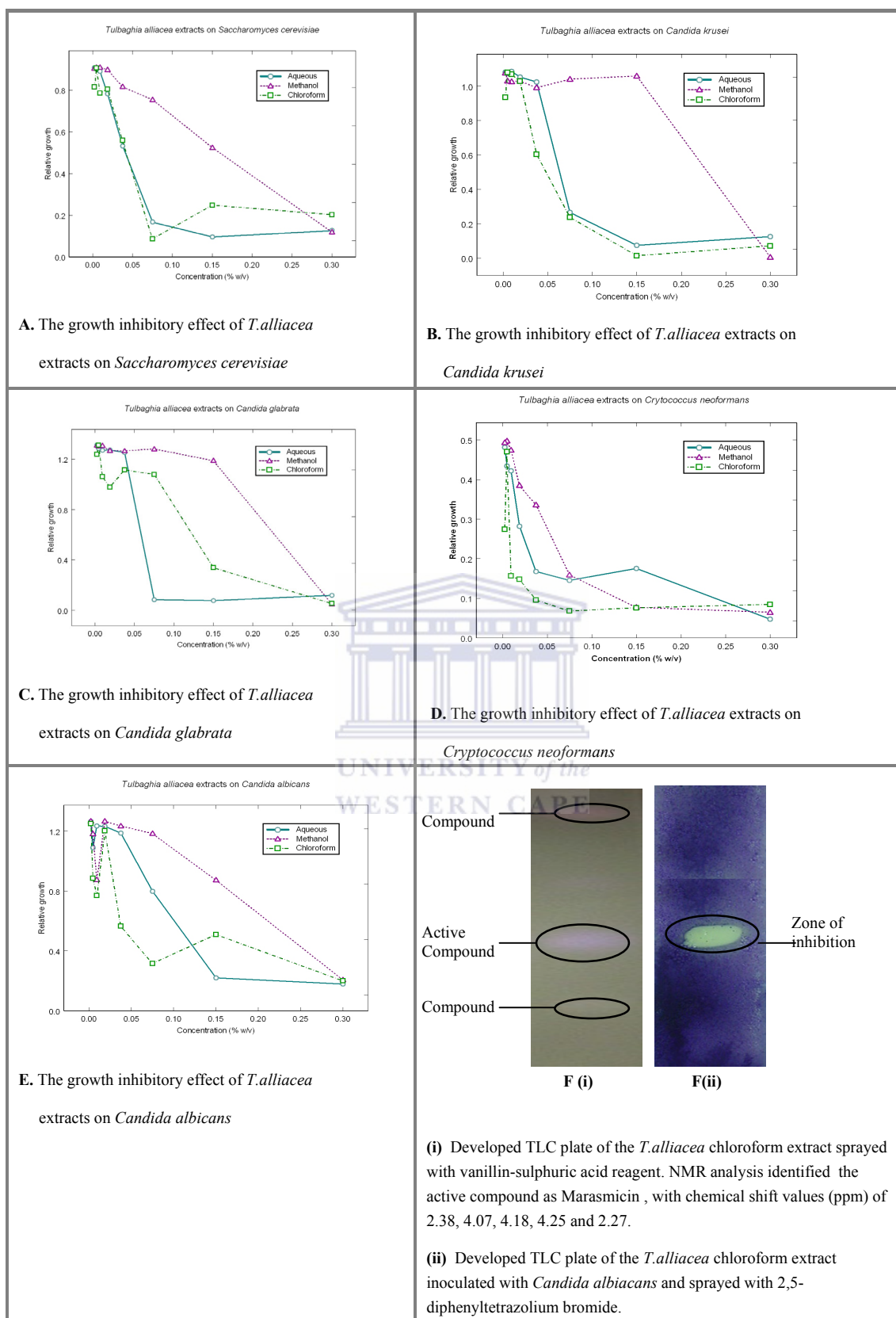
Cell densities after 24 hour growth in the presence of extracts were estimated by 630 nm light scattering. According to the estimated IC<sub>50</sub> concentrations (Table 3), *C. neoformans* was the most sensitive strain to the *T. alliacea* extracts because its growth was inhibited at the lowest concentrations. In contrast, *C. glabrata* was slightly more resistant. In all cases, the methanol extracts were the poorest in inhibiting microbial growth. On the other hand, the chloroform and aqueous extracts appeared to be similarly potent. With all but the exception of the *C. glabrata* strain, the chloroform extract was slightly more potent than the aqueous extract.

**Table 3:** IC<sub>50</sub> concentrations of *Tulbaghia alliacea* extracts for five fungal species

Fungal species	<i>Tulbaghia alliacea</i> extracts		
	Aqueous (H <sub>2</sub> O)	Methanol (MeOH)	Chloroform (CHCl <sub>3</sub> )
<i>Saccharomyces cerevisiae</i>	0.038	0.150	0.038
<i>Candida albicans</i>	0.075	0.150	0.028
<i>Candida glabrata</i>	0.056	0.230	0.110
<i>Candida krusei</i>	0.056	0.230	0.038
<i>Cryptococcus neoformans</i>	0.019	0.056	0.007



Figure 1 (A-E) provides the data for the effect of the various aqueous, methanol and chloroform concentrations of *Tulbaghia alliacea* extracts, on the growth of *Saccharomyces cerevisiae*, *Candida albicans*, *Candida glabrata*, *Candida krusei* and *Cryptococcus neoformans*, and includes a TLC profile of the phytotherapy. These data indicate that the IC<sub>50</sub> concentrations of *Tulbaghia alliacea* extracts were between 0.007 – 0.038% (w/v), and identifies Marasmicin as the active compound with the most potent antifungal effect, using NMR analyses.



**Fig.1.** The antifungal activity (A-E) and TLC profile (F) of *T. alliacea* phytotherapy.

### 2.4.3. Nuclear Magnetic Resonance (NMR) analysis

NMR spectral analysis of the *T. alliacea* inhibitory fraction (Fig.1F), showed prominent signals at 2.38, 4.07, 4.18, 4.25 and 2.27, which are due to the methyl and methylene groups of Marasmicin. These chemical shift values are in agreement with those reported for the same compound, previously isolated from the related *Tulbaghia violacea* (Kubec *et al.* 2002).

### 2.4.4. Mechanism of antifungal action of *T.alliacea* extract

Preliminary experiments were performed to analyze the killing mechanism of the *T. alliacea* extract. An *S. cerevisiae* culture was prepared with minimum concentration of aqueous extract (0.075%, w/v) that allowed no detectable growth.

Cell viability was measured by comparing the total cells (by hemacytometer) and viable cells (colony forming units). It was found that after five hours of exposure, cell viability had decreased to about one-half. After 24 hours, there was a greater than 1000-fold drop in viable cells. Had the action of the extract been fungistatic, no reduction in the number of viable cells would have been observed. Therefore, *T. alliacea* extracts kill yeast cells rather than merely inhibiting growth. Furthermore, the death rate that would reduce the viable cells by 1000-fold in 24 hours, gave a half-life of about two hours. These outcomes are stated in orders of magnitude, since these experiments were not quantitative.

### 2.4.5. Antifungal activity of *T.alliacea* extracts on TLC

The aqueous, methanol and chloroform extracts of *T.alliacea* separated with one major inhibitory fraction, which was indicated as zones of growth inhibition on the bioautograms. TLC separations showed the compounds present in the extracts under UV-366nm are the same as the antifungal compounds visible on the bioautograms, based on the distance they traveled on the TLC plates (Fig. 1F).

## 2.5. Discussion

This investigation aimed at comparing the antifungal effect of the indigenous South African garlic plants *T.alliacea* and *T.violacea*, to the commercial variety *A.sativum*. This comparison was made because of the reputed antimicrobial effects of the commercial garlic species (Uchida, Takahashi, Sato, 1975) and traditional use of the indigenous varieties. *Allium sativum* is quite expensive while the indigenous forms (*Tulbaghia alliacea* and *Tulbaghia violacea*), are significantly more affordable to the poorer communities afflicted by opportunistic fungal infections like candidiasis.

Table 1. reflects the anti-infective value of the aqueous, methanol and chloroform extracts of *Tulbaghia alliacea*, using the disk diffusion assay. There is a dose-dependant increase in the anti-infectivity of the extracts, with the most striking inhibition of *Candida albicans* being induced by:

- (1) 0.030% *T.alliacea* aqueous extract (8.33mm);  $P \leq 0.0031$  vs *A.sativum*,  
 $P \leq 0.0001$  vs *T.violacea*.
- (2) 0.030% *T.alliacea* methanol extract (7.33mm);  $P \leq 0.0132$  vs *A.sativum*,

$P \leq 0.0011$  vs *T.violacea*

(3) 0.030% *T.alliacea* chloroform extract (10.67mm);  $P \leq 0.0004$  vs

*A.sativum*;  $P \leq 0.00001$  vs *T.violacea*

The disk diffusion method was well suited for preliminary screening and has the advantage of allowing for the use of small sample sizes (Rios, Recio, Villar, 1988). Chloroform extracts of *T.alliacea* were more potent than *A.sativum* and *T.violacea*, in inhibiting the growth of *C.albicans* ( $P < 0.05$ ).

Further analyses of fungal growth inhibition, were carried out using the more sensitive broth dilution susceptibility test. The broth micro-dilution method has been shown to be reproducible and clinically useful, with good *in vitro-in vivo* correlation in the setting of oropharyngeal candidiasis in HIV-infected patients (Revankar, Kirkpatrick, McAtee, Fothergill, Redding, Rinaldi, Patterson, 1998). The chloroform extract of *T.alliacea* was more potent than the aqueous and methanol extract in inhibiting the growth of the yeast strains (*Saccharomyces cerevisiae*, *Candida albicans*, *Candida glabrata*, *Candida krusei* and *Cryptococcus neoformans*).

Bioautography is a method that facilitates the localization of antimicrobial activity on a chromatogram, and provides qualitative information about the active components of plants. By separating extracts on TLC plates, it is possible to secure information on the bioactive compounds present in the mixture. Aqueous, methanol and chloroform extracts of *T.alliacea* showed one major inhibiting fraction, indicated as a zone of inhibition on



the bioautograms. TLC separations showed that the fraction present in the extract under UV-366nm was the same as the compound exhibiting antifungal activity, which was visible on the bioautograms (Fig. 1F)). NMR spectral analysis of the *T. alliacea* inhibitory fraction indicated the presence of the methyl and methylene groups of Marasmicin. These chemical shift values are in agreement with those reported for the same compound, previously isolated from the related *Tulbaghia violacea* (Kubec, Velisek, Musah, 2002).

A preliminary viability assay revealed *T.alliacea* extract to be fungicidal (kills yeast cells rather than merely inhibiting growth). Future experiments that address the mechanism of *T. alliacea* fungicidal activity, will require the comprehensive assessment of mutants resistant to the extracts. Initial experiments showed that the assay plates that were incubated for up to three days did not show any increase in cell density. This observation is consistent with the fungicidal action of the extracts. It also shows that spontaneous mutants resistant to the extract occur at a frequency less than  $10^{-6}$ . These observations are indeed very important in the development of a mutant isolation scheme, and creation of a scientifically proven phytotherapeutic remedy for candidiasis.

This investigation has indicated that *T. alliacea* might be of value in the continuing struggle to control opportunistic fungal infections, which are especially important in HIV and AIDS. Furthermore, it also verifies the traditional use of indigenous garlic as an anti-infective herbal medicine. This study provides useful leads for the development of novel pharmaceuticals, in the ongoing search for effective and affordable anti-fungal remedies.

## 2.6. References

- Agarwal AK, Rogers PD, Baerson SR, Jacob MR, Barker KS, Cleary JD, Walker LA, Nagle DG, Clark AM (2003). Genome-wide expression profiling of the response to polyene, pyrimidine, azole, and echinocandin antifungal agents in *Saccharomyces cerevisiae*. *Biol Chem* 278: 34998-5015.
- Ankri S, Mirelman D (1999). Antimicrobial properties of allicin from garlic. *Microbes Infect* 1: 125-129.
- Beugue WJ, Kline RM (1972). The use of tetrazolium salts in bioautographic procedures, *J Chromatogr* 64: 182-184.
- Blignaut E, Messer S, Hollis RJ, Pfaller MA (2002). Antifungal susceptibility of South African oral yeast isolates from HIV/AIDS patients and healthy individuals. *Diagn Microbiol Infect Dis* 44: 169-174.
- Bruce AJ, Rogers RS (2004). Oral manifestations of sexually transmitted diseases. *Clin Dermatol* 22: 520-527.
- Drouhent E, Dupont B (1989). Fluconazole for the treatment of fungal diseases in immunosuppressed patients. *Ann N Y Acad Sci* 544: 564-570.
- Ellepola ANB, Samaranayake LP (2000). Antimycotic agents in oral candidosis: an overview. 1. Clinical variants. *Dent Update* 27: 111-116.
- Eloff JN (1998). Which extractant should be used for the screening and isolation of antimicrobial components from plants. *J Ethnopharmacol* 60: 1-8.

- Fichtenbaum CJ, Koletar S, Yiannoutsos C, Holland F, Pottage J, Cohn SE, Walawander A, Frame P, Feinberg J, Saag M, Van der Horst C and Powderly WG. (2000). Refractory mucosal candidiasis in advanced human immunodeficiency virus infection. *Clin Infect Dis* 30: 749-756.
- Glick M, Muzyka BC, Lurie D, Salkin LM (1994). Oral manifestations associated with HIV-related disease as markers for immune suppression and AIDS. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 77: 344–345.
- Guthrie C, Fink GR (1991). *Guide to Yeast Genetics and Molecular Biology* (Methods in Enzymology, Vol. 194). Academic Press: San Diego.
- Koks CHW, Crommentuyn KML, Mathot RAA, Mulder JW, Meenhorst PL, Beijnen JH (2002). Prognostic factors for the clinical effectiveness of fluconazole in the treatment of oral candidiasis in HIV-1-infected individuals. *Pharmacol Res* 46: 89-94.
- Kubec R, Velisek J and Musah RA (2002). The amino acid precursors and odor formation in society garlic (*Tulbaghia violacea*), *Phytochemistry*, 60: 21-25.
- Matsuse IT, Lim YA, Hattori M, Correa M. Gupta MP (1999). A search for antiviral properties in Panamanian medicinal plants - the effect on HIV and its essential enzymes. *J Ethnopharmacol* 64: 15–22.
- Mekkawy SE, Meselhy MR, Kusumoto IT, Kadota S, Hattori M, Namba T (1995). Inhibitory effects of Egyptian folk medicines on human immunodeficiency virus (HIV) reverse transcriptase. *Chem Pharm Bull* 43: 641–648.

- Munoz P, Moreno S, Berenuer J, Bernaldo de Quiros JC, Bouza E (1991). Fluconazole-related hepatotoxicity in patients with acquired immunodeficiency syndrome. *Arch Intern Med* 5: 1020-1021.
- Ng TT, Denning DW (1993). Fluconazole resistance in *Candida* in patients with AIDS - a therapeutic approach. *J Infect* 26: 117–125.
- Pfaller MA (1994). Epidemiology and control of fungal infections. *Clin Infect Dis* 19: S8–13.
- Revankar SG, Kirkpatrick WR, McAtee RK, Fothergill AW, Redding SW, Rinaldi MG, Patterson TF (1998). Interpretation of Trailing Endpoints in Antifungal Susceptibility Testing by the National Committee for Clinical Laboratory Standards Method. *J Clin Microbiol* 36: 153-156.
- Rios JL, Recio MC, Villar A (1998). Screening methods for natural products with antimicrobial activity: a review of the literature. *J Ethnopharmacol* 23:127-149.
- Samaranyake LP, Scully C (1989). Oral candidosis in HIV infection *Lancet* ii: 1491–1492.
- Samaranyake LP (1992). Oral mycoses in human immunodeficiency virus infection: a review. *Oral Surg Oral Med Oral Path Oral Radiol Endod* 73: 171–180.
- Samaranyake LP, Cheung LK, Samaranyake YH (2002). Candidiasis and other fungal diseases of the mouth. *Dermatol Ther* 15: 252–270.
- Soysa NS, Samaranyake LP, Ellepola ANB (2004). Cytotoxic drugs, radiotherapy and oral candidiasis. *Oral Oncol* 40: 970-978.

- Stegmann J (2001). The new South Africa-challenge to the church. *Grace and Truth* 18: 22–27.
- Tavitian A, Raufman JP, Rosenthal LE (1986). Oral candidiasis as a marker for esophageal candidiasis in the acquired immunodeficiency syndrome. *Ann Intern Med* 104: 54-55.
- Uchida Y, Takahashi T, Sato N (1975). The characteristics of the antibacterial activity of garlic. *Jpn J Antibiot* 28:638-642.
- Vanden Berghe DA, Vlietinck AJ (1991). Screening methods for antibacterial and antiviral agents from higher plants. In Dey PM, Harborne JB. *Methods in Plant Biochemistry* (vol 6). Academic Press Limited: London.
- Van Wyk B, Van Oudtshoorn B, Gericke N (2000). *Medicinal Plants of South Africa*. Briza Publications: South Africa.
- Vlietinck AJ, Bruyne TD, Apers S, Pieters LA (1998). Plant derived leading compounds for chemotherapy of human immunodeficiency virus infection. *J Ethnopharmacol* 64: 97–109.
- Wagner H, Bladt S (1996). *Plant Drug Analysis – A Thin Layer Chromatography Atlas* (2<sup>nd</sup> edn). Springer-Verlag: Berlin Heidelberg.

## **CHAPTER 3**

*Tulbaghia alliacea:*

**A potential antimycobacterial and  
immune-modulating phytotherapy**

WESTERN CAPE

### 3.1. Abstract

Tuberculosis (TB) is the leading cause of morbidity and mortality among HIV-1-infected patients. Conventional medication for this TB epidemic is expensive, and limited patient compliance leads to the development of MDR-TB and XDR-TB. Consequently, safe, effective and affordable medicine is required to combat the disease. Very little is known about the antimycobacterial properties of *Tulbaghia alliacea*, a species of garlic that is indigenous to South Africa and has been used in folk medicine as a treatment for TB. This study compares the *in vitro* antimycobacterial effect of *Allium sativum* and *Tulbaghia alliacea*, whilst also profiling the active antimycobacterial compounds of these medicinal plants, and assessing their ability to stimulate the expression of IFN- $\gamma$ . Extracts of varying polarities of *A.sativum* and *T.alliacea* were comparatively tested for *in vitro* antimycobacterial activity against *Mycobacterium smegmatis*. *T.alliacea* does have antimycobacterial effects, as was shown by growth inhibition zones on *M.smegmatis* inoculated agar plates. Aqueous and ethanolic extracts of *T. alliacea* at higher concentrations exhibited greater inhibition of *M.smegmatis* than *A.sativum* ( $P<0.003$  and  $P<0.002$ ). The 10mg/ml chloroform extract of *T. alliacea* most potently inhibited the development of the pathogen ( $P<0.0001$ ), when compared to *A.sativum*. This inhibitory effect is due to three active compounds in *T. alliacea* that were observed using TLC. One of these compounds was identified as Marasmicin ( $R_f$  0.44), a potent anti-infective compound previously isolated from *T. alliacea*. Cytokine measurements in whole blood cultures after exposure to the plant extracts showed that the *T.alliacea* aqueous extract is more potent in stimulating the expression of IFN- $\gamma$  than the chloroform extract ( $P<0.05$ ). This investigation confirms that extracts of *T. alliacea* that are used as indigenous TB medicines, do exhibit antimycobacterial activity *in vitro*, and are also capable of stimulating the immune response via IFN- $\gamma$  expression.

**Key words:** HIV, Tuberculosis, Garlic, *Allium sativum*, *Tulbaghia alliacea*, *Mycobacterium smegmatis*, antimycobacterial, Marasmicin.

## 3.2. Introduction

Tuberculosis (TB), a chronic contagious disease caused by infection with *Mycobacterium* species, has become an increasingly serious worldwide health concern in recent years (McGaw, Lall, Meyer, Ellof, 2008). TB is largely a disease of poverty, with the highest incidence of the disease (more than 80% of cases) occurring in Asia and Africa (Zager and McNerney, 2008). In sub-Saharan Africa, nine countries recently reported estimated annual incidences over 600 cases per 100 000 (Corbett, Marston, Churchyard, De Cock, 2006). Globally, there was an estimated 9.2 million new cases and 1.7 million deaths in related to TB in 2006 (World Health Organisation, 2008).

Together with the spread of HIV infection and the emergence of multi-drug resistant (MDR) and more recently, extremely drug resistant (XDR) strains of *Mycobacterium tuberculosis (Mtb)* (Jones, Hesketh, Yudkin, 2008), TB is becoming rampant, especially in countries lacking adequate health care systems to provide the required expensive and lengthy treatment (World Health Organization, 2008; Zager and McNerney, 2008). Adherence to treatment of TB is limited by a multitude of clinical, social, financial and behavioral factors (Ginsburg, Grossett, Bishai, 2003). Failure of patients to comply with conventional drug therapy has resulted in treatment failure and the emergence of drug resistance. This is compounded by bacterial mutation and the extrusion of



chemotherapeutics, via multi-drug resistant protein pumps in these pathogens (Walsh, 2000). In addition, fewer new antibiotics are being produced, owing partly to the high costs of development and licensing.

The World Health Organization estimates that one third of the world's population are latent carriers of the TB agent but only 10% of them develop clinical tuberculosis (WHO, 2008). More than 90% of infected individuals are therefore thought to develop a protective response to *Mtb* and successfully control *Mtb* growth (Chan and Kaffman, 1994). The risk of developing disease is greatly increased by acquired immunodeficiency syndrome (AIDS) and other immune-compromising conditions, demonstrating that protective immunity works in the majority of *M.tuberculosis*-infected individuals to suppress the infection (Korbel, Schneider, Schaible, 2008).

The protective response of hosts against *Mtb* is mediated by cell-mediated immunity, in which several cytokines including IFN- $\gamma$  play a crucial role (Ottenhof, Verreck, Hoeve, van de Visse, 2005). *Mycobacterium tuberculosis* is a facultative intracellular pathogen that resides and multiplies within human macrophages. IFN- $\gamma$  is the predominant inducer of macrophage-mediated microbicidal functions (Singhal, 2007) and has been shown to be required for the prevention of progressive *Mtb* infection (Cooper, Dalton, Stewart, Griffin, Russell, Orme, 1993; Flynn, Chan, Triebold, Dalton, Stewart, Bloom, 1993). Individuals who have genetic defects in their IFN- $\gamma$  system are known to be highly sensitive to mycobacterial infection (Ottenhof, Verreck, Hoeve, van de Vosse, 2005). Thus, *Mtb* infection generally induces *Mtb*-antigen specific IFN- $\gamma$  responses to protect hosts from developing TB.

The global prevalence of drug-resistant TB (Jones *et al.*, 2008) and the impact of HIV worldwide (Gutierrez-Lugo and Bewley, 2008) make the identification of potent new agents for the treatment of TB critically important.

Medicinal plants have been used, since time immemorial, in virtually all cultures as a source of medicine, and are recognized as a useful source of highly active antimycobacterial metabolites (Gibbons, 2005; Pauli, Case, Inui, Wang, Cho, Fischer, Franzblau, 2005). Many medicinal plants are also believed to enhance the natural resistance of the body to infections (Atal, Sharma, Kaul, Khajuria, 1986; Tiwari, Rastogi, Singh, Saraf, Vyas, 2004). The modulation of the immune response with the aid of various bioactives, in order to alleviate certain diseases is an active area of interest. (Tiwari, Rastogi, Singh, Saraf, Vyas, 2004). A number of plant products have been investigated for immune response modifying activity (Estrada, Katselis, Laarveld, Barl, 2000; Mediratta, Sharma, Singh, 2002) and a plethora of plant-derived materials (such as proteins, lectins, polysaccharides etc.) have been shown to stimulate the immune system (Tzianabos, 2000).

The commercial garlic species, *Allium sativum* L. (Liliaceae) has generated significant interest throughout human history as a medicinal panacea. A wide range of microorganisms including bacteria, fungi, protozoa and viruses has been shown to be sensitive to crushed garlic preparations (Ankri and Mirelman, 1999). Garlic contains a collection of sulfur and selenium aromatic compounds that have developed in the plant to

protect it from predators such as animals and soil-borne organisms (Sendl, 1995). It may well be the aforementioned natural actives that are responsible for garlic's natural antibiotic effects.

In a review of Indian medicinal plants as a source of antimycobacterial agents, Gautam, Saklani, and Jachak (2007) reported that, interestingly, many of the plant species surveyed showed a strong positive correlation between antimycobacterial activity results and ethnomedical use for TB and TB-related diseases. This provides support for investigating plants customarily used in other cultures to treat symptoms relating to TB.

In South Africa, members of the genus *Tulbaghia* are commonly referred to as “indigenous garlic,” which are invariably species of the sub-family Alliaceae. Interestingly, it is believed that the indigenous garlic, *Tulbaghia alliacea* L.f. (Liliaceae) might have some antimycobacterial activity since it is traditionally used for fever, colds, asthma and tuberculosis (TB) more specifically (Van Wyk *et al.*, 2000; Bamuamba, Gammon, Meyers, Dijoux-Franca, Scott, 2008).

*Tulbaghia alliacea* extracts have been shown to have anti-infective activity against a variety of Candidal species *in vitro*, and a very potent antifungal compound called Marasmicin was recently discovered in *T.alliacea* (Thamburan, Klaasen, Mabusela, cannon, Folk, Johnson, 2006). This investigation therefore aimed to assess the antimycobacterial properties of *T.alliacea* phytotherapy, as well assessing its ability to modulate the cytokine IFN- $\gamma$ .

### **3.3. Materials and Methods**

#### **3.3.1. Plant material**

Bulbs of *T. alliacea* were obtained from Rastafarian herbal traders in Cape Town, South Africa, while a commercial garlic brand of *A. sativum* was purchased from a retail store. Both species were verified by a botanist at the University of the Western Cape Herbarium where voucher specimens (Thamburan001 for *T.alliacea* and Thamburan003 for *A.sativum*) were deposited.

#### **3.3.2. Preparation of plant extracts**

Bulbs of *T. alliacea* were washed with distilled water and chopped into tiny pieces, whilst cloves of *A. sativum* were peeled and finely chopped (Eloff, 1998). Solvent extractions of different concentrations of the bulbs and cloves were prepared using solvents of differing polarity (water, methanol, ethanol, hexane, and chloroform). Extractions were prepared by soaking the chopped plant material in the solvents overnight and then vacuum filtering it.

### **3.3.3. Analysis of the antimycobacterial activity of**

#### ***Tulbaghia alliacea* extracts**

##### **3.3.3.1. Microorganism and growth media**

For the antimycobacterial screening, *Mycobacterium smegmatis* was used as a test model organism due to its similarity to *Mycobacterium tuberculosis* genetically, but lack of virulence as an infectious organism (Mitscher and Baker, 1998; Gillespie, Morrissey, Everett, 2001, Gibbons, 2005). A culture of *M.smegmatis* was obtained from the Department of Medical BioSciences at the University of the Western Cape, South Africa, and maintained on Mycobacteria 7H11 agar.

Subcultures of *M. smegmatis* were inoculated in nutrient broth and spread onto prepared agar plates using sterile swab sticks under aseptic conditions. Plated cultures were incubated at 37°C for 48 hr. The agar plate method for plant extract susceptibility testing is well standardized and widely used (Vanden Berghe and Vlietinck, 1991).

##### **3.3.3.2. Disk Diffusion Assay**

All solvent (aqueous, methanol, ethanol, hexane, and chloroform) extracts of *T.alliacea* and *A.sativum* were comparatively tested for *in vitro* antimycobacterial activity against *M.smegmatis*, using the agar plate disk diffusion method. The pharmaceutical drug, Ciprofloxacin (40 µg/disk) was used as a positive control, as it is a broad-spectrum antibiotic. 9mm filter paper disks (Schleider and Schuell) were autoclaved at 120°C in a

Speedy Autoclave (Lasec) for 20 min, and then incubated at 37°C overnight. Each disk was impregnated with 50µl of a particular extract and was allowed to dry overnight. Ciprofloxacin served as a positive control, and the extraction solvents (water, methanol, ethanol, hexane, and chloroform) served as a negative control. The dried impregnated disks were placed on the surface of agar plates inoculated with the mycobacterial culture. Each plate consisted of the following disks: a positive control (Ciprofloxacin), a negative control (particular solvent) and a different extraction of each solvent (e.g. 0.30% water). Each extract was tested in triplicate. After being accurately labeled, the agar plates were inverted and incubated at 37°C for 48 hr. After incubation, inhibition zones were recorded as the diameter of the growth-free zones around the disk.

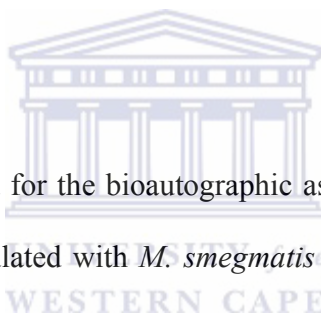


### **3.3.3.3. Minimum Inhibitory Concentrations (MIC)**

The MIC's for each extract were calculated by preparing serial dilutions of the original extractions. The extract concentration exhibiting a 1mm zone of inhibition was taken to be the MIC value for that solvent extraction, as a 1mm zone is the smallest zone that can be detected as growth inhibition of microorganisms *in vitro* using the agar plate disk diffusion method. The disk diffusion method is well suited for preliminary screening and has the advantage of allowing for the use of small sample sizes (Rios, Recio, Villar, 1988).

#### **3.3.3.4. Thin-layer chromatography (TLC) – Bioautographic Assay**

For direct bioassay on TLC plates, a 30µl aliquot of the chloroform extract was applied to 5 x 20 cm silica F254 (Merck) glass backed plates in duplicate for fingerprinting. All plates were developed in hexane: ethyl acetate (1:2 v/v) (Wagner and Blade, 1996). The developed TLC plates were dried overnight and then viewed under ultraviolet (UV) light (366 nm). Thereafter, one set of plates (of each extract) was sprayed with vanillin-sulphuric acid (VSA) reagent, made up by dissolving 3g vanillin in 30ml ethanol, to which 5ml concentrated sulphuric acid was added. Some compounds that were not visible under UV light became visible upon spraying and heating (for approximately 20 min) of the developed plates.



The duplicate plates were used for the bioautographic assay (inoculated TLC), whereby Mycobacteria 7H11 agar inoculated with *M. smegmatis* was poured over the developed plates under aseptic conditions. These plates were placed on damp paper towels in a plastic tray, covered with cling wrap and incubated at 37 °C for 48 hours.

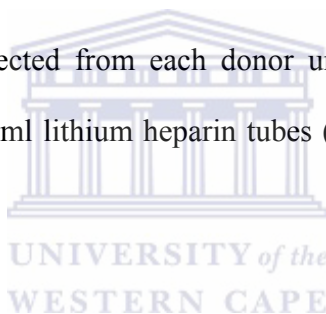
To detect the antimycobacterial activity on the plates, they were sprayed with a 0.2mg/ml solution of 2,5-diphenyltetrazolium bromide (MTT) (Sigma Chemicals Co.). Clear zones on the chromatogram indicated inhibition of growth (Beugue and Kline, 1972).

### **3.3.4. Assessment of the immune-modulation**

#### **characteristics of *Tulbaghia alliacea* extracts**

##### **3.3.4.1. Whole blood culture assay**

This study received ethical clearance from the University of the Western Cape. Brief histories were taken from 8 volunteers to confirm that they were in good general health, had had no recent medication or alcohol, and were non-smokers. Informed consent was received from the donors, who consisted of males and females between the ages of 20 – 30 years old. Blood was collected from each donor under good clinical practice, by means of venous puncture in 6ml lithium heparin tubes (BD Vacutainer Systems) at the UWC Campus Clinic.

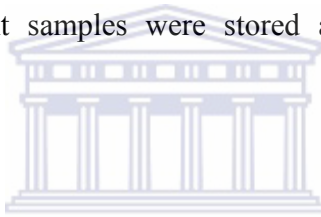


The blood was immediately processed in the tissue culture laboratory, where cell culture was undertaken under sterile conditions within a laminar flow. Blood was diluted 1 in 9 with RPMI-1640 (Sigma-Aldrich) medium, and plated in sterile 96-well flat-bottom tissue culture plates (Corning Costar, Cambridge, MA). Cells were stimulated in duplicate with antigen, mitogen or with medium. Each well consisted of 180µl of diluted whole blood, 10µl of stimulant and 10µl of plant extract, making up a final volume of 200µl/well.



Phytohaemagglutinin (PHA; Sigma-Aldrich) a common T cell mitogen, was used to non-specifically stimulate the cells. Purified protein derivative from *Mycobacterium tuberculosis* (PPD) obtained from Statens Serum Institut (SSI), Copenhagen, Denmark, was used for antigenic-specific stimulation. Negative control wells consisted of 180µl of diluted blood, 10µl of stimulant and 10 µl of RPMI-1640 medium.

All samples were incubated at 37°C with 5% carbon dioxide. Supernatants from the PHA-stimulated wells were harvested on day 3 after a 72 hour incubation period, while supernatants from the PPD-stimulated samples were harvested on day 5 after a 96 hour incubation period. Supernatant samples were stored at -20°C until used for cytokine analysis.



### **3.3.3 Cytokine Measurement: Enzyme-linked Immunosorbent Assay**

A sandwich Enzyme Linked Immunosorbent Assay (ELISA) kit (DuoSet, R&D Systems) was used in this experiment to measure human IFN- $\gamma$  concentrations in cell culture supernatant. The DuoSet ELISA is designed to detect and quantify IFN- $\gamma$  captured in a specific antibody-antigen interaction. The assay was performed according to the manufacturer's instructions. A 96-well plate (Nunc Maxisorp, Amersham) was coated with 50µl of mouse anti-human IFN- $\gamma$  capture antibody, diluted to a working concentration of 4.0µg/ml in phosphate buffered saline (PBS), and incubated overnight at room temperature. Plates were then washed in wash buffer (0.05% Tween-20 in PBS) to remove non-specifically bound material. Unoccupied sites were blocked with 300µl of block buffer (1% BSA, 0.05% NaN<sub>3</sub> in PBS) per well, and incubated at room temp for 1 hour.

After incubation of one hour, the blocking buffer was discarded and the plate was washed thrice with wash buffer (0.05% Tween-20 in PBS). Cell culture supernatants and serially diluted recombinant human IFN- $\gamma$  standard (0 – 1000 pg/ml) were then added to the plates at 50 $\mu$ l per well in duplicate, and incubated at room temperature for 2 hours. The plate was washed thrice after incubation, followed by adding 50 $\mu$ l of detection goat anti-human IFN- $\gamma$  antibody diluted in reagent diluent at a concentration of 175ng/ml. After incubation of 2 hours, 50 $\mu$ l of streptavidin conjugated to horseradish-peroxidase (HRP) enzyme was added to the wells and incubated in the dark for 20 minutes.

A colour substrate solution (H<sub>2</sub>O<sub>2</sub> + Tetramethylbenzidine) was then added and incubated in the dark for 20 minutes. Without washing, 50 $\mu$ l of stop (2 N H<sub>2</sub>SO<sub>4</sub>) solution was then added to each well. The intensity of the color change from blue to yellow is proportional to the levels of IFN- $\gamma$  present in the samples. The optical density was determined immediately using a microplate reader (Titertek Multicsan) at 450nm.

### **3.3.7. Statistical analysis**

For the antimycobacterial analyses, inhibition zones were statistically compared by conducting an unpaired *t*-test using the MedCalc (version 7.1, 2000) statistical programme. Means  $\pm$  SEM (standard error of the mean) were considered significant at  $P < 0.05$ . For the immunological analyses, modulatory effects were statistically compared using the Wilcoxon Rank Test. Differences between the medians were considered significant at  $P < 0.05$ .

## 3.4. Results

### 3.4.1. Disk diffusion assays

Inhibition zones caused by *A. sativum* and *T. alliacea* were compared to those of the conventional antibiotic, Ciprofloxacin, which also served as a positive control (Table 1). Ciprofloxacin significantly inhibited *M. smegmatis* when compared to *A. sativum* and *T. alliacea* across all solvent systems ( $P < 0.0001$ ).

Aqueous extracts of *T. alliacea* exhibited no antimycobacterial activity at a concentration of 2mg/ml, whereas *A. sativum* showed a small zone of inhibition. However, at a concentration of 5mg/ml and 10mg/ml, *T. alliacea* exhibited a statistically bigger zone of inhibition than *A. sativum* ( $P < 0.05$ ).

Ethanollic extracts of *T. alliacea* did not exhibit activity at 2mg/ml, whilst *A. sativum* did ( $P < 0.001$ ). Both *A. sativum* and *T. alliacea* showed similar inhibitory effects at 5mg/ml concentration, however, *T. alliacea* had a statistically bigger inhibitory effect than *A. sativum* at 10mg/ml ( $P < 0.002$ ). For methanol extracts; *A. sativum* and *T. alliacea* exhibited no activity at a concentration of 2mg/ml, but *A. sativum* at 5mg/ml had a bigger zone of inhibition ( $P < 0.001$ ) than *T. alliacea*, and both plants at 10mg/ml exhibited the same antimycobacterial activity (12mm zone).

*T.alliacea* hexane extracts did not show any activity at 2mg/ml, compared to *A.sativum*, which had a 2.66mm zone. Furthermore, no significantly different patterns of inhibition were detected at 5mg/ml and 10mg/ml concentration of hexane extracts.

The chloroform extract: *T.alliacea* did not show activity at 2mg/ml, but had statistically greater inhibition than *A.sativum* at 5mg/ml ( $P<0.002$ ) and 10mg/ml ( $P<0.0001$ ) concentrations. In general, the antimycobacterial activity of *T.alliacea* for the 10mg/ml chloroform extract (17.66mm) was greater than that of the 10mg/ml aqueous (9.33mm), ethanol (12.66mm), methanol (12.00mm), and hexane (10.66mm) extracts.



**Table 1.** The antimycobacterial activity of *A. sativum* and *T. alliacea* extracts.

Extract	Conc. (mg/ml)	Zone of Inhibition (mm ± SEM)			Statistical significance*		
		Ciprofloxacin (a)	<i>A. sativum</i> (b)	<i>T. alliacea</i> (c)	ab	ac	bc
<b>Aqueous (H<sub>2</sub>O)</b>	2	24.00 ± 0.00	1.66 ± 0.33	0	0.0001	0.0001	0.0075
	5	24.00 ± 0.00	3.33 ± 0.33	5.00 ± 0.00	0.0001	0.0001	0.0075
	10	24.00 ± 0.00	6.33 ± 0.33	9.33 ± 0.33	0.0001	0.0001	0.0031
<b>Ethanol (EtOH)</b>	2	24.00 ± 0.00	2.66 ± 0.33	0	0.0001	0.0001	0.0013
	5	24.00 ± 0.00	4.33 ± 0.33	4.00 ± 0.00	0.0001	0.0001	0.3739
	10	24.00 ± 0.00	7.33 ± 0.33	12.66 ± 0.66	0.0001	0.0001	0.0020
<b>Methanol (MeOH)</b>	2	24.00 ± 0.00	0	0	0.0001	0.0001	NS
	5	24.00 ± 0.00	5.33 ± 0.33	4.00 ± 0.00	0.0001	0.0001	0.0161
	10	24.00 ± 0.00	12.00 ± 0.00	12.00 ± 0.00	0.0001	0.0001	NS
<b>Hexane</b>	2	24.00 ± 0.00	2.66 ± 0.33	0	0.0001	0.0001	0.0013
	5	24.00 ± 0.00	5.44 ± 0.33	3.66 ± 0.33	0.0001	0.0001	0.1012
	10	24.00 ± 0.00	11.33 ± 0.66	10.66 ± 0.33	0.0001	0.0001	0.4216
<b>Chloroform (CHCl<sub>3</sub>)</b>	2	24.00 ± 0.00	2.66 ± 0.33	0	0.0001	0.0001	0.0013
	5	24.00 ± 0.00	5.00 ± 0.00	9.33 ± 0.33	0.0001	0.0001	0.0020
	10	24.00 ± 0.00	7.66 ± 0.33	17.66 ± 0.33	0.0001	0.0001	0.0001

\* *P* values for comparing main effects: ab = Ciprofloxacin and *A. sativum*, ac = Ciprofloxacin and *T. alliacea*, bc = *A. sativum* and *T. alliacea*. NS = No Significance.

# Negative controls (water, ethanol, methanol, hexane and chloroform) did not inhibit *M. smegmatis*, hence no zone of inhibition was observed.

An MIC value is defined as the lowest concentration, which visibly inhibits growth of the microorganism after incubation. *A. sativum* extracts were shown to have lower MIC values than *T. alliacea* (Table 2). The lowest MIC value against *M. smegmatis* was obtained with the 1.0mg/ml and 2.4mg/ml chloroform extract of *A. sativum* and *T. alliacea*, respectively.

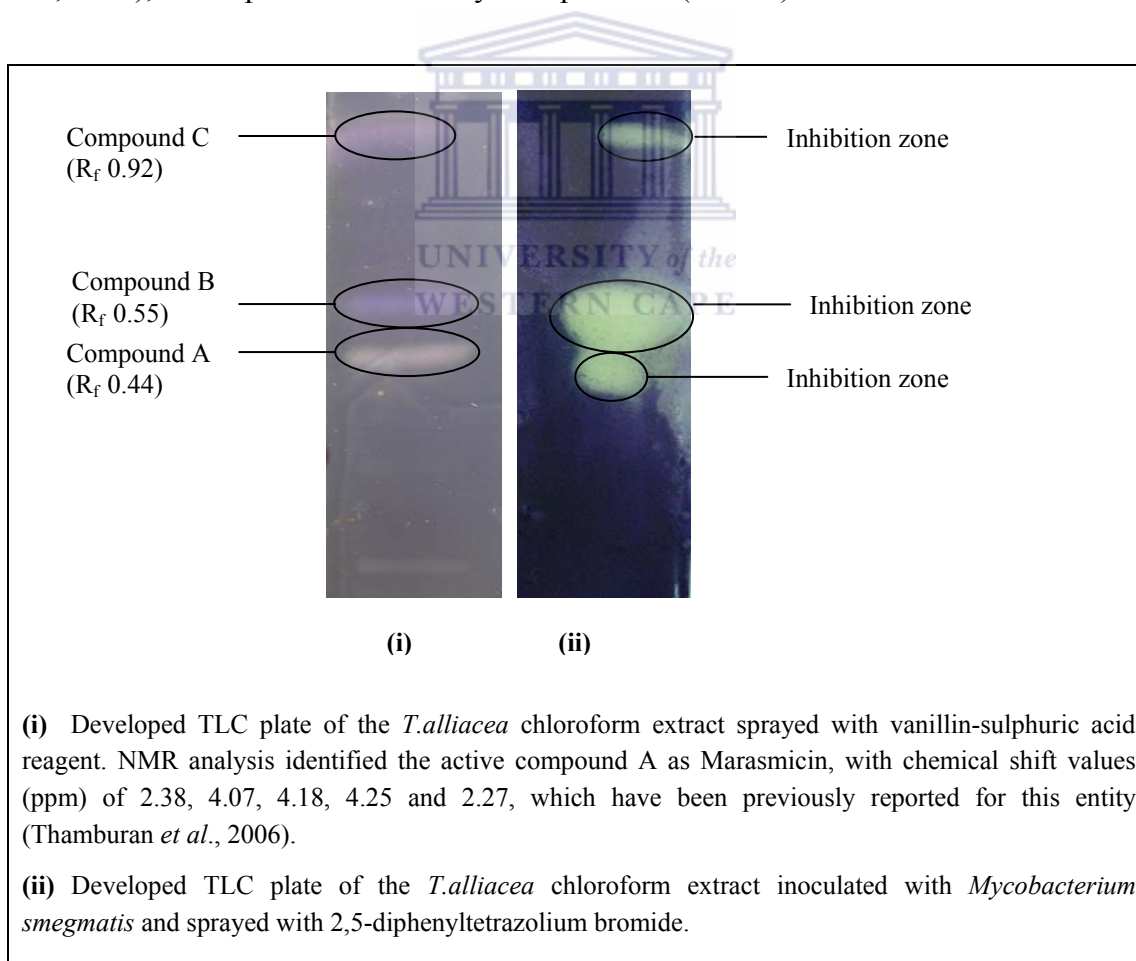
**Table 2.** The minimum inhibitory concentrations (MIC) for *Tulbaghia alliacea* and *Allium sativum* extracts against *M. smegmatis*.

Extract	Extract Concentration (mg/ml)		Zone of Inhibition (mm)
	<i>A. sativum</i>	<i>T. alliacea</i>	
Aqueous (H <sub>2</sub> O)	2.0	4.0	1
Methanol (MeOH)	3.0	4.4	1
Ethanol (EtOH)	2.0	4.4	1
Hexane	1.6	4.4	1
Chloroform (CHCl <sub>3</sub> )	1.0	2.4	1

### 3.4.2. Antimycobacterial activity of *T. alliacea* using

#### TLC–Bio-autography

The chloroform extract separated as three inhibitory fractions (Fig.1-i) that are indicated as zones of inhibition on the bioautogram. TLC separations (Fig.1-ii) sprayed with vanillin-sulphuric acid reagent showed the compounds present in the extract; these compounds are the same as the antimycobacterial compounds visible on the bioautogram, based on the distance they traveled on the TLC plates. Comparing the  $R_f$  values from a previous study that assessed the antifungal activity of *T.alliacea* extracts (Thamburan *et al.*, 2006), it was possible to identify Compound A ( $R_f$ 0.44) as Marasmicin.



**Fig. 1.** The antimycobacterial activity of *T. alliacea* chloroform extract on TLC-bioautography.

### 3.4.3. Assessment of the immune-modulating characteristics of *T.alliacea* extracts

#### 3.4.3.1. Standard curve

A standard curve was generated using eight different concentrations, in duplicate, of standard IFN- $\gamma$ , analysed with the same ELISA DuoSet kit employed for the analyses of the experimental samples (Table 3). Optical densities were read off a colorimetric reader at 450 nm. The concentrations of IFN- $\gamma$  (pg/ml) were plotted against the optical densities obtained. A correlation coefficient of 0.99 was obtained (Fig. 2).

**Table 3.** Standard curve for the evaluation of IFN- $\gamma$  in whole blood culture supernatants

IFN- $\gamma$ standard	Optical Density 1	Optical Density 2	Average Optical Density	Standard Deviation	Co-efficient of variance
1000	0.631	0.632	0.632	0.001	0.112
500	0.347	0.349	0.348	0.001	0.406
250	0.220	0.220	0.220	0.000	0.000
125	0.166	0.165	0.166	0.001	0.427
62	0.139	0.137	0.138	0.001	1.025
31	0.103	0.102	0.103	0.001	0.690
15	0.104	0.105	0.105	0.001	0.677
0	0.106	0.106	0.106	0.000	0.000



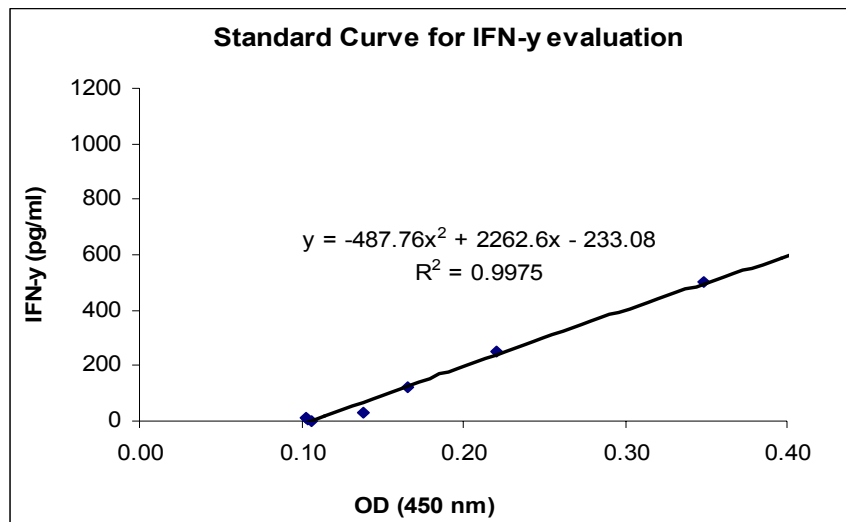
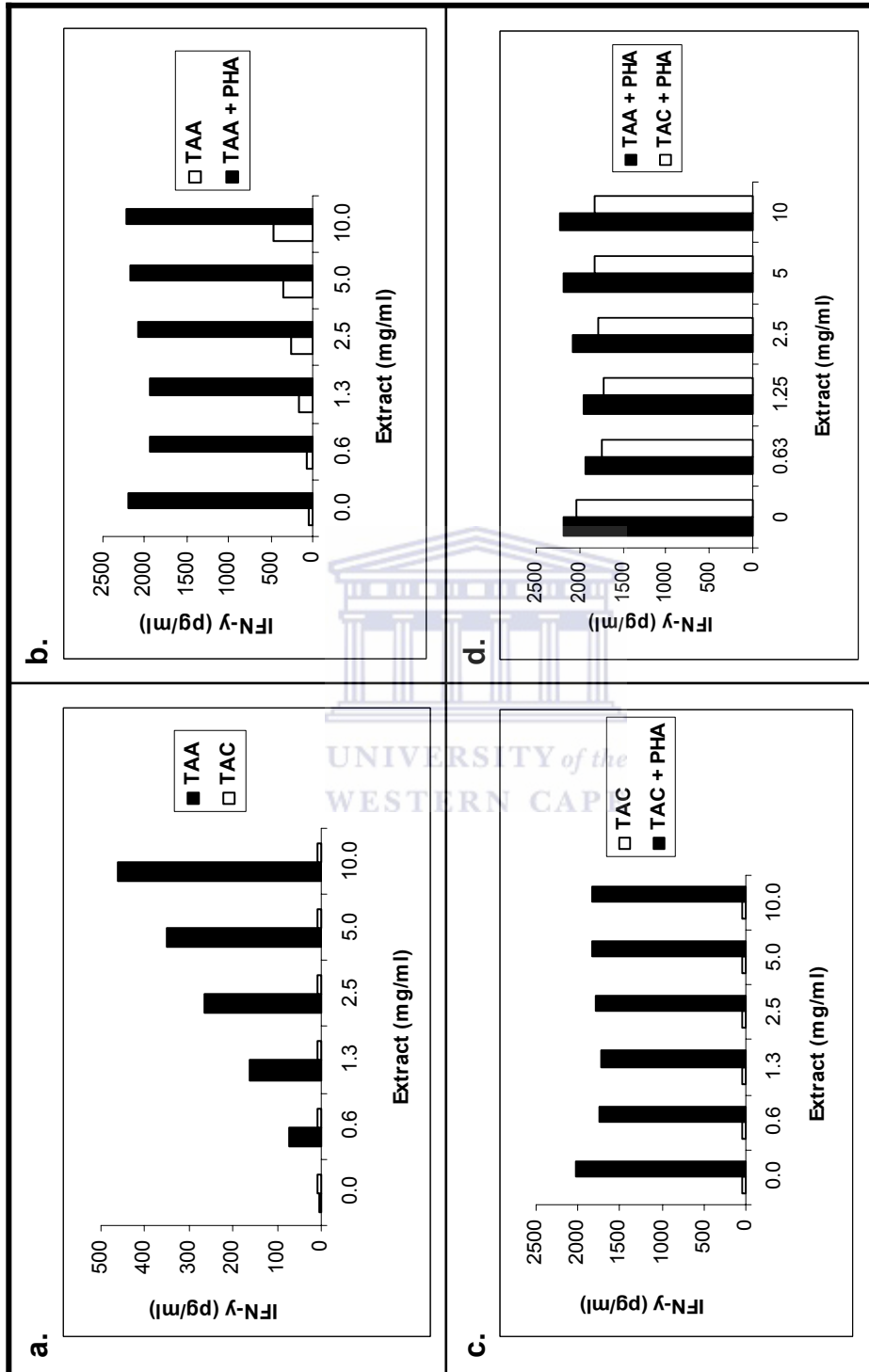


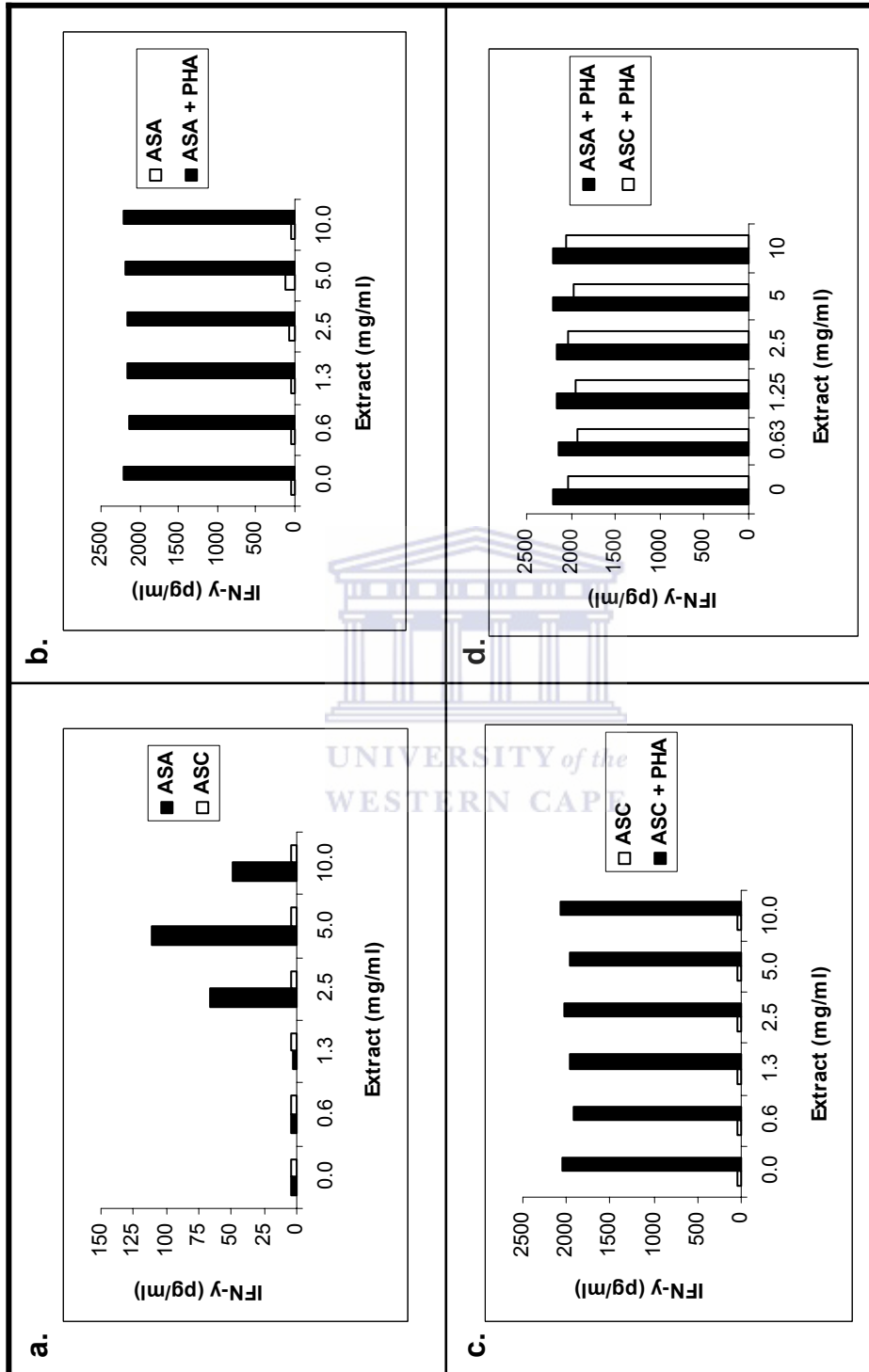
Fig.2. Standard curve of IFN- $\gamma$ .

### 3.4.3.2. Modulation of IFN- $\gamma$ expression in whole blood cell cultures stimulated with the mitogen, PHA

Whole blood cell cultures exposed to TAA extracts only, showed a small but dose-dependant increase in IFN- $\gamma$  expression levels; IFN- $\gamma$  increased from 2.48pg/ml at 0.63mg/ml of TAA extract to 460.89pg/ml at 10mg/ml (Fig 3). In contrast, exposure to the TAC extract did not induce IFN- $\gamma$  expression; cytokine levels remained at baseline (Fig 3). Cultures stimulated with PHA only induced ~2000pg/ml of IFN- $\gamma$ . Cultures stimulated with PHA and exposed to TAA and TAC extracts did not demonstrate much difference in the quantity of IFN- $\gamma$  expressed (Fig 3). However, at 10mg/ml, the TAA extract was more potent in inducing IFN- $\gamma$  expression than the TAC extract ( $P < 0.05$ ) (Table 4). The aqueous extracts of both plants *Tulbaghia alliacea* and *Allium sativum* had similar activity (Fig. 4), except at 10mg/ml where the chloroform extract of *A. sativum* (ASC) was statistically more potent than TAC ( $P < 0.05$ ) (Table 4).



**Fig 3.** The modulation of IFN- $\gamma$  by *Tulbaghia alliacea* extracts in unstimulated (a) and PHA-stimulated (b-d) whole blood cell cultures.



**Fig 4.** The modulation of IFN- $\gamma$  by *Allium sativum* extracts in unstimulated (a) and PHA-stimulated (b-d) whole blood cell cultures.

**Table 4.** Comparison of the median values for the modulation of IFN- $\gamma$  by different garlic extracts in unstimulated whole blood cells (3-day incubation).

Plant extract (mg/ml)	IFN- $\gamma$ expression (pg/ml)				Statistical significance*			
	TAA (a)	TAC (b)	ASA (c)	ASC (d)	ab	ac	bd	cd
0.00	2	0	0	0	0.465	0.465	0.108	0.108
0.63	74	0	0	0	<b>0.027</b>	<b>0.027</b>	0.285	0.285
1.25	162	0	3	0	<b>0.017</b>	<b>0.027</b>	0.179	0.715
2.50	264	0	67	0	<b>0.017</b>	<b>0.026</b>	1.000	0.079
5.00	350	0	111	0	<b>0.017</b>	<b>0.236</b>	0.285	<b>0.027</b>
10.00	461	0	149	0	<b>0.017</b>	<b>0.017</b>	0.654	<b>0.043</b>

\* *P* values for comparing main effects:

ab = *T.alliacea* aqueous extract and *T.alliacea* chloroform extract

ac = *T.alliacea* aqueous extract and *A.sativum* aqueous extract

bd = *T.alliacea* chloroform extract and *A.sativum* chloroform extract

cd = *A.sativum* aqueous extract and *A.sativum* chloroform extract

**Table 5.** Comparison of the median values for the modulation of IFN- $\gamma$  by different garlic extracts in PHA-stimulated whole blood cells

Plant extract (mg/ml)	IFN- $\gamma$ expression (pg/ml)				Statistical significance *			
	TAA+PHA (a)	TAC+PHA (b)	ASA+PHA (c)	ASC+PHA (d)	ab	ac	bd	cd
<b>0.00</b>	2193	2033	2203	2038	0.593	0.472	0.312	0.289
<b>0.63</b>	1940	1748	2141	1924	0.320	0.472	0.125	0.078
<b>1.25</b>	1947	1713	2155	1954	0.074	0.421	0.074	0.187
<b>2.50</b>	2081	1778	2168	2027	0.054	0.421	0.074	0.343
<b>5.00</b>	2176	1825	2195	1965	0.191	0.628	0.054	0.343
<b>10.00</b>	2225	1826	2203	2060	0.039	0.230	0.027	0.593

\* *P* values for comparing main effects:

ab = *T.alliacea* aqueous extract + PHA and *T.alliacea* chloroform extract + PHA

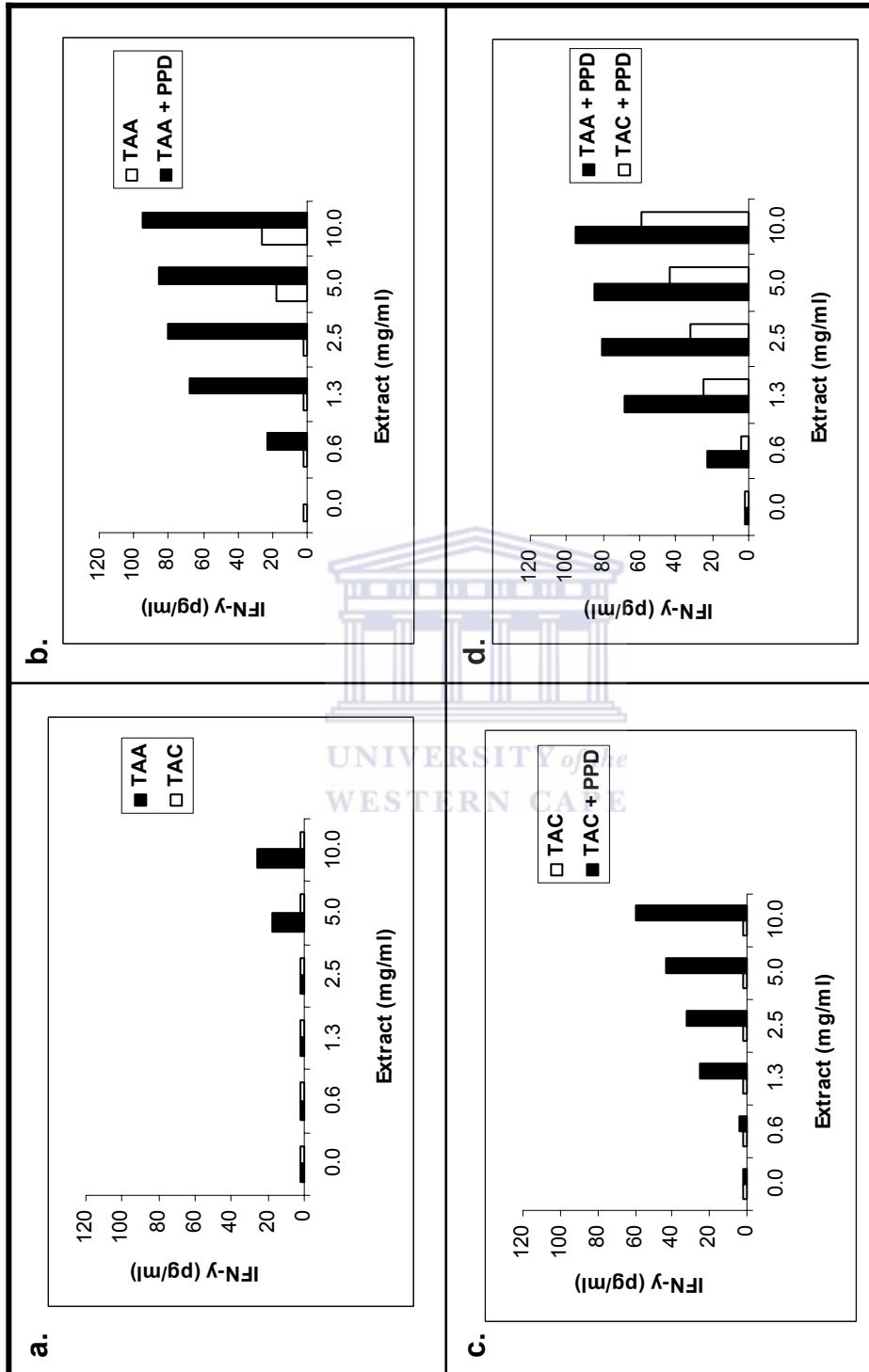
ac = *T.alliacea* aqueous extract + PHA and *A.sativum* aqueous extract + PHA

bd = *T.alliacea* chloroform extract + PHA and *A.sativum* chloroform extract + PHA

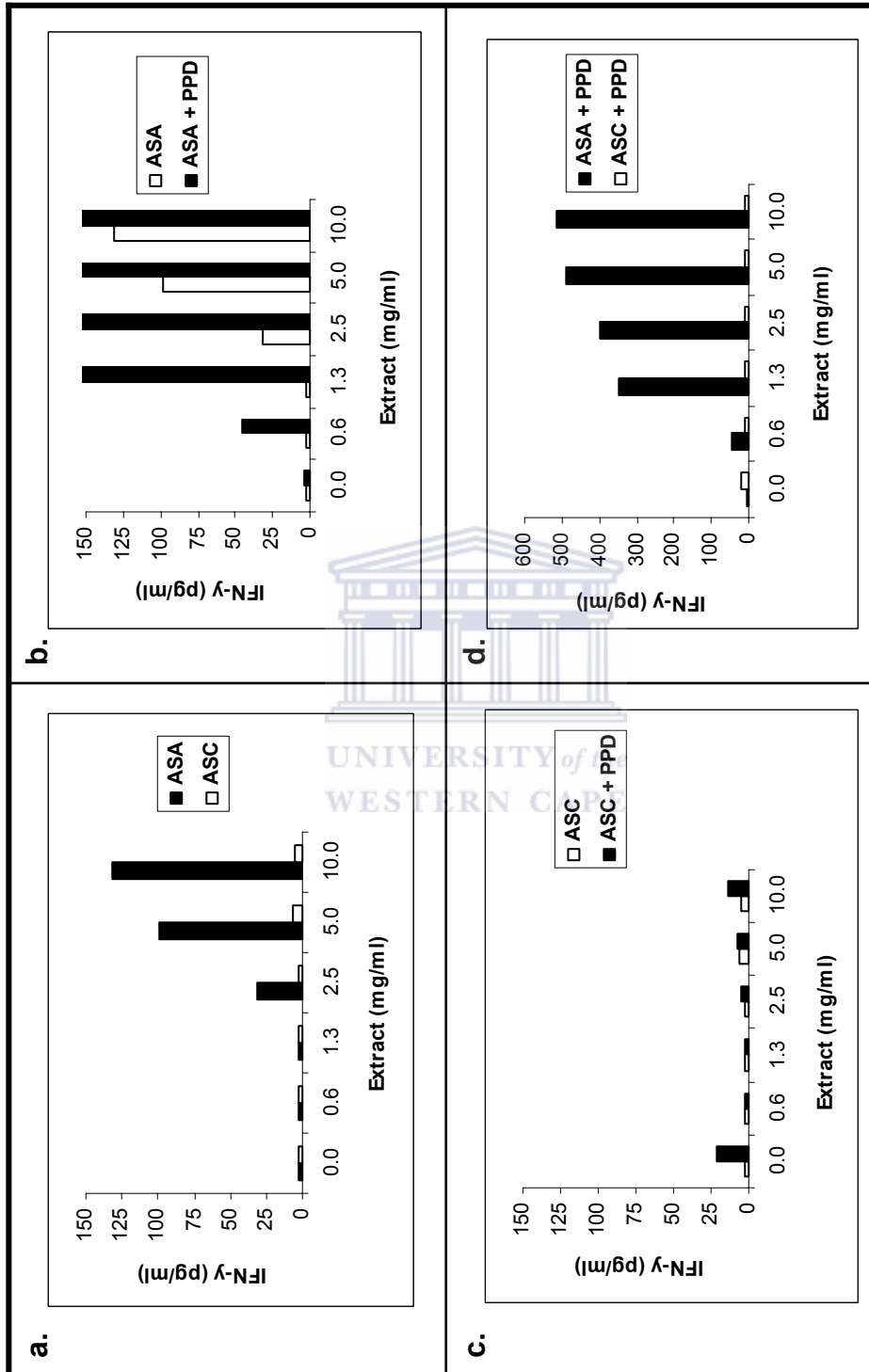
cd = *A.sativum* aqueous extract + PHA and *A.sativum* chloroform extract + PHA

### **3.4.3.3. Modulation of IFN- $\gamma$ expression in whole blood cell cultures stimulated with the antigen, PPD**

Blood cultures stimulated with PPD and exposed to varying concentrations of TAA and TAC extracts showed a dose-dependant increase in the expression levels of IFN- $\gamma$  (Fig. 5). At concentrations of 1.25mg/ml, 5mg/ml, and 10mg/ml, the TAA extract induced greater expression of IFN- $\gamma$  than TAC ( $P<0.05$ ) (Table 5). Similarly, the aqueous and chloroform extracts of *Allium sativum* differed greatly in their ability to modulate expression of IFN- $\gamma$  (Fig. 6); at extract concentrations of 0.63mg/ml, 1.25mg/ml, 5mg/ml, and 10mg/ml, ASA was more active than ASC ( $P<0.05$ ) and hence produced higher levels of IFN- $\gamma$  (Table 5). The ASA extract was also more active than the TAA extract at those concentrations ( $P<0.05$ ) (Table 5).



**Fig 5.** The modulation of IFN- $\gamma$  by *Tulbaghia alliacea* extracts in unstimulated (a) and PPD-stimulated (b-d) whole blood cell cultures.



**Fig 6.** The modulation of IFN- $\gamma$  by *Allium sativum* extracts in unstimulated (a) and PPD-stimulated (b-d) whole blood cell cultures.



**Table 6.** Comparison of the median values for the modulation of IFN- $\gamma$  by different garlic extracts in unstimulated whole blood cells (5-day incubation).

Plant extract (mg/ml)	IFN- $\gamma$ expression (pg/ml)				Statistical significance *			
	TAA (a)	TAC (b)	ASA (c)	ASC (d)	ab	ac	bd	cd
0.00	0	0	0	0	0.317	0.317	0.317	0.317
0.63	0	0	0	0	0.179	0.179	0.317	0.319
1.25	0	0	0	0	0.179	0.175	0.317	0.108
2.50	0	0	31	0	0.179	0.498	0.179	<b>0.017</b>
5.00	18	0	98	7	0.144	<b>0.042</b>	0.361	0.236
10.00	26	0	131	5	<b>0.043</b>	<b>0.042</b>	0.108	<b>0.027</b>

\* *P* values for comparing main effects:

ab = *T.alliacea* aqueous extract and *T.alliacea* chloroform extract

ac = *T.alliacea* aqueous extract and *A.sativum* aqueous extract

bd = *T.alliacea* chloroform extract and *A.sativum* chloroform extract

cd = *A.sativum* aqueous extract and *A.sativum* chloroform extract

**Table 7.** Comparison of the median values for the modulation of IFN- $\gamma$  by different garlic extracts in PPD-stimulated whole blood cells

Plant extract (mg/ml)	IFN- $\gamma$ expression (pg/ml)				Statistical significance*			
	TAA+PPD (a)	TAC+PPD (b)	ASA+PPD (c)	ASC+PPD (d)	ab	ac	bd	cd
<b>0.00</b>	1	0	3	21	0.187	0.437	0.187	0.406
<b>0.63</b>	23	4	45	0	0.312	<b>0.031</b>	0.687	<b>0.015</b>
<b>1.25</b>	68	25	350	1	<b>0.011</b>	<b>0.007</b>	0.718	<b>0.003</b>
<b>2.50</b>	80	32	399	5	0.054	0.007	0.781	0.003
<b>5.00</b>	85	43	490		<b>0.039</b>	<b>0.007</b>	0.343	<b>0.003</b>
<b>10.00</b>	95	59	514	14	<b>0.019</b>	<b>0.007</b>	0.687	<b>0.003</b>

\* *P* values for comparing main effects:

ab = *T.alliacea* aqueous extract + PPD and *T.alliacea* chloroform extract + PPD

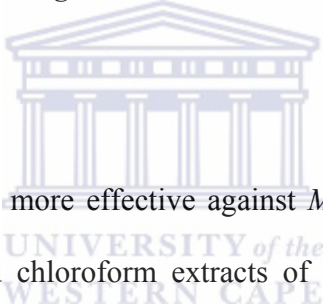
ac = *T.alliacea* aqueous extract + PPD and *A.sativum* aqueous extract + PPD

bd = *T.alliacea* chloroform extract + PPD and *A.sativum* chloroform extract + PPD

cd = *A.sativum* aqueous extract + PPD and *A.sativum* chloroform extract + PPD

### 3.5. Discussion

The domestic garlic plant, *Allium sativum*, is well known for its antimicrobial characteristics (Uchida, Takahashi, Sato, 1975). Whilst there is much indigenous knowledge about the traditional use of the South African garlic plant *Tulbaghia alliacea*, as an anti-infective remedy, there is very limited scientific information about this phytotherapy. Therefore, this investigation aimed at understanding the antimycobacterial effect of *Tulbaghia alliacea*, and its recently identified active ingredient Marasmicin (Thamburan, *et al.*, 2006). More specifically, our investigation compared the antimycobacterial effects of *Tulbaghia alliacea* to *Allium sativum* and the synthetic antibiotic, Ciprofloxacin.



Ciprofloxacin was found to be more effective against *M. smegmatis* than the aqueous, ethanol, methanol, hexane and chloroform extracts of *Tulbaghia alliacea* and *Allium sativum*. However, it must be remembered that Ciprofloxacin, as a pharmaceutical drug, has been completely purified whilst the garlic extracts may still contain compounds that masks the true potency of this medicinal herb (Johnson, Weitz, Mabusela, Klaasen, Coldrey, Gabielse, 2002). It is generally reported that MIC's of plant antimicrobials are typically in the range of 100 to 1000µg/ml, orders of magnitude weaker than commercial broad-spectrum antibiotics. However, it was discovered that the activity of principal antimicrobial plant compounds was potentiated 100- to 2000 fold depending on the bacterial species, by disabling multi-drug resistance pumps, which could suggest that plants might have developed other means of delivering their antimicrobials more effectively into bacterial cells (Tegos, Stermitz, Lomovskaya, Lewis, 2002).

Chloroform extracts of *T.alliacea* were statistically more potent than the *A.sativum*, in inhibiting the growth of *M.smegmatis*. Consequently, the *T.alliacea* chloroform extracts were subjected to phytochemical analyses, to better understand which compounds may be responsible for combating *M. smegmatis in vitro*. Through bioautography, we determined the localization of antimicrobial activity on chromatograms, and provided important qualitative information about the active components of the phytotherapy. These outcomes were further enhanced, by separating the phytotherapy extracts on TLC plates, which provided additional information about the bioactive compounds present in the mixture.

These analyses showed that the chloroform extract of *Tulbaghia alliacea* contained three major inhibitory fractions that were indicated as zones of inhibition on the bioautogram. Compound A had the same  $R_f$  value (0.44) as Marasmicin, a previously isolated compound from *Tulbaghia alliacea* that exhibited antifungal activity (Thamburan *et al.*, 2006). This suggests that Marasmicin also has antimycobacterial activity. In addition to Marasmicin, a further two compounds that exhibit strong antimycobacterial activity, have now been located in *Tulbaghia alliacea*, and the structural analyses of these moieties are the subject of our next advanced study. Consequently, Marasmicin may act singularly or in concert with synergistic compounds in *Tulbaghia alliacea*, as a very significant natural antibiotic.

The crisis in antimicrobial therapy, which has stemmed from antibiotic overuse, misuse and the limited number of new antimicrobial drugs on the near horizon, is well documented (Spellberg, Powers, Brass, Miller, Edwards, 2004). However, another area that limits the utility of antimicrobial drug-based therapy is that antimicrobial agents are frequently ineffective in individuals with impaired immunity, often despite being highly active *in vitro* or in individuals with intact immunity (Pirofski and Casadevall, 2006).

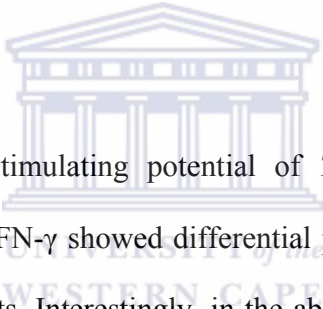
Approximately one-third of the world's population is latently infected with *Mycobacterium tuberculosis*, and 90% of these individuals will never develop active disease, indicating that the human immune system is capable of controlling *M. tuberculosis* infection effectively (Kursar, Koch, Mittrucker, Nouailles, Bonhagen, Kamrdat, Kaufmann, 2007). It has long been recognized that cell-mediated immune (CMI) responses predominate in TB infection; specifically, a type-1 T-cell response that is characterized by production of IFN- $\gamma$  (Andersen, Doherty, Pai, Wedingh, 2007). IFN- $\gamma$  has been identified as an essential cytokine for controlling and containing TB infection (Cooper, Dalton, Stewart, Griffin, Russell, Orme, 1993; Dalton, Pitts-Meek, Keshav, Figari, Bradley, Stewart, 1993; Flynn, Chan, Triebold, Dalton, Stewart, Bloom, 1993; Kamijo, Le, Shapiro, 1993). Animals and humans that lack the ability to produce or respond to IFN- $\gamma$  show extreme susceptibility and increased mortality to a TB infection (Cooper *et al.*, 1993; Dalton *et al.*, 1993, Flynn *et al.*, 1993; Kamijo *et al.*, 1993; Jouanguy, Altare, Lamhamedi, 1996; Doffinger, Helbert, Barcenas-Morales, 2004; Goldsack and Kirman, 2007).

As IFN- $\gamma$  is required for a Th1 immune response to *Mtb* infection, this cytokine has been measured *ex vivo* in serum (Vankayalapati, Wizel, Weis, Klucar, Shams, Samten, Barnes, 2003), bronchoalveolar lavage fluids (Barry, Lipman, Bannister, Johnson, Janossy, 2003) and pleural effusions of TB patients (Hirsch, Toossi, Johnson, Luzze, Ntambi, Peters, McHugh, Okwera, Joloba, Mugenyi, 2001), or in culture supernatants of lymphocytes isolated from these body fluids and stimulated *in vitro* with mycobacterial antigens (Veenstra, Crous, Brahmhatt, Lukey, Beyers, van Helden, Walzl, 2007). In the majority of the latter studies, secreted IFN- $\gamma$  was measured by ELISA.

To assess the immune-modulatory effects of *Tulbaghia alliacea* extracts on IFN- $\gamma$  expression, diluted whole blood was stimulated with the non-specific mitogen PHA and the TB-specific antigen PPD, and exposed to varying concentrations of *Tulbaghia alliacea* aqueous and chloroform extracts. The use of whole blood cultures for cytokine studies was first described in 1982 (Kirchner, Kleinicke, Digel). Performing a whole blood experiment rather than examining peripheral blood mononucleocytes (PBMCs) or separated blood cells provides the major advantage that all cellular components of peripheral blood are present in their natural proportions and that no preactivation or desensitization of immune cells, often brought about by separating PBMCs on a density gradient, would occur (Breidhardt, Frohn, Luhm, Kirchner, Brand, 2002; Van Crevel, Ven-Jongekrijg, Netea, de Lange, Kullberg, van der Meer, 1999). The dilution of blood and incubation in tissue culture wells facilitates the use of much smaller volumes of blood and longer incubation periods, both of which may be advantageous for the

detection of cytokines with a low production rate (Van Crevel *et al.*, 1999). The study of whole blood *in vitro*, thus, has both practical and theoretical advantages.

PHA was used as a non-specific stimulant, as it is a potent T cell stimulant that can activate T cells to produce cytokines like IFN- $\gamma$  (Goldsby, Kindt, Osborne, 2000; Chang, Chiang, Chang, Yeh, Shyur, Kuo, Wu, Yang, 2007). Antigenic-specific stimulation was provided by the use of PPD. PPD is prepared by precipitation of proteins from heat-killed cultures of *M tuberculosis*, and has been used for *in vitro* detection of specific IFN- $\gamma$ -secreting lymphocytes sensitised by mycobacterial antigens (Andersen, Munk, Pollock, Doherty, 2000).



Assessment of the immune stimulating potential of *Tulbaghia alliacea* extracts in modulating the expression of IFN- $\gamma$  showed differential patterns of activity between the aqueous and chloroform extracts. Interestingly, in the absence of mitogenic or antigenic stimulus, TAA induced a dose-dependant increase in IFN- $\gamma$  expression levels (2.48 pg/ml at 0.63mg/ml of TAA extract to 460.89 pg/ml at 10mg/ml), whereas the TAC extract at all concentrations did not induce IFN- $\gamma$  expression (0 pg/ml).

Mitogenic stimulation of whole blood cultures with PHA and exposure to TAA and TAC extracts, resulted in similar quantities of IFN- $\gamma$  being expressed (Fig 3a-b), except at 10mg/ml, where the TAA extract was more potent in inducing IFN- $\gamma$  expression than the TAC extract ( $P<0.05$ ) (Table 4). Comparison of the two garlic plants *Tulbaghia alliacea* and *Allium sativum*, also showed similar activity (Fig. 4a-b), except at the highest extract

concentration of 10mg/ml where the chloroform extract of *A.sativum* (ASC) was statistically more potent than TAC ( $P<0.05$ ) (Table 4).

Following antigenic stimulation of whole blood cultures with PPD and subsequent exposure to the plant extracts, it was observed that TAA and TAC extracts induced a dose-dependant increase in the expression levels of IFN- $\gamma$  (Fig. 5a-b). The TAA extract again induced greater expression of IFN- $\gamma$  than TAC ( $P<0.05$ ) (Table 5). Similarly, the aqueous and chloroform extracts of *Allium sativum* differed greatly in their ability to modulate expression of IFN- $\gamma$  (Fig. 6a-b); ASA was more active than ASC ( $P<0.05$ ) (Table 5). Comparing the two garlic plants revealed the ASA extract to be more active than the TAA extract ( $P<0.05$ ) (Table 5).

A noteworthy finding in this study is that the aqueous extract of *Tulbaghia alliacea* is more active than the chloroform extract, in stimulating the expression of IFN- $\gamma$ . This is significant because an aqueous extract is the form in which this phytotherapy is used in indigenous cultures, as a remedy for various ailments including TB and TB-related conditions (Van Wyk *et al.*, 2000; Bamuamba, Gammon, Meyers, Dijoux-Franca, 2008). The aqueous extract also showed considerable antimycobacterial activity against *Mycobacterium smegmatis in vitro*, indicating that administration of the phytotherapy during infection could work either by eradicating the pathogen, or by stimulating the host's immune system to mount a protective response via expression of IFN- $\gamma$ .



### **3.6. Conclusion**

This investigation has indicated that *Tulbaghia alliacea* could have potential as an anti-tuberculosis remedy, as it has been shown to possess both antimycobacterial activity as well as immune-modulatory effects on IFN- $\gamma$  expression. This study also validates the traditional use of the aqueous extract of this widely-used indigenous phytotherapy.



### 3.7. References

- Andersen P, Doherty TM, Pai M, Wedingh K (2007). The prognosis of latent tuberculosis: can disease be predicted? *Trends in Molecular Medicine* 13: 175 – 182.
- Andersen P, Munk ME, Pollock JM, Doherty TM (2000). Specific immune-based diagnosis of tuberculosis. *Lancet* 356: 1099–104.
- Ankri S, Mirelman D (1999). Antimicrobial properties of allicin from garlic. *Microbes Infect* 1: 125-129.
- Atal CK, Sharma ML, Kaul A, Khajuria A (1986). Immunomodulating agents of plant origin. *Journal of Ethnopharmacology* 41: 185 – 192.
- Bamuamba K, Gammon DW, Meyers P, Dijoux-Franca M-G, Scott G (2008). Antimycobacterial activity of five plant species used as traditional medicines in the Western Cape Province (South Africa). *Journal of Ethnopharmacology* 117: 385-390.
- Barry SM, Lipman MC, Bannister B, Johnson MA, Janossy G (2003). Purified protein derivative-activated Type 1 cytokine-producing CD4<sup>+</sup> T lymphocytes in the lung: a characteristic feature of active pulmonary and non-pulmonary tuberculosis, *J. Infect. Dis* 187: 243–250.
- Beugue WJ, Kline RM (1972). The use of tetrazolium salts in bioautographic procedures, *J Chromatogr* 64: 182-184.
- Breidhardt T, Frohn C, Luhm J, Kirchner H, Brand JM (2002). Prolactin induces enhanced interferon gamma release in peripheral whole blood after stimulation with either PHA or LPS. *Immunobiol* 206: 424 – 431.

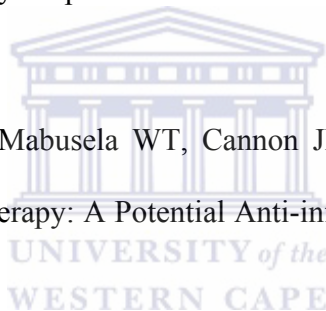
- Chan J, Kaufmann SHF. Immune mechanisms of protection (1994). In: Bloom BR, editor. Tuberculosis: pathogenesis, protection, and control. Washington, DC: ASM Press; p. 389–415.
- Chang SL, Chiang YM, Chang CLT, Yeh HH, Shyur LF, Kuo YH, Wu TK, Yang WC (2007). Flavonoids, centaurin and centaureidin, from *Bidens plosa*, stimulate IFN- $\gamma$  expression. *Journal of Ethnopharmacology* 112: 232 – 236.
- Cooper AM, Dalton DK, Stewart TA, Griffin JP, Russell DG, Orme IM (1993). Disseminated tuberculosis in interferon gamma gene-disrupted mice. *J Exp Med* 178: 2243–7.
- Cooper AM, Dalton DK, Stewart TA, Griffin JP, Russell DG, Orme IM (1993). Disseminated tuberculosis in interferon- $\gamma$  gene disrupted mice. *J. Exp. Med.* 178: 2243–2247.
- Corbett EL, Marston B, Churchyard GJ, De Cock KM (2006). Tuberculosis in sub-Saharan Africa: opportunities, challenges, and change in the era of antiretroviral treatment. *Lancet* 367: 926-937.
- Costa MS, Boechat N, Rangel EA, da Silva FC, de Souza AMT, Rodrigues CR, Castro HC, Junior IN, Lourenço MCS, Wardell SMSV and Ferreira VF (2006). Synthesis, tuberculosis inhibitory activity, and SAR study of N-substituted-phenyl-1,2,3-triazole derivatives. *Bioorganic and Med Chem* 14: 8644 – 8653.
- Dalton DK, Pitts-Meek S, Keshav S, Figari IS, Bradley A, Stewart TA (1993). Multiple defects of immune cell function in mice with disrupted interferon-gamma genes. *Science* 259: 1739–42.

- Doffinger R, Helbert MR, Barcenás-Morales G (2004). Autoantibodies to interferon- $\gamma$  in a patient with selective susceptibility to mycobacterial infection and organ-specific autoimmunity. *Clin Infect Dis* 38:e10–4.
- Eloff JN (1998). Which extractant should be used for the screening and isolation of antimicrobial components from plants. *J Ethnopharmacol* 60: 1-8.
- Espinal MA, Laszlo A, Simonsen L (2001). Global trends in resistance to anti-tuberculosis drugs. World Health Organization, Geneva, 1294–1303.
- Estrada A, Katselis GS, Laarveld B, Barl B (2000). Isolation and evaluation of immunological adjuvant activities of saponins from *Polygala senega*. *Microbiology and Infectious Diseases* 23: 27 -43.
- Flynn JL, Chan J, Triebold KJ, Dalton DA, Stewart TA, Bloom BR (1993). An essential role for interferon- $\gamma$  in resistance to *Mycobacterium tuberculosis* infection. *J. Exp. Med.* 178: 2249–2254.
- Gautam R, Saklani A, Jachak SM (2007). Indian medicinal plants as a source of antimycobacterial agents. *Journal of Ethnopharmacology* 110: 200 – 234.
- Gibbons S (2005). Plants as a source of bacterial resistance modulators and anti-infective agents. *Phytochemistry Reviews* 4: 63 – 78.
- Gillespie SH, Morrissey I, Everett D (2001). A comparison of the bactericidal activity of quinolone antibiotics in a *Mycobacterium fortuitum* model. *Journal of Medical Microbiology* 50: 565-570.
- Ginsburg AS, Grosset JH, Bishai WR (2003). Fluoroquinolones, tuberculosis, and resistance. *Lancet Infect Dis* 3: 432-442.

- Goldsack L, Kirman JR (2007). Half-truths and selective memory: Interferon gamma, CD4+ T cells and protective memory against tuberculosis. *Tuberculosis* 87: 465 – 473.
- Goldsby RA, Kindt TJ, Osborne BA (2000). *Kuby Immunology*. W.H. Freeman and Company, New York, p. 239.
- Gutierrez-Lugo M-T, Bewley CA (2008). Natural products, small molecules, and genetics
- Hirsch CS, Toossi Z, Johnson JL, Luzze H, Ntambi L, Peters P, McHugh M, Okwera A, Joloba M, Mugenyi P (2001). Augmentation of apoptosis and interferon- $\gamma$  production at sites of active *Mycobacterium tuberculosis* infection in human tuberculosis. *J. Infect. Dis.* 183: 779–788.
- Johnson, Q, Weitz F, Mabusela W, Klaasen J, Coldrey N, Gabrielse V (2002). The effect of wild garlic extracts on AIDS related fungal and bacterial pathogens. XIV International AIDS Conference, Spain.
- Jones KDJ, Hesketh T, Yudkin J (2008). Extensively drug-resistant tuberculosis in sub-Saharan Africa: an emerging public-health concern. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 102: 219-224.
- Jouanguy E, Altare F, Lamhamedi S (1996). Interferon-gamma receptor deficiency in an infant with fatal bacille Calmette–Guerin infection. *N Engl J Med* 335:1956–61.
- Kamijo R, Le J, Shapiro D (1993). Mice that lack the interferon-gamma receptor have profoundly altered responses to infection with *Bacillus Calmette–Guerin* and subsequent challenge with lipopolysaccharide. *J Exp Med* 178:1435–40.

- Kirchner H, Kleinicke C, Digel W (1982). A whole-blood technique for testing production of human interferon by leukocytes. *J. Immunol. Meth.* 48: 213.
- Korbel DS, Schneider B, Schaible UE (2008). Innate immunity in tuberculosis: myths and truths. *Microbes and Infection* doi:10.1016/j.micinf.2008.07.039 Article in press.
- Kursar M, Koch M, Mittrucker HW, Nouailles G, Bonhagen K, Kamrdat T, Kaufmann SHE (2007). *Journal of Immunology* 178: 2661 – 2665.
- McGaw LJ, Lall N, Meyer JJM, Eloff JN (2008). The potential of South African plants against *Mycobacterium* infections. *Journal of Ethnopharmacology* 119: 482-500.
- Mediratta PK, Sharma KK, Singh S (2002). Evaluation of immunomodulatory potential of *Ocimum sanctum* seed oil and its possible mechanism of action. *Journal of Ethnopharmacology* 80: 15 – 20.
- Mitscher LA, Baker WR (1998). A search for novel chemotherapy against tuberculosis amongst natural products. *Pure Appl Chem* 70: 365-371.
- Mukadi YD, Maher D, Harries A (2001). Tuberculosis case fatality rates in high HIV prevalence populations in sub-Saharan Africa. *AIDS* 15: 143–152
- Ottenhoff TH, Verreck FA, Hoeve MA, van de Vosse E (2005). Control of human host immunity to mycobacteria. *Tuberculosis* 85: 53–64.
- Pauli GF, Case RJ, Inui T, Wang Y, Cho S, Fischer NH, Franzblau SG (2005). New perspectives on natural products in TB drug research. *Life Sciences* 78: 485-494.
- Pirofski LA, Casadevall A (2006). Immunomodulators as an antimicrobial tool. *Current Opinion in Microbiology* 9: 489 – 495.

- Rios JL, Recio MC, Villar A (1998). Screening methods for natural products with antimicrobial activity: a review of the literature. *J Ethnopharmacol* 23:127-149.
- Sendl A (1995). *Allium sativum* and *Allium ursinum*: Part 1. Chemistry, analysis, history, botany. *Phytomedicine* 4: 323-339.
- Spellberg B, Powers JH, Brass EP, Miller LG, Edwards JE (2004). Trends in antimicrobial drug development: implications for the future. *Clin Infect Dis* 38: 1279-1286.
- Tegos G, Stermitz FR, Lomovskaya O, Lewis K (2002). Multidrug pump inhibitors uncover remarkable activity of plant antimicrobials. *Antimicrob Agents Chemother* 46: 3133-3141.
- Thamburan S, Klaasen J, Mabusela WT, Cannon JF, Folk W, Johnson Q (2006). *Tulbaghia alliacea* Phytotherapy: A Potential Anti-infective Remedy for Candidiasis. *Phytother Res* 20: 844-850
- Tiwari U, Rastogi B, Singh P, Saraf DK, Vyas SP (2004). Immunomodulatory effects of aqueous extract of *Tridax procumbens* in experimental animals. *Journal of Ethnopharmacology* 92: 113 – 119.
- Tzianabos AO (2000). Polysaccharide immunomodulators as therapeutic agents: structural aspects and biological function. *Clinical Microbiology Reviews* 13: 523 – 533.
- Uchida Y, Takahashi T, Sato N (1975). The characteristics of the antibacterial activity of garlic. *Jpn J Antibiot* 28: 638-642.



- Van Crevel R, Ven-Jongekrijg J, Netea MG, de Lange W, Kullberg BJ, van der Meer JWM (1999). Disease-specific ex vivo stimulation of whole blood for cytokine production: applications in the study of tuberculosis. *Journal of Immunological Methods* 222: 145 – 153.
- Van Wyk B, Van Oudtshoorn B, Gericke N (2000). *Medicinal Plants of South Africa*. Briza Publications: South Africa.
- Vanden Berghe DA, Vlietinck AJ (1991). Screening methods for antibacterial and antiviral agents from higher plants. In Dey PM, Harborne JB. *Methods in Plant Biochemistry* (vol 6). Academic Press Limited: London.
- Vankayalapati R, Wizel B, Weis SE, Klucar P, Shams H, Samten B, Barnes PF (2003). Serum cytokine concentrations do not parallel *Mycobacterium tuberculosis*-induced cytokine production in patients with tuberculosis. *Clin. Infect. Dis* 36: 24–28.
- Veenstra H, Crous I, Brahmabhatt S, Lukey P, Beyers N, van Helden PD, Walzl G (2007). Changes in the kinetics of intracellular IFN- $\gamma$  production in TB patients during treatment. *Clinical Immunology* 124: 336–344
- Wagner H, Blatt S (1996). *Plant Drug Analysis – A Thin Layer Chromatography Atlas* (2<sup>nd</sup> edn). Springer-Verlag: Berlin Heidelberg.
- Walsh C (2000). Molecular mechanisms that confer antibacterial drug resistance. *Nature* 406: 775-781.
- WHO (2008). WHO Report: Global tuberculosis control. WHO, Geneva.
- Zager EM, McNerney R (2008). Multidrug-resistant tuberculosis. *BioMedCentral Infectious Diseases* 8: 10.



## **CHAPTER 4**



UNIVERSITY of the  
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***Tulbaghia alliacea* phytotherapy:**

**A potential anti-cancer remedy**

## 4.1. Abstract

*Tulbaghia alliacea* is used in traditional medicine as an anti-cancer remedy. However the use of this plant to treat cancer has not yet been tested in a scientific study. Hence the objective of this work was to evaluate the potential use of extracts of this plant to treat cancer. Since the cytotoxic effects of anti-cancer drugs is often associated with the induction of apoptosis, this study aimed to evaluate the anti-cancer activity of the plant extracts by assessing the ability of the extracts to induce apoptosis during in vitro cell culture conditions. Two extracts (TAA or aqueous extract and TAC or chloroform extract) were prepared. Several cell lines including five human cancer cell lines were treated with these plant extracts and the induction of apoptosis was assessed using various assays that detect biochemical changes that have been associated with the activation of apoptosis. Using phosphatidyl serine externalisation as a marker of apoptosis this study shows that both extracts induce apoptosis in three of these cell lines (Jurkat, MCF7 and MG63) while the other two cell lines (HeLa and H157) were completely resistant to the effects of the extracts. Three other markers of apoptosis, which included, caspase-3 cleavage, mitochondrial depolarisation, and DNA fragmentation, was used to further characterise the induction of apoptosis in Jurkat cells. All three these assays demonstrated that the two plant extracts contained compounds with pro-apoptotic activity. Furthermore a comparison between the two extracts shows that there is a significant difference in the activity of the two extracts with the aqueous extract (TAA) showing higher pro-apoptotic activity than the chloroform extract (TAC). This study also demonstrates that mechanism of apoptosis induction is likely to involve the mitochondrial pathway. An investigation into gene expression levels by real time PCR

revealed that the plant extracts induce the expression of several genes (*Bax*, *caspase-3*, and *caspase-9*) previously implicated in apoptosis, which further supports the idea that the extracts induce apoptosis via the mitochondrial pathway. This study demonstrated that extracts of *T. alliacea* induce apoptosis in human cancer cells during in vitro conditions and therefore provide supporting evidence that this plant may be used as remedy to treat certain forms of cancer.

**Keywords:** Garlic, *Tulbaghia alliacea*, apoptosis, cancer, cell cycle, caspase-3, DNA fragmentation, mitochondrial depolarization.



## 4.2. Introduction

Apoptosis is a biochemical process by which cells die through a highly regulated program resulting in the removal of damaged or unwanted tissue. Apoptosis make sure that cellular homeostasis is maintained and prevents the development of cancer (Lowe and Lin, 2000). Apoptosis is associated with distinct biochemical and morphological changes (Lawen, 2003) characterized by chromatin condensation, membrane blebbing, cell shrinkage, and DNA fragmentation (Wyllie, Kerr, Currie, 1980). Numerous studies have demonstrated that the evasion of apoptosis is one of the most important mechanisms of uncontrolled growth of tumour cells (Hager and Hanahan, 1999; Hanahan and Weinberg, 2000; Igney and Krammer, 2002; Schulze-Bergkamen and Krammer, 2004). The elimination of neoplastic cells through the activation of apoptosis thus represents a protective mechanism against cancer (Owuor and Kong, 2002) and considerable attention has been given to the manipulation of apoptosis as a novel and promising strategy for cancer chemoprevention and therapy (Cal, Garban, Jazirehi, Yeh, Mizutani, Bonavida, 2003; Dong, 2003; Fesus, Szondy, Uray, 1995; Holzman, 1996; Krzystyniak, 2002). Several anti-cancer drugs have been shown to exert their effects through the activation of apoptosis and a substantial number of these drugs have been isolated from medicinal plants.

*Allium sativum*, a member of the Lily family and commonly known as garlic, has been cultivated as a food plant for over 10, 000 years. Egyptian records dating back to about 1550 BC make reference to garlic as a remedy for a variety of diseases (Block, 1985) and

more recently epidemiological studies showed that the enhanced consumption of garlic is closely related to reduced risk of cancer incidence (Buiatti, Palli, Decarli, Amadori, Avellini, Bianchi, Biserni, Cipriani, Cocco, Giacosa, Mrubini, Puntoni, Vindig, Frumeni, Blot, 1989; Fleischauer and Arab, 2001; Haenszel, Kurihara, Segi, Lee, 1972; Hsing, Chokkalingam, Goa, Madigan, Deng, Gridley, Fraumeni, 2002; Steinmetz, Kushi, Bostick, Folsom, Potter, 1994; You, Blot, Chang, Ershow, Yang, An, Henderson, Fraumeni, Wang, 1989). The anti-tumour activity of this plant has been associated with the induction of apoptosis in tumour cells (Knowles and Milner, 2001; Ledezma, Apitz-Castro, Cardier, 2004; Oommen, Anto, Srinivas, Karunakaran, 2004; Robert, Mouille, Mayeur, Michaud, Blachier, 2001; Wu, Sheen, Chen, Tsai, Lii, 2001). The major active principles in garlic, most of which are organosulfur compounds have been shown to inhibit the proliferation of cancer cells, and some of them induced apoptosis in tumour cells of different tissue origin (Dirsch, Gerbes, Vollmar, 1998; Kwon, Yoo, Ryu, Yang, Rho, Kim, Park, Kim, Park, 2002; Pinto, Lapsia, Shah, Santiago, Kim, 2001; Shirin, Pinto, Kawabata, Soh, Delohery, Moss, Murty, Rivlin, Holt, Weinstein, 2001; Sigounas, Hooker, Anagnostou, Steiner, 1997).

Another member of the indigenous garlic family *T. violacea*, also known as wild garlic, is used in traditional medicine as a remedy for the treatment of fever, asthma, hypertension and oesophageal cancer (Hutchings, Scott, Lewis, 1996; Van Wyk and Wink., 2004). A study by Bungu, Frost, Brauns and Van de Venter (2006), demonstrated that extracts of *T. violacea* exhibited anti-proliferative and pro-apoptotic effects in four human cancer cell lines. It was previously shown that another species of *Tulbaghia*, *T. alliacea* has

significant anti-fungal activity against the medically important opportunistic pathogen *C. albicans* (Thamburan *et al.*, 2006). The anti-fungal agent was identified as *marasmicin* in a chloroform extract of *T. alliacea*. The chemical shift values for *marasmicin* isolated from *T. alliacea* were in agreement with those reported for the same compound, previously isolated from the related species, *T. violacea* (Kubec *et al.* 2002). In view of the fact that both *T. alliacea* and *T. violacea* belong to the same Family (*Alliaceae*) and Genus (*Tulbaghia*) (Van Wyk *et al.*, 1997), and since similar chemical characteristics have been reported for both plants (Thamburan *et al.*, 2006; Kubec *et al.* 2002), the potential anti-tumour properties of *T. alliacea* extracts was investigated through evaluating the ability of these extracts to induce apoptosis in several human cancer cell lines.



## **4.3. Materials and Methods**

### **4.3.1. Plant material**

Bulbs of *Tulbaghia alliacea* were obtained from Rastafarian herbal traders in Cape Town, South Africa. The species was verified by a botanist at the University of the Western Cape Herbarium where a voucher specimen (Thamburan001) was deposited.

### **4.3.2. Preparation of plant extracts**

Bulbs of *T. alliacea* were washed with distilled water, peeled and chopped into small pieces (Eloff, 1998). Aqueous and chloroform extracts were prepared by soaking the chopped plant material in the solvents overnight, and then vacuum filtering it. After filtration, the solvents were evaporated, and the extracts reconstituted in DMSO and stored at -20°C.

### **4.3.3. Cell culture**

All available laboratory cell lines were used in this study. Chinese Hamster Ovary (CHO) cells were cultured in Hams F-12 medium containing 10% (v/v) foetal calf serum and 0.2% (v/v) streptomycin-penicillin. MCF7 (human breast adenocarcinoma), MG63 (human osteosarcoma), H157 (human small lung cell carcinoma) and HeLa (human cervical carcinoma) cells were cultured in DMEM medium supplemented with 10% (v/v) foetal calf serum, and 0.2% (v/v) streptomycin-penicillin. Jurkat (human leukemia T cells) were cultured in RPMI medium with GlutaMAX-1, 10% (v/v) foetal calf serum,

and 0.2% (v/v) streptomycin-penicillin. All cell lines were maintained at 37°C in an atmosphere of 5% CO<sub>2</sub>. Cells were plated in 6-well tissue culture plates at a cell density of  $2.5 \times 10^5$  cells per well, or in 24-well tissue culture plates at a density of  $1.5 \times 10^5$  cells per well. After 24 h the medium was replaced with medium containing the test extracts. The cells were treated for the indicated times, after which the cells were harvested and the extent of apoptosis was assessed.

#### **4.3.4. APOPercentage™ assay**

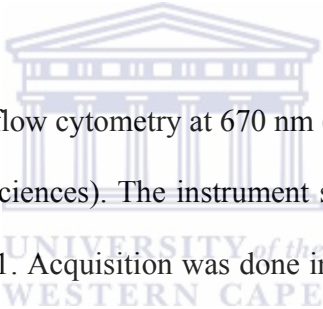
##### **4.3.4.1. Microscopy**

CHO cells were cultured in 24-well cell culture plates for 24 h; thereafter the cells were treated with increasing doses (0, 2, 4, 6, 8, and 10mg/ml) of *T. alliacea* extracts. Following a 6 h treatment, the cells were stained with APOPercentage™ dye (Biocolor Ltd.) as described by the manufacturer. Briefly, the medium containing the plant extracts was removed and replaced with medium containing the APOPercentage™ dye. The cells were incubated for 30 min at 37°C. The dye was removed and the cells were washed with PBS to remove excess dye. The cells were evaluated by light microscopy. Photographs were taken with a Leica digital camera using a Nikon inverted light microscope at 20 × magnification.



#### **4.3.4.2. Flow cytometry**

The cells were plated at a cell density of  $1.5 \times 10^5$  cells per well in a 24-well cell culture plate, and were cultured for 24 h before treatment with either aqueous (TAA) or chloroform (TAC) extracts of *T. alliacea*. Staurosporine (Stp), a known inducer of apoptosis was used as a positive control. Following a 6 h treatment, the cells were removed using trypsin, washed with PBS, and stained with APOPercentage™ dye for 30 min at 37°C. The cells were washed with PBS and analysed by FACS as described previously (Meyer *et al.*, 2008).



Cell staining was measured by flow cytometry at 670 nm (FL3) using a Becton Dickinson FACScan instrument (BD Biosciences). The instrument settings were as follow: voltage set at 470 and AmpGain set at 1. Acquisition was done in Log mode. A minimum of 10,000 cells per sample were acquired and analysed using CELLQuest PRO software (BD Biosciences).

#### **4.3.5. Caspase-3 assay**

The activation of caspase-3 was detected using a monoclonal antibody specific for the cleaved form of caspase-3 (BD Biosciences). Jurkat cells were seeded in 6-well tissue culture plates. The cells were treated with 6mg/ml of *T. alliacea* extracts or Stp (positive control) and incubated for different time periods (6, 12, 24 h). After incubation, the cells were washed with PBS, and re-suspended in Cytofix/Cytoperm™ (BD Biosciences).

Following 20 min of incubation on ice, the cells were washed twice with Perm/Wash™ buffer (BD Biosciences) and stained for 30 min at room temperature with a FITC-conjugated monoclonal antibody specific for active caspase-3. Cell staining was measured by flow cytometry at 530 nm using a Becton Dickinson FACScan instrument (BD Biosciences). A minimum of 10, 000 cells per sample were acquired and analyzed using CELLQuest PRO software (BD Biosciences).

#### **4.3.6. TMRE (Tetramethylrhodamine) assay**

Jurkat cells were plated in 6-well cell culture plates. The cells were treated with 6mg/ml of *T. alliacea* extracts or staurosporine (positive control) and incubated for different time periods (30 min, 1 h, and 2 h). The cells were incubated for 30 min in medium containing 1 µM TMRE dye (Molecular Probes). The cells were washed with PBS and analyzed by flow cytometry using a Becton Dickinson FACScan instrument (BD Biosciences). Cell fluorescence was measured at 670 nm (FL3). A minimum of 10, 000 cells per sample were acquired and analysed using CELLQuest PRO software (BD Biosciences).

### **4.3.7. Terminal deoxynucleotide transferase dUTP Nick**

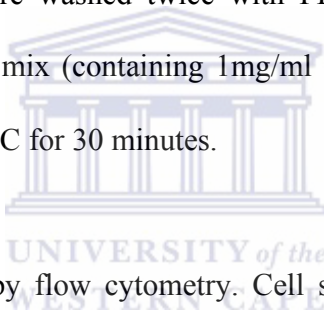
#### **End Labeling (TUNEL) assay**

To analyse the occurrence of DNA fragmentation, the Terminal deoxynucleotide transferase dUTP Nick End Labeling (TUNEL) assay (BD Biosciences) was used. Jurkat cells were plated in 6-well cell culture plates at a cell density of  $2.5 \times 10^5$  cells per well. The cells were treated with 6mg/ml of *T. alliacea* extracts or staurosporine (positive control) and incubated for different time periods (6, 12, 18, 24 h). After incubation, the cells were washed twice with PBS and fixed for 30 min in 1% paraformaldehyde.

The cells were then washed twice with PBS and permeabilized for at least 48 h in 70% ethanol at -20°C. Subsequently, the cells were labelled with FITC-dUTP and propidium iodide (PI) as described in the manufacturer's manual (BD Biosciences). Cell staining was measured by flow cytometry at 530 nm and 585 nm using a Becton Dickinson FACScan instrument (BD Biosciences). A minimum of 10, 000 cells per sample were acquired and analysed using CELLQuest PRO software (BD Biosciences). Dual parameter analysis (DNA area signal on the Y-axis and DNA width on the X-axis) was used to exclude DNA doublet events.

### 4.3.8. Cell cycle analysis

Jurkat cells were plated in 6-well cell culture plates at a cell density of  $2.5 \times 10^5$  cells per well. The cells were treated with 6mg/ml of *T. alliacea* extracts and incubated for different time periods (24h, 48h, and 72h). A negative control sample (untreated cells) was prepared for each time point. After treatment, the cells were washed twice with PBS and fixed for 30 min in 1% paraformaldehyde. Thereafter the cells were washed twice with PBS again and permeabilized for at least 48 h in 70% ethanol at  $-20^{\circ}\text{C}$ . After permeabilization, the cells were washed twice with PBS. The cell pellets were then resuspended in 1ml PI master mix (containing 1mg/ml Propidium iodide and 10mg/ml RNase A), and incubated at  $37^{\circ}\text{C}$  for 30 minutes.



Samples were then analyzed by flow cytometry. Cell staining was measured by flow cytometry at 530 nm using a Becton Dickinson FACScan instrument (BD Biosciences). A minimum of 10, 000 cells per sample were acquired and analyzed using CELLQuest PRO software (BD Biosciences). Dual parameter analysis (DNA area signal on the Y-axis and DNA width on the X-axis) was used to exclude DNA doublet events.

### 4.3.9. RNA isolation

Jurkat cells were plated in 6-well cell culture plates at a cell density of  $2.5 \times 10^5$  cells per well. The cells were treated with 6mg/ml of *T. alliacea* extracts and incubated for different time periods (6, 12, and 24 h). Total RNA was isolated from the cells using TRIzol LS (Gibco BRL). Cells were homogenized by the addition of 0.3 - 0.4ml TRIzol per 10 cm<sup>3</sup> of culture flask. The homogenate was transferred to a 15ml conical centrifuge tube and 0.2ml chloroform per 0.75ml of TRIzol LS reagent was added. The solution was vigorously shaken for 15 sec and allowed to incubate at room temperature for 10 min. The sample was centrifuged for 30min at  $3000 \times g$  at 4°C to allow phase separation. The top aqueous phase was transferred to a new conical tube and mixed with 0.5ml isopropyl alcohol per 0.75ml of TRIzol LS reagent. The sample was incubated at room temperature for 15min and centrifuged for 30min at  $3000 \times g$  at 4°C. The resulting pellet was washed with 1ml 70% ethanol per 0.75ml of TRIzol LS reagent. The pellet was allowed to dry at room temperature and resuspended in 100µl DEPC treated water. The RNA was stored as 20µl aliquots at -80°C.

### 4.3.10. cDNA synthesis

The first strand cDNA synthesis (AMV) kit for RT (Roche Applied Science) was used.

The reaction for the first strand synthesis consisted of the following reagents:

**Table 1.** Reagents used for first strand cDNA synthesis.

Reagent	Volume per sample	Final Concentration
10× Reaction Buffer	2 $\mu$ l	1×
25mm MgCl <sub>2</sub>	4 $\mu$ l	5mM
10mm dNTPs	2 $\mu$ l	1mM
Oligo dT-Primer	Variable	1 picoM
RNase inhibitor	1 $\mu$ l	50 Units
AMV reverse transcriptase	0.8 $\mu$ l	20 Units
Sterile water	To final volume of 20 $\mu$ l	
RNA	Variable	1 $\mu$ g

The mixture was thoroughly mixed and centrifuged briefly. The reaction mixture was incubated at 25°C for 10 minutes and then 42°C for 60 minutes. The AMV reverse transcriptase was denatured by incubating the reaction mixture at 99°C for 5 minutes. The first strand cDNA preparation was stored at -20°C.

### 4.3.11. Real-Time Quantitative RT-PCR

A standard PCR mixture contained 4 $\mu$ l of LightCycler FastStart DNA Master<sup>PLUS</sup> SYBR Green Reaction Mix (Roche Applied Science) 200ng of the pre-synthesized cDNA was used as template and 0.5 $\mu$ M of each primer in a final volume of 20 $\mu$ l. The final concentration of MgCl<sub>2</sub> was adjusted to 3mM. A negative control was included which contained the same mixture with the cDNA replaced by water. The PCR mixture was subsequently transferred to a Lightcycler® capillary tube (Roche Applied Science) in a pre-cooled adapter block and centrifuged at 700  $\times$  g for 5 sec. The capillaries were placed into the LightCycler carousel instrument (Roche Applied Science) and cycled through the following parameters:



**Table 2.** PCR conditions used in Real-Time Quantitative PCR

<b>Program</b>	<b>Step</b>	<b>Temp.</b>	<b>Time</b>	<b>Temperature Transition Rate</b>	<b>Fluorescence acquisition</b>
Pre-incubation		95°C	10 min	20°C/sec	None
Amplification	Denaturation	95°C	10 sec	20°C/sec	None
Amplification	Annealing	60°C	10 sec	20°C/sec	None
Amplification	Extension	72°C	10 sec	20°C/sec	Single
Melting curve	Denaturation	95°C	0	20°C/sec	None
Melting curve	Annealing	65°C	10 sec	20°C/sec	None
Melting curve	Melting	95°C	0	0.1°C/sec	Continuous
Cooling		40°C	30 sec	20°C/sec	None

Note: The Amplification program was repeated for 40 cycles.

### **4.3.12. Primer sequences**

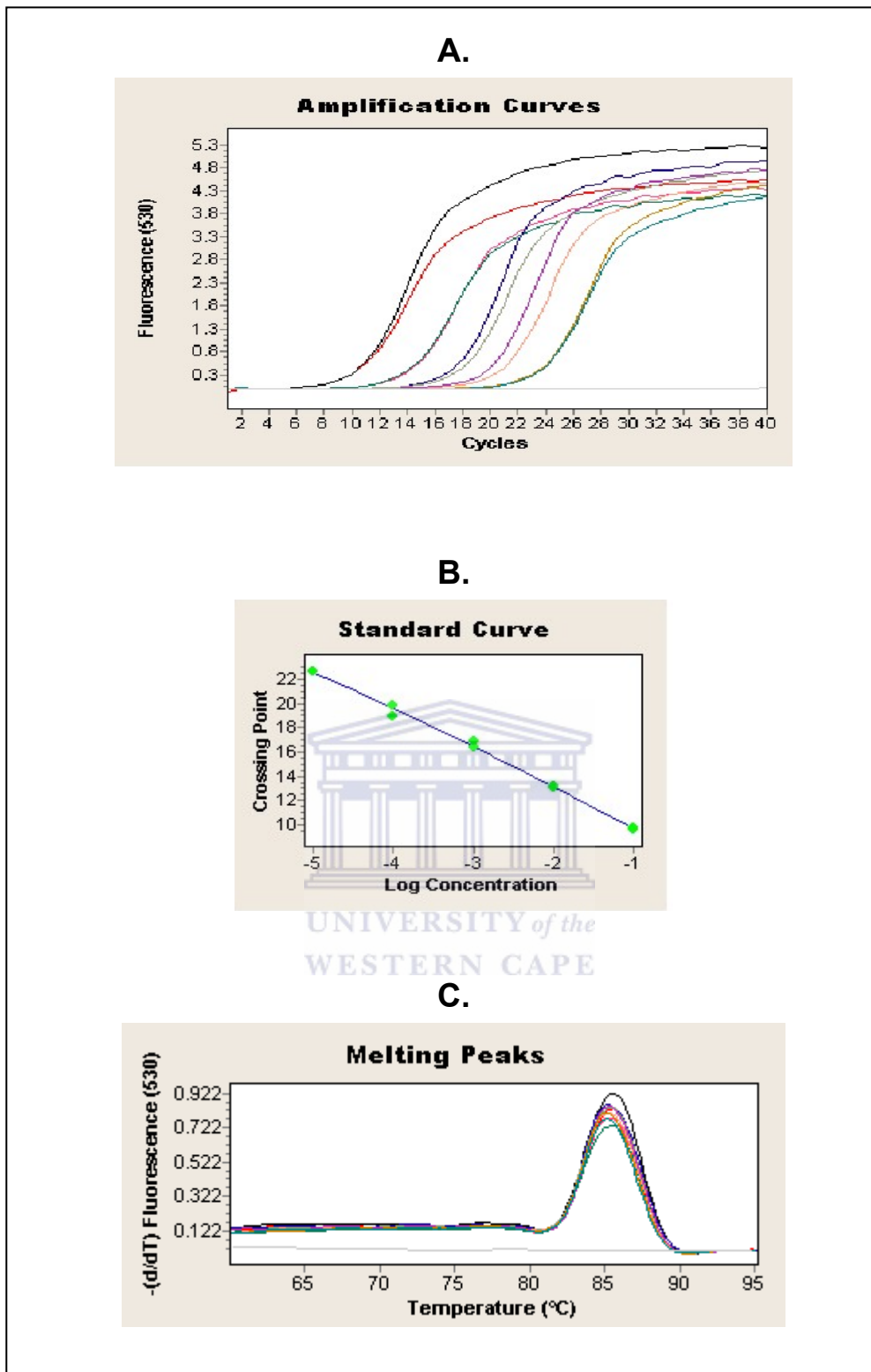
The primer sequences were designed using the Primer3 program (<http://Frodo.wi.mit.edu>). All primer sets were designed in the exon regions across intron-exon boundaries. This was done to eliminate additional products that could be amplified from genomic DNA. Primers were tested by performing a BLAST search against the Human Genome reference database.



1. *Caspase-3* Forward: 5'- AAC CAG ATC ACA AAC TTC TGC-3'
2. *Caspase-3* Reverse: 5'- TGG AGT CCA GTG AAC TTT CTT C-3'
3. *Caspase-8* Forward: 5'- TTC TCC CTA CAG GGT CAT GC-3'
4. *Caspase-8* Reverse: 5'- GCA GGC TCA AGT CAT CTT CC-3'
5. *Caspase-9* Forward: 5'- AGG ATA TTC AGC AGG CAG GA -3'
6. *Caspase-9* Reverse: 5'- GGC CTG TGT CCT CTA AGC AG -3'
7. *Bax* Forward: 5'- GCC CTT TTG CTT CAG GGT TT -3'
8. *Bax* Reverse: 5'- TCC AAT GTC CAG CCC ATG AT-3'
9. *Bcl-2* Forward: 5'- GAC AGA AGA TCA TGC CGT CC-3'
10. *Bcl-2* Reverse: 5'- GGT ACC AAT GGC ACT TCA AG-3'
11. *GAPDH* Forward: 5'- ACC CAC TCC TCC ACC TTT G-3'
12. *GAPDH* Reverse: 5'- CTC TTG TGC TCT TGC TGG G-3'



To assess the efficiency of PCR amplification, a serial dilution of the cDNA was prepared and used as template for the amplification of the house-keeping gene, *GAPDH*. The house-keeping gene is not expected to be affected by the experimental condition (i.e. treatment with the plant extracts). The expression levels of the genes that were of interest (*Bax*, *Bcl2*, *caspase-3*, *caspase-8*, *caspase-9*) were aligned to the expression levels of *GAPDH* in order to normalize cellular expression levels of these genes. Figure 1A shows the amplification curves for *GAPDH* using cDNA that was serially diluted 10-fold (starting at 200 ng) as the template for the PCR. Figure 1B shows a standard curve using the amplification cycle number at the crossing point were amplification plateau off and logarithmic value of the cDNA concentration generated for the amplification curves of

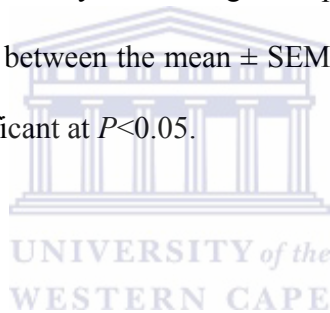


**Fig 1. Evaluating the efficiency of PCR amplification**

*GAPDH*. The efficiency of PCR amplification was calculated using the Second Derivative Maximum Method (Lightcycler 4.5, Roche Applied Science). PCR efficiencies between 91 and 96% were considered to be sufficient. This allowed for very accurate comparison of expression ratios between the *GAPDH* and the target genes. Figure 1C shows the melting peak analysis for *GAPDH* produced from the serially diluted cDNA. This type of analysis was performed for all 6 PCR primer pairs.

### **4.3.13. Statistical analysis**

Effects were statistically compared by conducting an unpaired *t*-test using the Microsoft Excel programme. Differences between the mean  $\pm$  SEM (standard error of the mean) of samples were considered significant at  $P < 0.05$ .

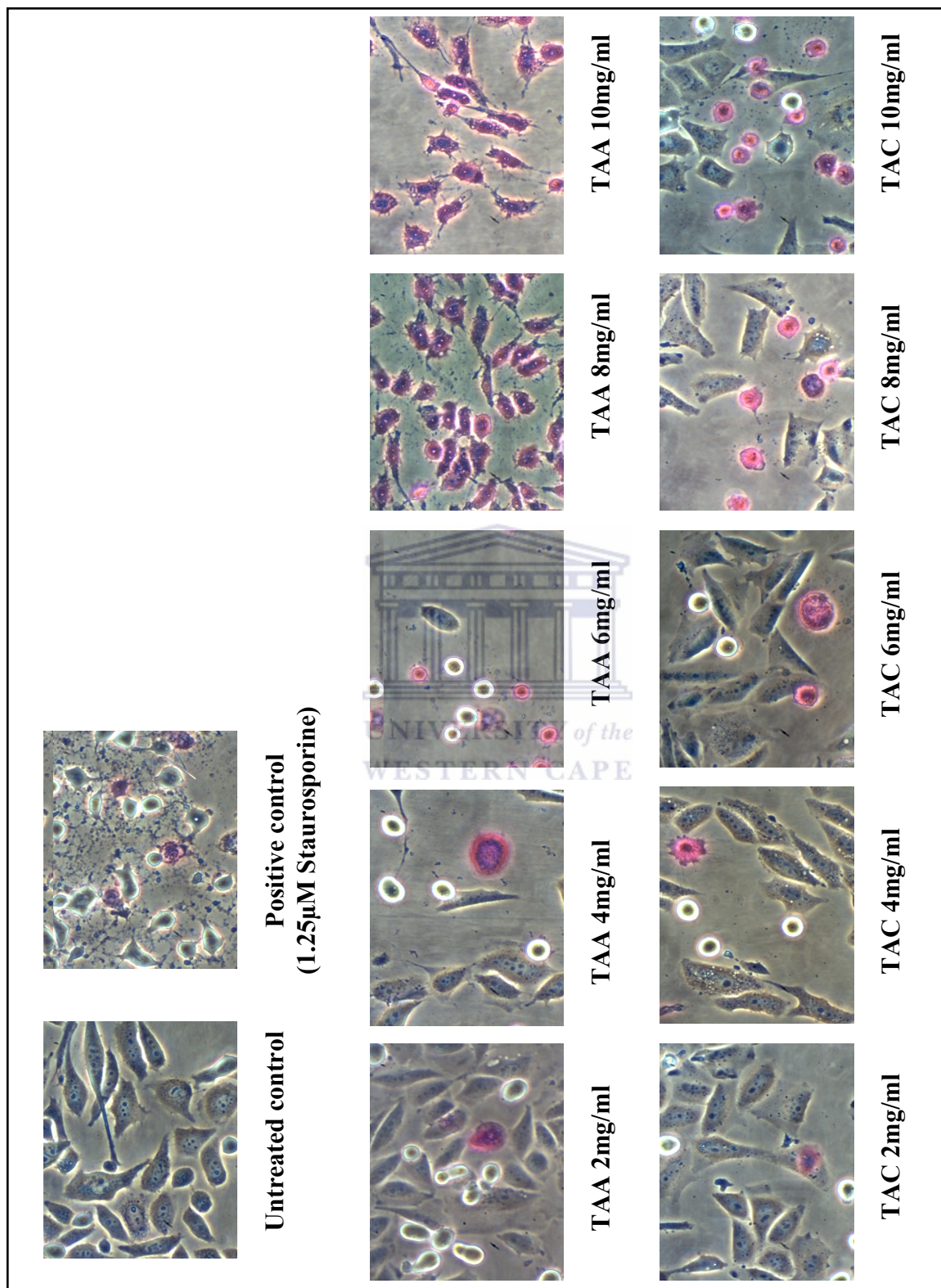


## 4.4. RESULTS

### 4.4.1. APOPercentage™ assay

#### 4.4.1.1. Dose-response study on CHO cells

A dose-response study was undertaken to establish if *T. alliacea* extracts had any pro-apoptotic activity and to determine the LD<sub>50</sub> of these extracts. The pro-apoptotic activity of the extracts was evaluated using the APOPercentage™ assay as described in Section 2.4.1. CHO cells were treated for 6h with increasing doses (0 – 10mg/ml) of the extracts. Two extracts of *T. alliacea*; an aqueous extract (TAA) and a chloroform extract (TAC) were prepared and tested. Staurosporine, a known inducer of apoptosis was used as a positive control. The cells were stained with the APOPercentage™ dye and evaluated under a light microscope (Figure 2) The extent of apoptosis induction was evaluated by flow cytometry as described in Section 2.4.2 and is shown in Figure 3.



**Fig 2.** CHO cells treated with increasing doses of *Tulbaghia alliacea* extracts and stained with APOPercentage dye

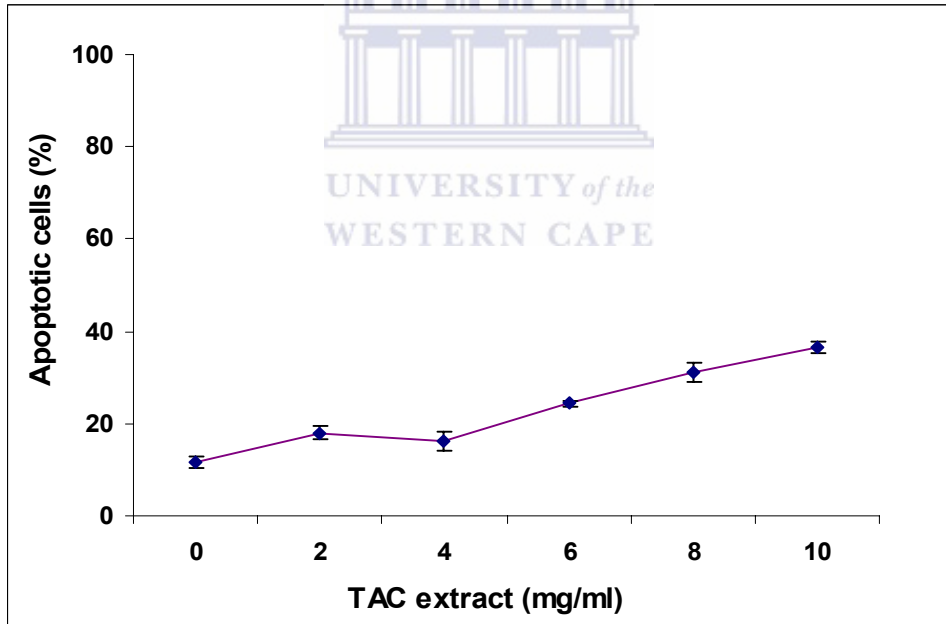
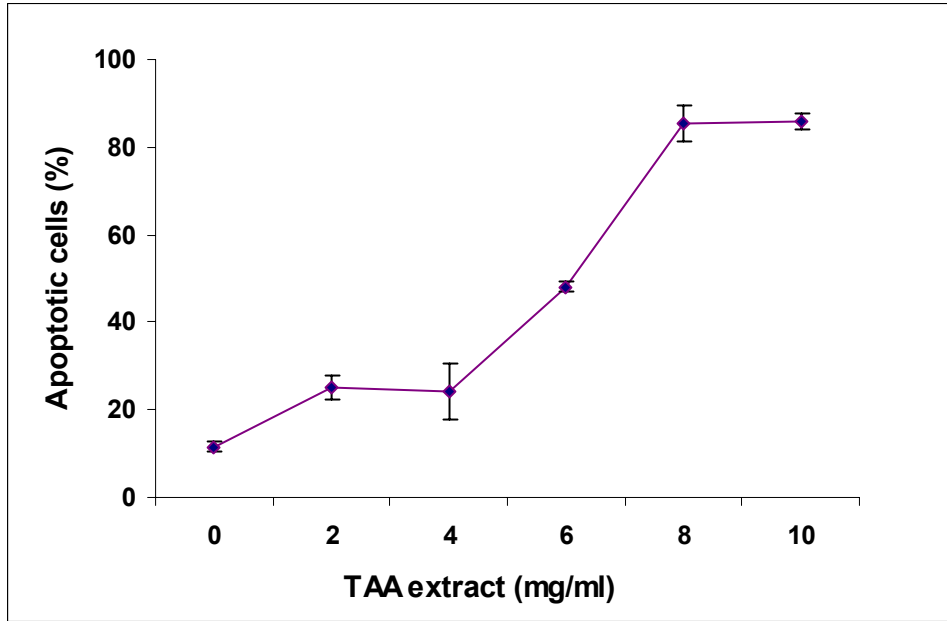
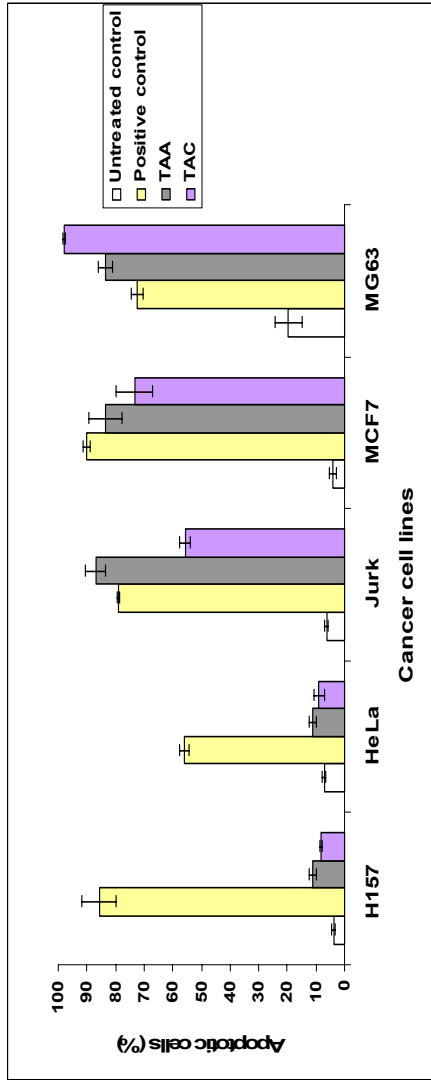


Fig 3. Dose-response apoptotic effect of *Tulbaghia alliacea* extracts on CHO cells

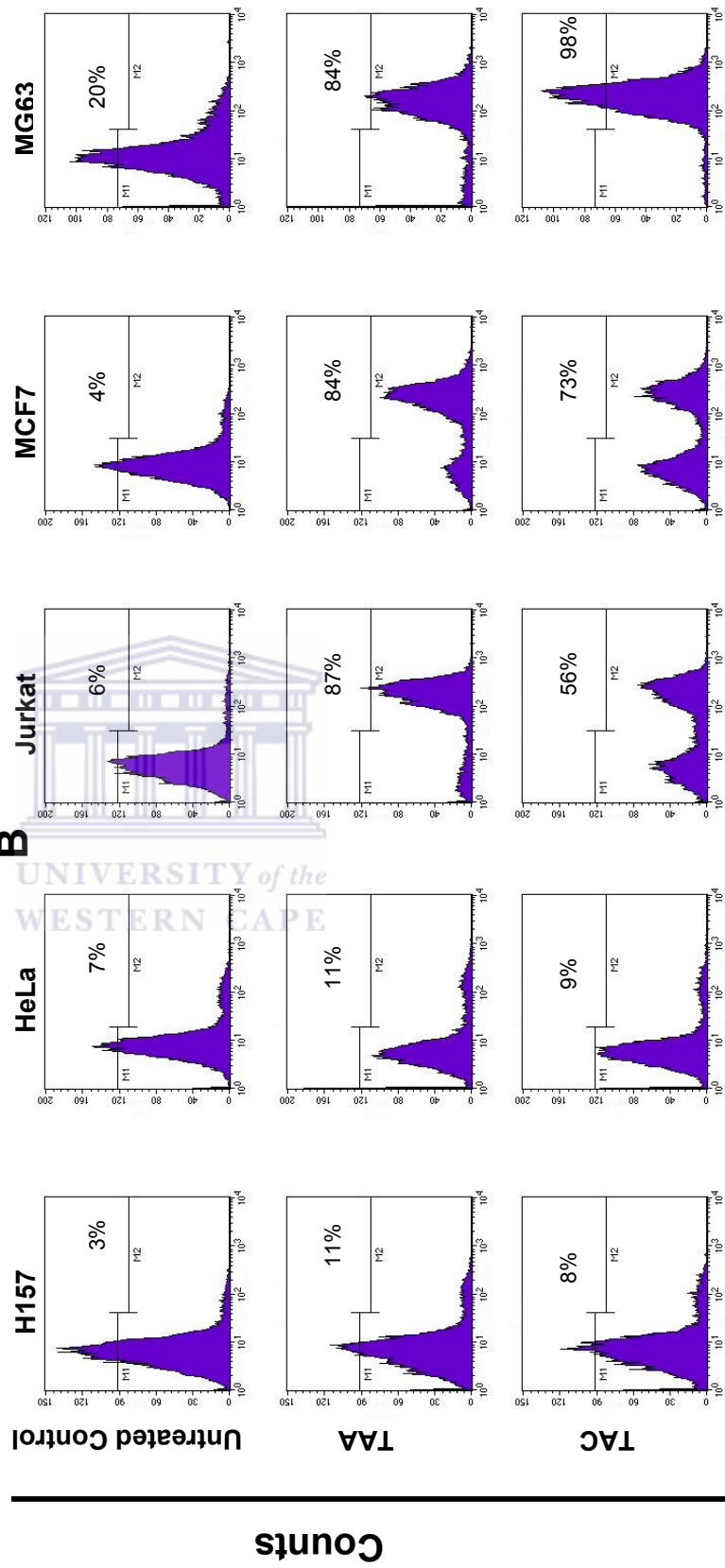
#### **4.4.1.2. Screening different human cancer cell lines for the induction of apoptosis**

The apoptosis inducing potential of *T. alliacea* extracts was evaluated in several human cancer cell lines (H157, HeLa, Jurkat, MCF7 and MG63). The cells were treated for 6h with TAA and TAC extracts (6mg/ml). The level of apoptosis was measured by flow cytometry using the *APOPercentage*<sup>TM</sup> assay as described in Section 2.4.2. These experiments were performed in triplicate and the results are shown in Figure 4. The histograms in Figure 4 are examples of the flow cytometric results that were acquired. The numbers on the histograms represent the number of events demarcated by the M2 marker and refer to the number of apoptotic cells staining positive for the *APOPercentage*<sup>TM</sup> dye, while M1 refers to the unstained viable cells. The activity of the TAA and TAC extracts were statistically analysed by conducting an unpaired *t*-test using the Microsoft Excel programme (Table 3). The untreated controls of the various cell lines were also compared to each other.

**A**



**B**



**APOPercentage**

**Fig 4. Apoptosis induction of *Tulbaghia alliacea* extracts in different cancer cell lines**



**Table 3.** The apoptosis inducing activity of *Tulbaghia alliacea* extracts on different cell lines

Cell line	Apoptotic cells (mean $\pm$ SEM)			Statistical significance ( <i>P</i> )		
	Untreated control (a)	Aqueous extract (TAA) (b)	Chloroform extract (TAC) (c)	ab	ac	bc
<b>CHO</b>	17 $\pm$ 1.06	31 $\pm$ 1.13	45 $\pm$ 1.51	0.0004	0.0001	0.0034
<b>H157</b>	4 $\pm$ 0.57	11 $\pm$ 1.33	8 $\pm$ 0.40	0.0146	0.0035	0.1595
<b>HeLa</b>	7 $\pm$ 0.65	11 $\pm$ 1.26	9 $\pm$ 1.99	0.0649	0.4767	0.4159
<b>Jurkat</b>	6 $\pm$ 0.63	87 $\pm$ 3.46	56 $\pm$ 1.89	0.0019	0.0016	0.0042
<b>MCF7</b>	4 $\pm$ 1.18	83 $\pm$ 5.69	73 $\pm$ 6.38	0.0053	0.0086	0.2977
<b>MG63</b>	20 $\pm$ 4.84	84 $\pm$ 2.41	98 $\pm$ 0.37	0.0013	0.0038	0.0270

Data for positive control [Staurosporine]: (apoptotic cells  $\pm$  SEM)

H157: 85  $\pm$  5.90

HeLa: 56  $\pm$  1.53

Jurkat: 79  $\pm$  0.58

MCF7: 90  $\pm$  1.16

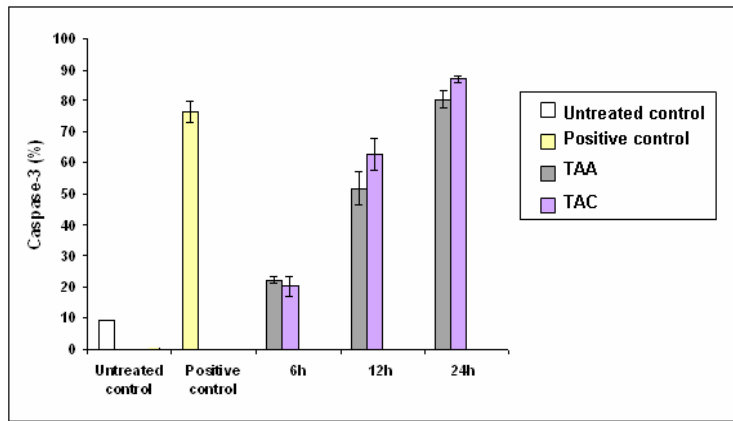
MG63: 72.33  $\pm$  2.03

### 4.4.2. Assessing the activation of caspase-3

Caspase-3 is a key regulator of apoptosis. However, the activation of apoptosis can occur independent of caspase-3 activation. The activation of caspase-3 was assessed using a monoclonal antibody specific for the cleaved form of caspase-3. Jurkat T cells were treated for different time points (6, 12 and 24 h) with TAA and TAC extracts (6mg/ml). Staurosporine was used as a positive control. The cells were fixed and stained with an anti-active caspase-3 antibody as described in Section 2.5. Cell fluorescence was measured by flow cytometry.

These experiments were performed in triplicate and the results are displayed in Figure 5A. The histograms in Figure 5B are examples of the flow cytometry results that was acquired. The numbers on the histograms (M2) refer to the number of cells staining positive for the anti-active caspase-3 antibody. M1 refers to the unstained viable cells. The activity of the TAA and TAC extracts were statistically analysed by conducting an unpaired *t*-test using the Microsoft Excel programme (Table 4).

A.



B.

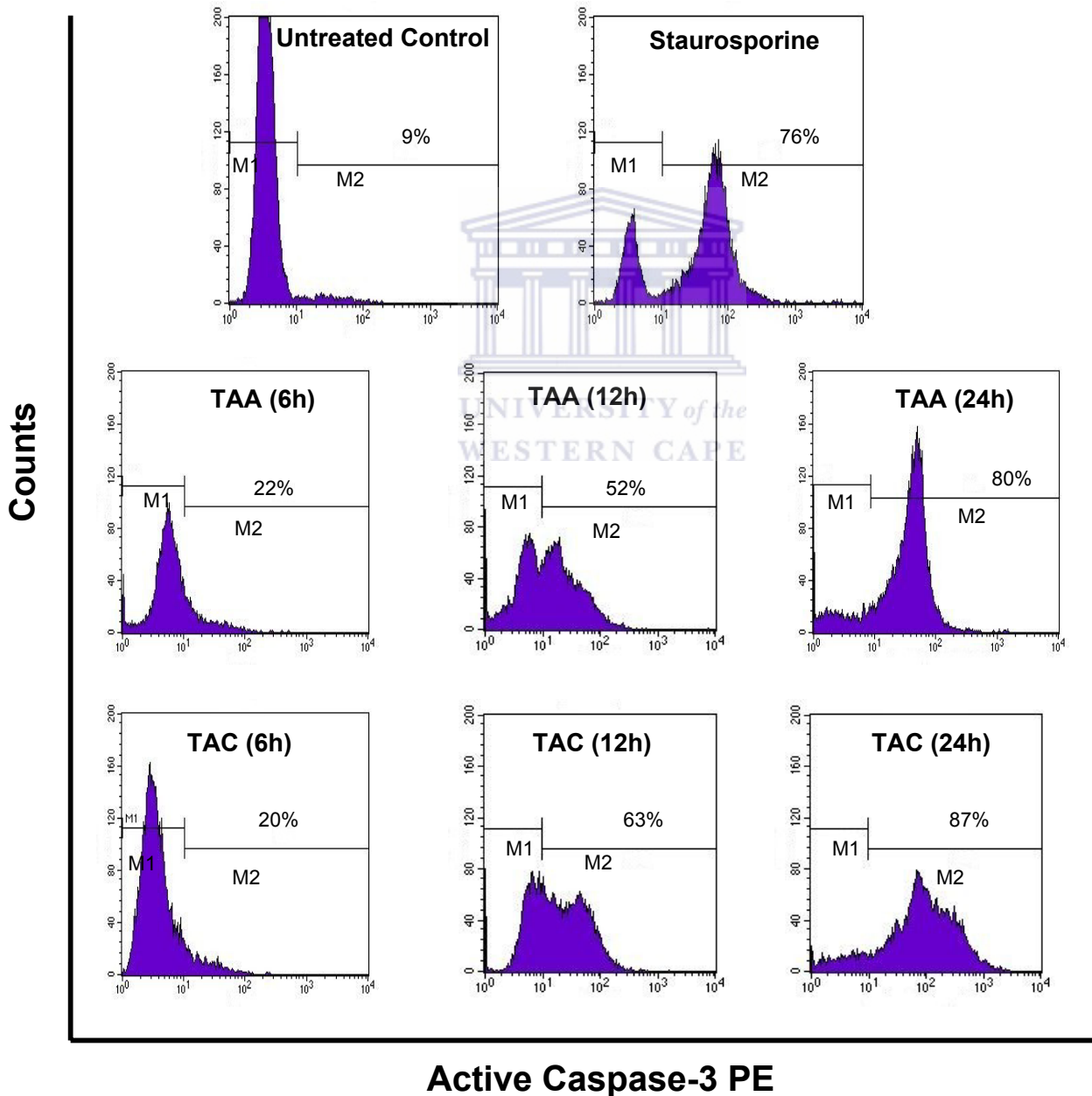
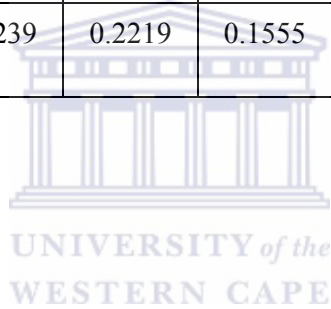


Fig 5. Activation of caspase-3 by *Tulbaghia alliacea* extracts in Jurkat cells during a time-course experiment.

**Table 4.** Caspase-3 activation by *Tulbaghia alliacea* extracts

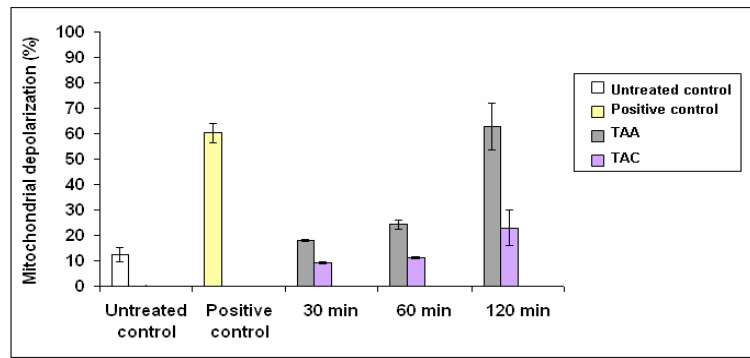
Extracts	TAA			TAC				
	6h (a)	12h (b)	24h (c)	6h (d)	12h (e)	24h (f)		
<b>% cells with cleaved caspase-3 (mean ± SEM)</b>	22 ± 1.31	51 ± 5.45	80 ± 2.81	20 ± 3.30	62 ± 5.02	86 ± 0.93		
<b>Statistical significance (P)</b>								
<b>ab</b>	<b>ac</b>	<b>ad</b>	<b>Bc</b>	<b>be</b>	<b>cf</b>	<b>de</b>	<b>df</b>	<b>ef</b>
0.0339	0.0003	0.6256	0.0239	0.2219	0.1555	0.0059	0.0026	0.0409



### **4.4.3. Evaluating mitochondrial depolarization using the Tetramethylrhodamine (TMRE) assay**

Decrease in mitochondrial potential is also associated with the activation of apoptosis. The TMRE assay was used to assess the mitochondrial depolarization potential in Jurkat cells following treatment with the extracts. Jurkat cells were treated for different time points (30, 60, and 120 min) with TAA and TAC extracts (6mg/ml). Staurosporine was used as a positive control. The cells were stained with TMRE dye and cell fluorescence was measured by flow cytometry as described in Section 2.6. These experiments were performed in triplicate and the results are displayed in Figure 6A. The histograms in Figure 6B are examples of the flow cytometry results that were acquired. The numbers (M2) on the histograms refer to the number of cells with depolarized mitochondria. The activity of the TAA and TAC extracts were statistically analysed by conducting an unpaired *t*-test using the Microsoft Excel programme (Table 5)

A.



B

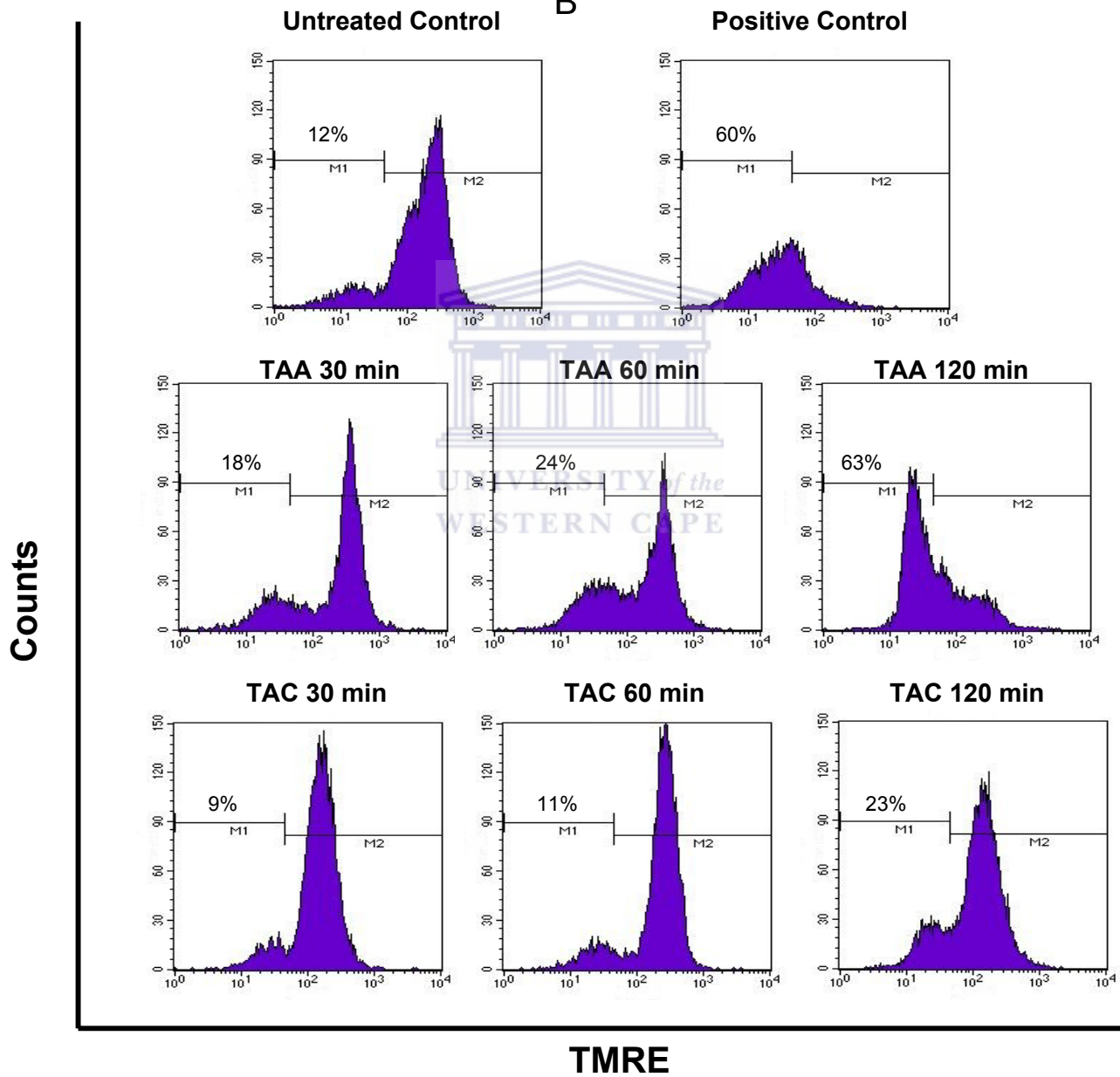


Fig 6. Mitochondrial depolarization by *Tulbaghia alliacea* extracts in Jurkat cells during a time-course experiment.

**Table 5.** Changes in mitochondrial depolarization potential of Jurkat cells in response to *Tulbaghia alliacea* extracts

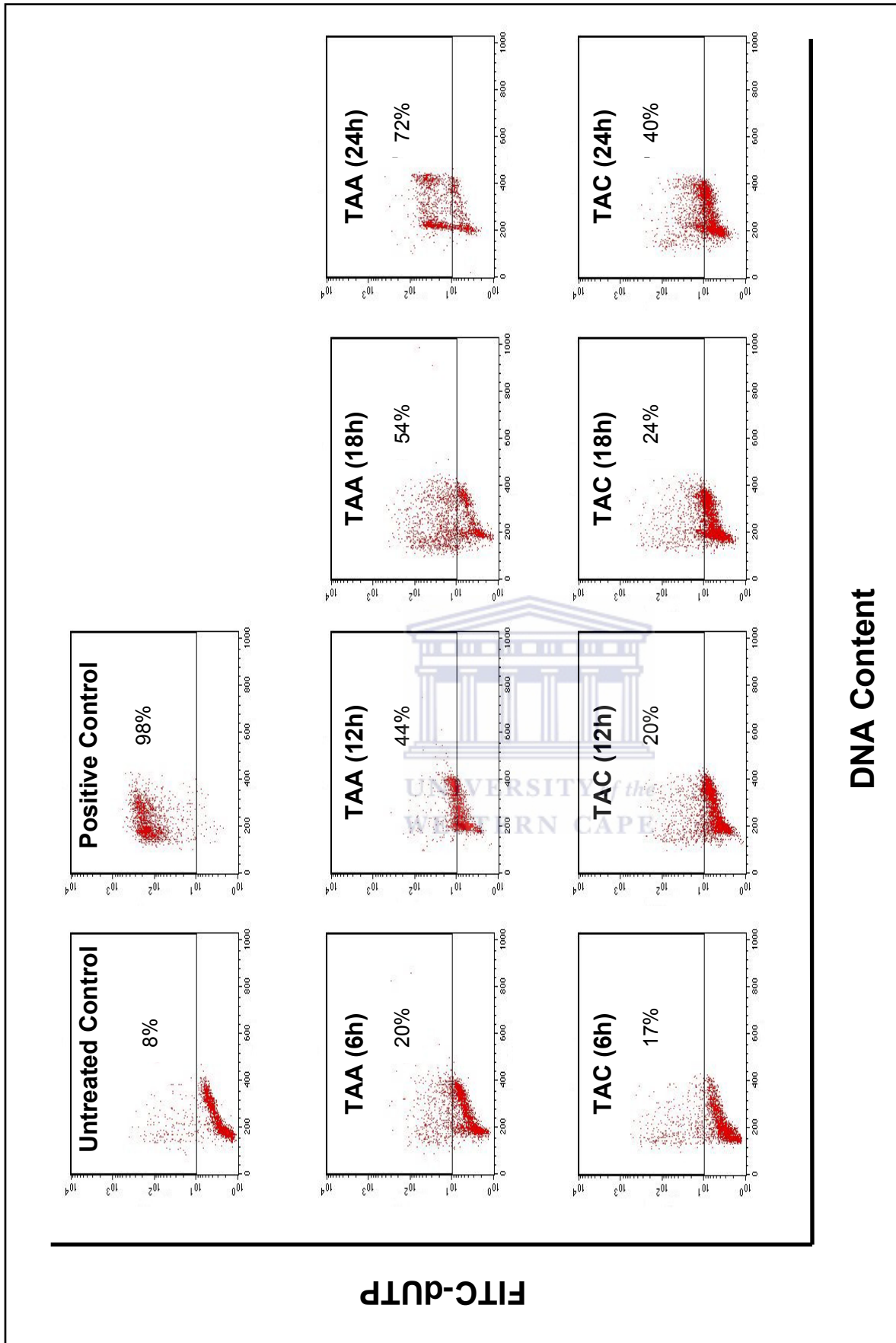
Extracts	TAA			TAC				
Treatment Time	30 min (a)	60 min (b)	90 min (c)	30 min (d)	60 min (e)	90 min (f)		
% cells with depolarized mitochondria (mean ± SEM)	18 ± 0.58	24 ± 1.86	63 ± 9.25	9 ± 0.33	11 ± 0.33	23 ± 7.10		
<b>Statistical significance (P)</b>								
ab	ac	ad	bc	be	cf	de	df	ef
0.0219	0.0089	0.4676	0.0054	0.0972	0.0023	0.2829	0.2278	0.6886

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#### **4.4.4. Evaluating DNA fragmentation using the Terminal deoxynucleotide transferase dUTP Nick End Labeling (TUNEL) assay**

The fragmentation of genomic DNA is another hallmark of apoptosis. The extent of DNA fragmentation was analysed using the TUNEL assay. Jurkat T cells were treated for different time points (6, 12, 18, 24h) with TAA and TAC extracts (6mg/ml). Staurosporine was used as a positive control. The cells were permeabilized and labelled with FITC-dUTP and propidium iodide, as described in Section 2.7. The dot plots in Figure 7 compares the cell fluorescence of FITC-dUTP and propidium iodide (DNA content). The events in the R1 region of the dot plot represent cells that are negative for FITC-dUTP staining and therefore do not display any DNA fragmentation, while events in the R2 region represent cells that are positive for FITC-dUTP and DNA fragmentation. The numbers in the R2 region refers to the number of cells containing fragmented DNA

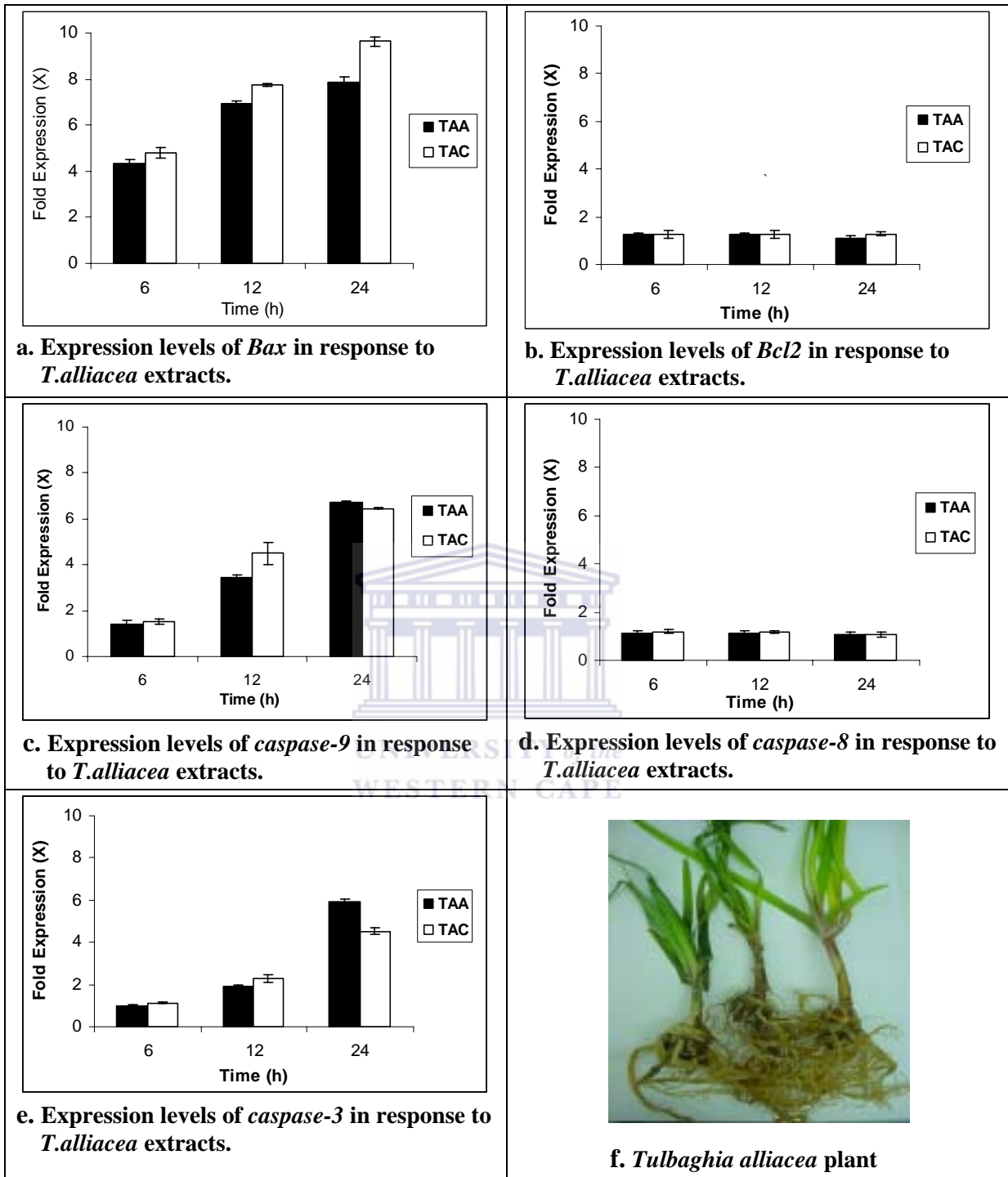




**Fig 7.** DNA fragmentation induced by *Tulbaghia alliacea* extracts in Jurkat cells.

#### 4.4.5. Quantitative PCR

Several genes are involved in the activation and regulation of apoptosis. In order to evaluate whether apoptosis induced by the plant extracts is associated with the up-regulation of certain apoptotic genes, the expression levels of several genes (*Bax*, *Bcl2*, *capase-3*, *caspace-8*, *caspace-9*) previously implicated in the regulation and execution of apoptosis were evaluated by real time quantitative PCR. Jurkat cells were treated for different time points (6h, 12h, and 24h) with the plant extracts. Thereafter total RNA was isolated from the cells as described in Section 2.9 and 1 µg of this RNA was used to synthesize cDNA as described in Section 2.10. The expression levels of *Bax*, *Bcl2*, *caspace-3*, *caspace-8*, *caspace-9* were assessed by quantitative real time PCR as described in Section 2.11 and normalized against the expression levels of the housekeeping gene, *GAPDH*. Figure 8 shows the expression levels of these genes in Jurkat cells treated with the plant extracts. The presentation of the expression levels of all these genes are relative to the expression levels in the untreated control cells. The activity of the TAA and TAC extracts were statistically analysed by conducting an unpaired *t*-test (Tables 6.1 – 6.5).



**Fig 8. Quantitative PCR analysis of apoptosis-related genes (a-e) in response to treatment with *T.alliacea* (f) extracts during a time-course experiment.**

**Table 6.1.** The expression levels of Bax in Jurkat cells treated with *T.alliacea* extracts.

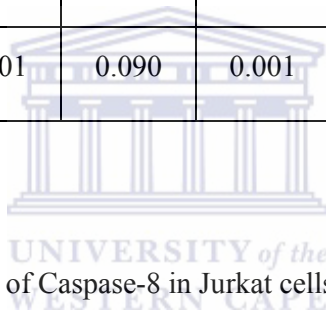
Extracts	TAA			TAC				
Treatment Time	6h (a)	12h (b)	24h (c)	6h (d)	12h (e)	24h (f)		
Fold expression (X) (mean ± SEM)	4.33 ± 0.19	6.93 ± 0.15	7.86 ± 0.21	4.80 ± 0.23	7.76 ± 0.06	9.64 ± 0.21		
<b>Statistical significance (P)</b>								
ab	ac	ad	bc	be	cf	de	df	ef
0.0003	0.0003	0.2197	0.0323	0.0062	0.0053	0.0002	0.0000	0.0009

**Table 6.2.** The expression levels of Bcl2 in Jurkat cells treated with *T.alliacea* extracts.

Extracts	TAA			TAC				
Treatment Time	6h (a)	12h (b)	24h (c)	6h (d)	12h (e)	24h (f)		
Fold expression (X) (mean ± SEM)	1.24 ± 0.02	1.25 ± 0.02	1.10 ± 0.08	1.26 ± 0.18	1.26 ± 0.18	1.28 ± 0.08		
<b>Statistical significance (P)</b>								
ab	ac	ad	bc	be	cf	de	df	ef
1.000	0.100	0.945	0.100	0.945	0.160	1.000	0.936	0.936

**Table 6.3.** The expression levels of Caspase-9 in Jurkat cells treated with *T.alliacea* extracts.

Extracts	TAA			TAC				
	6h (a)	12h (b)	24h (c)	6h (d)	12h (e)	24h (f)		
<b>Fold expression (X) (mean ± SEM)</b>	1.43 ± 0.15	3.42 ± 0.15	6.74 ± 0.03	1.53 ± 0.09	4.50 ± 0.46	6.46 ± 0.03		
<b>Statistical significance (P)</b>								
<b>ab</b>	<b>ac</b>	<b>ad</b>	<b>bc</b>	<b>be</b>	<b>cf</b>	<b>de</b>	<b>df</b>	<b>ef</b>
0.006	0.001	0.587	0.001	0.090	0.001	0.003	0.001	0.013



**Table 6.4.** The expression levels of Caspase-8 in Jurkat cells treated with *T.alliacea* extracts.

Extracts	TAA			TAC				
	6h (a)	12h (b)	24h (c)	6h (d)	12h (e)	24h (f)		
<b>Fold expression (X) (mean ± SEM)</b>	1.11 ± 0.13	1.11 ± 0.13	1.09 ± 0.10	1.20 ± 0.07	1.17 ± 0.07	1.08 ± 0.10		
<b>Statistical significance (P)</b>								
<b>ab</b>	<b>Ac</b>	<b>ad</b>	<b>bc</b>	<b>be</b>	<b>cf</b>	<b>de</b>	<b>df</b>	<b>ef</b>
1.000	0.907	0.717	0.907	0.717	0.904	1.000	0.001	0.013

**Table 6.5.** The expression levels of Caspase-3 in Jurkat cells treated with *T.alliacea* extracts.

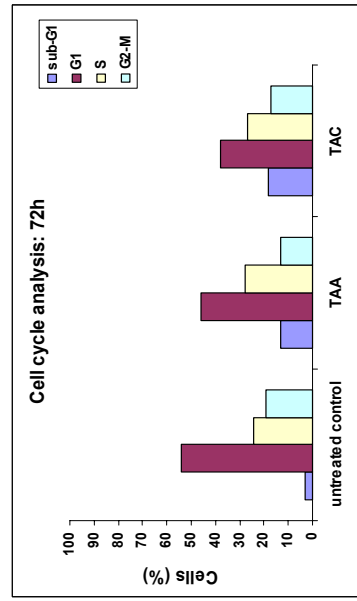
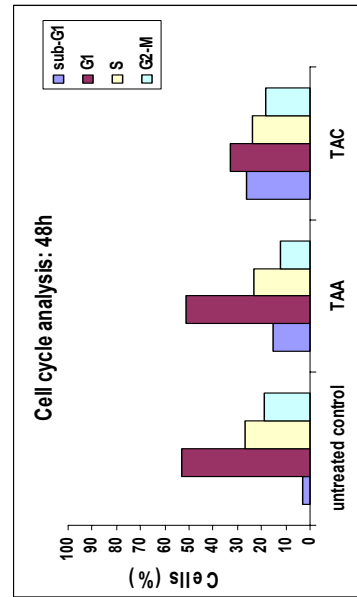
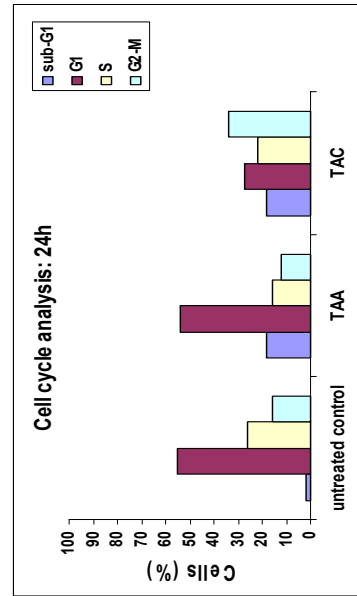
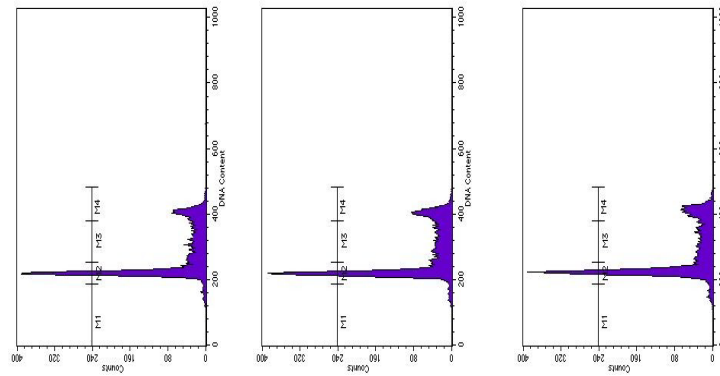
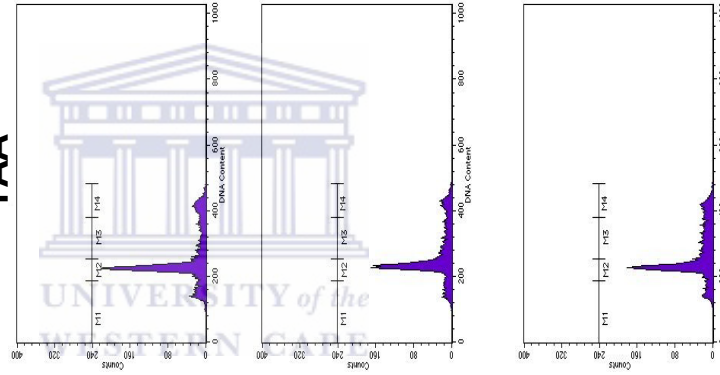
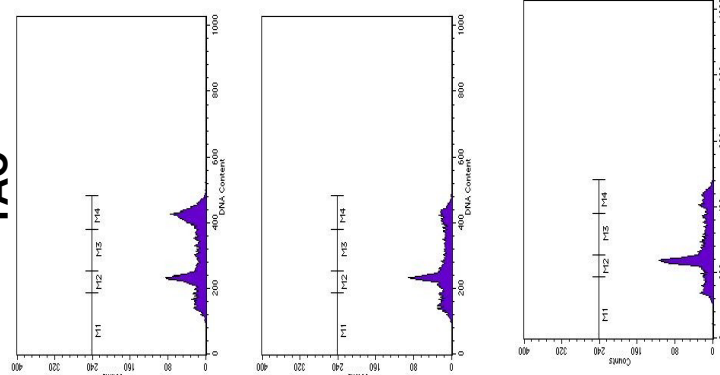
Extracts	TAA			TAC				
	6h (a)	12h (b)	24h (c)	6h (d)	12h (e)	24h (f)		
<b>Fold expression (X) (mean ± SEM)</b>	0.97 ± 0.07	1.90 ± 0.10	5.91 ± 0.15	1.13 ± 0.03	2.27 ± 0.18	4.53 ± 0.15		
<b>Statistical significance (P)</b>								
<b>ab</b>	<b>ac</b>	<b>ad</b>	<b>bc</b>	<b>be</b>	<b>cf</b>	<b>de</b>	<b>df</b>	<b>ef</b>
0.001	0.001	0.089	0.001	0.144	0.002	0.003	0.001	0.001



#### **4.4.6. Cell cycle analysis**

The anti-tumour activity of several anti-cancer agents can also be associated with anti-proliferative activity. In order to assess whether the plant extracts have an effect on cell cycle progression, Jurkat cells were treated with a sub-lethal dose (0.5mg/ml) of the plant extracts and the cell cycle profile of the cells were evaluated over a 72h period at 24h intervals. Cell cycle progression was investigated by studying four phases (G1, S, G2M and sub-G1) of cell cycle progression. The cell populations in the different phases were quantified by flow cytometry, and are displayed in Figure 9A. The flow cytometry data is shown in Figure 9B with the M1, M2, M3 and M4 markers representing sub-G1, G1, S, and G2-M cell populations, respectively.



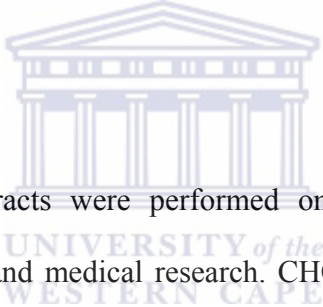
**A****B****Untreated Control****TAA****TAC**

**Fig 9. The effect of *Tulbaghia alliacea* extracts on the cell cycle of Jurkat cells**



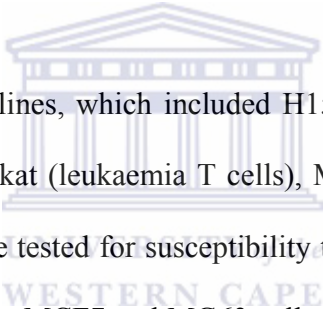
## 4.5. Discussion

*Tulbaghia alliacea* is used traditionally for a variety of ailments (Van Wyk *et al.*, 2000). Some communities also claim to use it for the treatment of oesophageal cancer. This investigation aimed at assessing the potential of *T. alliacea* extracts as an anti-cancer phytotherapy. The cytotoxic effects of several anti-cancer drugs involve the activation of apoptosis in cancerous cells. To evaluate the anti-cancer potential of *T. alliacea*, the pro-apoptotic activity of the aqueous (TAA) and chloroform (TAC) extracts were assessed using several bioassays, which detect biochemical and morphological changes that are associated with apoptosis.



Initial screenings of the extracts were performed on CHO cells. These cells are commonly used in biological and medical research. CHO cells are non-cancerous cells that are susceptible to apoptosis induced by a wide range of anti-cancer agents (including ceramide, staurosporine, doxorubicin, cisplatin, etc.), suggesting that the apoptosis apparatus in these cells is intact, and that this cell line is an appropriate control to evaluate the pro-apoptotic potential of the plant extracts. CHO cells were treated with both extracts and the level of apoptosis was assessed using the APOPercentage™ assay, which detects apoptosis at the stage of phosphatidyl serine exposure. The extracts induced morphological changes (cell shrinkage and cell detachment) in CHO cells; that were indicative of cells undergoing apoptosis (Figure 2).

The APOPercentage™ assay confirmed that cell death was as a result of apoptosis since the cells treated with the plant extracts stained positive with the APOPercentage™ dye. The quantification of apoptosis by flow cytometry demonstrated that both extracts induced a dose-dependant increase in the number of apoptotic cells (Figure 3). At a concentration of 6mg/ml, the TAA extract induced apoptosis in ~50% of the cell population. In comparison, the TAC extract was less effective, inducing apoptosis in ~25% of the cells. A concentration of 6mg/ml was used in subsequent assays to assess the potential of both these extracts to induce apoptosis in several human cancer cell lines.



A panel of human cancer cell lines, which included H157 (small lung cell carcinoma), HeLa (cervical carcinoma), Jurkat (leukaemia T cells), MCF7 (breast adenocarcinoma), and MG63 (osteosarcoma) were tested for susceptibility to these extracts. Three of these cell lines, which included Jurkat, MCF7 and MG63 cells, were highly susceptible to both the extracts, while HeLa and H157 cells were resistant (Figure 4).

Since the polarity of chloroform and water is significantly different, it is expected that the chemical entities present in the chloroform extract (TAC) of the plant will be different from that in the aqueous extract (TAA). Consequently, the bioactivity of these two extracts may differ. A comparison of the effects of these two extracts shows that the MCF7 cells were equally susceptible to both the TAA and TAC extract, while Jurkat cells were more susceptible ( $P<0.05$ ) to the TAA extract. The MG63 cells were more susceptible ( $P<0.05$ ) to the TAC extract (Figure 4 and Table 3).

This suggests that the plant extracts show selectivity towards particular human cancer cell lines. The difference in the bioactivity may also be as a result of genetic variance between the different cell lines.

Cancer cell lines are known to have mutations in genes that are involved in apoptosis and as a result these cells may be more resistant to particular apoptosis-inducing agents. Apoptosis can be activated through several pathways and it is therefore possible that the two resistant cell lines (H157 and HeLa) may have acquired genetic mutations in genes that are present in the pathways activated by the pro-apoptotic compounds in the extracts.

Jurkat is one of the two cell lines that displayed differential susceptibility to the two extracts (TAA and TAC). Hence, this cell line was selected to further characterise the pro-apoptotic activity of these extracts. Three other markers of apoptosis (mitochondrial depolarisation, caspase-3 cleavage, and DNA fragmentation) were used to assess the bioactivity of the plant extracts. Caspases are a family of cysteine proteases that are involved in the induction of cell death by apoptosis (Grimm, 1996; Polverino, 1997). The activation of caspase-3 is central to both apoptosis pathways. Caspase-3 has many substrates and is known to cleave structural proteins that generate the characteristic apoptotic morphology (Kothakota, Azuma, Reinhard, Klippel, Tang, Chu, McGarry, Kirschner, Koths, Kwiatkowski, Williams, 1997); Lazebnik, Kaufmann, Desnoyers, Poirier, Earnshaw, 1994; Mashima, Naito, Noguchi, Miller, Nicholson, Tsuruo, 1997).

Jurkat cells were treated with TAA and TAC extracts and analysed for caspase-3 activation using an anti-active caspase-3 PE conjugated antibody. Both extracts induced a time-dependant increase in caspase-3 activity. In contrast to the APOPercentage™ assay, which showed that the TAA extract was more active than the TAC extract, the caspase-3 assay showed that both extracts induced similar levels of caspase-3 cleavage (Figure 5). This could possibly point to different mechanisms of apoptosis induction for these two extracts.

Mitochondria have been implicated as sensors and executioners in the cell's decision to live or die (Kroemer, Zamzami, Susin, 1997; Murphy, Fiskum, Beal, 1999; Reed, 1997) and may even influence the mode of cell death chosen, which is either necrosis or apoptosis, depending on their functional state (Nicotera and Leist, 1997; Tsujimoto, 1997). It has been shown that mitochondria release pro-apoptotic factors into the cytosol. The release of these proteins from the mitochondria is triggered by the loss of the mitochondrial potential. Using the potentiometric fluorescence probe, TMRE, to evaluate the mitochondrial potential in Jurkat cells treated with the plant extracts, it was demonstrated that both extracts induce mitochondrial depolarisation, but that the TAA extract is significantly more active ( $P < 0.05$ ) (Figure 6).

Cleavage of chromosomal DNA into oligonucleosomal size fragments is a biochemical hallmark of apoptosis (Wyllie, Kerr, Currie, 1980). Several nucleases have been associated with DNA fragmentation during apoptosis. Amongst these nucleases is the Caspase Activated Deoxyribonuclease (CAD), which pre-exists in living cells as an

inactive complex with an inhibitory sub-unit, called ICAD. The cleavage of CAD/ICAD complex i.e. activation of CAD, occurs by means of caspase-3 mediated cleavage of ICAD. Since the caspase-3 assay confirmed that both the TAA and TAC extracts induce the activation of caspase-3, the TUNEL assay was used to evaluate DNA fragmentation in Jurkat cells treated with TAA and TAC extracts. Both extracts demonstrated a time-dependant increase in DNA fragmentation, but the TAA extract was significantly more active ( $P<0.05$ ) (Figure 7). This is in agreement with the APOPercentage™ assay and the TMRE assay, which showed that TAA is more active than the TAC extract.

Two major apoptotic pathways have been described; the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway. These two pathways are distinct and involve different genetic components. In an effort to understand and further characterise the pro-apoptotic activity of the plant extracts, the expression levels of five genes (*Bax*, *Bcl2*, *caspase-3*, *caspase-8* and *caspase-9*) that play key roles in the regulation and execution of apoptosis were evaluated by real time PCR. The expression levels of three of these genes (*Bax*, *caspase-3*, and *caspase-9*) were significantly up-regulated over a 24 h period in Jurkat cells treated with the extracts ( $P<0.05$ ), while the other two genes, *caspase-8* and the anti-apoptotic gene, *Bcl2* were only moderately (1-fold increase) increased in the treated cells (Figure 8).

A comparison between the two extracts demonstrated a significant difference between the two extracts with regard to the induction of *Bax*, *caspase-3*, and *caspase-9* expression ( $P<0.05$ ; Tables 6.1, 6.3, 6.5). At 12 h and 24 h, the up-regulation of *Bax* was higher for

the TAC extract than the TAA extract. On the other hand, the expression levels of *caspase-3* and *caspase-9* were higher for the TAA extract at the 24 h time point. This demonstrates that both extracts affect the expression levels of genes that are involved in the regulation of apoptosis and that the mechanism of apoptosis induction may involve the mitochondrial pathway. Furthermore, there is a distinct difference between the two extracts with regard to the mechanisms by which these extracts induce apoptosis.

Many conventional anti-cancer drugs are DNA damaging agents that cause double-stranded DNA breaks in cancer cells causing the cell cycle in these cells to stop and resulting in the elimination of these cells by apoptosis. Cellular proliferation involves four sequential phases: the S or synthesis phase when DNA synthesis occurs, the M or mitotic phase when the cell divides into two daughter cells, and two gap phases (G1 and G2) which separate the S and M phases (Garrett, 2001). G1 follows the M phase and is the stage when the cell is responsive to both positive and negative growth signals. G2 follows the S phase and is the stage when the cell prepares to enter the M phase. A fifth stage, the G0 or quiescence phase represents a stage into which cells may reversibly exit from G1 when growth conditions are not favourable. It is essential that the integrity of the genome is protected during cell division. This is achieved through three cell cycle checkpoints (G1/S, S, G2/M), which prevent cells with damaged DNA from proliferating and producing progeny with the same errors.

It was already demonstrated that apoptosis induced by the plant extracts involves chromosomal DNA fragmentation. In order to ascertain whether the plant extracts also affect cell cycle progression, Jurkat cells were treated with a sub-lethal dose (0.5mg/ml) of the extracts and the cell cycle progression was evaluated over a 72 hour period at 24 h intervals. At 24 h, both extracts induced apoptosis as is evident from the increased sub-G1 peaks (Figure 9). The TAA extract did not affect the cell cycle profile significantly except that the number of cells in the S phase dropped by about 10%, which may account for the increase in the sub-G1 population. The TAC extract on the other hand induced considerable changes in the cell cycle profile of the Jurkat cells. The G1 population decreased by about 25%, while the G2-M population increased by about 15%. The latter may suggest that the cell cycle progression halted in G2-M phase during the G2/M checkpoint. At 48 and 72 hours the cellular profile for the cells treated with the TAA extract still did not change significantly. However, the TAC extract demonstrated an increase in the sub-G1 population and a decrease in the G2-M population.

## 4.6. Conclusion

The ability of a compound or a plant extract to induce apoptosis in cancer cells can be used as a test to evaluate the potential use of such a compound or a plant extract to treat cancer. Since this study demonstrated that extracts of *T. alliacea* selectively induced apoptosis in human cancer cell lines, it can be concluded that extracts and in particular the aqueous extract (which is how the plant is traditionally used) shows potential as an anti-cancer remedy.

## 4.7. References

- Balasenthil S, Rao KS, Nagini (2002). Garlic induces apoptosis during 7,12-dimethylbenz[*a*]anthracene-induced hamster buccal pouch carcinogenesis. *Oral Oncol.* 38: 431–436.
- Behrend L (2005). Manganese Superoxide Dismutase Induces p53-Dependent Senescence in Colorectal Cancer Cells. *Journal of Molecular and Cellular Biology* 25: 7758–7769.
- Biswas SK, Huang J, Persaud S, Basu A (2004). Down-regulation of Bcl-2 is associated with cisplatin resistance in human small cell lung cancer H69 cells. *Journal of Molecular Cancer Therapeutics* 3: 327–334.
- Block E (1985). The chemistry of garlic and onions. *Sci. Am.* 252: 114–119.
- Buiatti E, Palli D, Decarli A, Amadori D, Avellini C, Bianchi S, Biserni R, Cipriani F, Cocco P, Giacosa A, Mrubini E, Puntoni R, Vindigi C, Frumeni J, Blot W (1989). A case-control study of gastric cancer, and diet in Italy. *Int J Cancer* 44: 611–616.
- Bungu L, Frost CL, Brauns SC, Van de Venter M (2006). Tulbaghia violacea inhibits growth and induces apoptosis in cancer cells in vitro. *Afr J Biotech* 5, 1936-1943.
- Buttke TM, Sandstrom PA (1994). Oxidative stress as a mediator of apoptosis. *Immunol Today* 15: 7–10.
- Cal C, Garban H, Jazirehi A, Yeh C, Mizutani Y, Bonavida B (2003). Resveratrol and cancer: chemoprevention, apoptosis, and chemo-immunosensitizing activities. *Curr. Med. Chem. Anti-cancer Agents* 3: 77–93.



- Calcabrini A (2006). Inhibition of proliferation and induction of apoptosis in human breast cancer cells by lauryl gallate. *Journal of Carcinogenesis*. 27: 1699–1712.
- Camerino DC; Tricarico D, Desaphy, JF (2007). Ion Channel Pharmacology. *The Journal of the American Society for Experimental NeuroTherapeutics* 4: 184–198.
- Chaturvedi R (2004). Induction of Polyamine Oxidase 1 by *Helicobacter pylori* Causes Macrophage Apoptosis by Hydrogen Peroxide Release and Mitochondrial Membrane Depolarization. *The Journal of Biological Chemistry* 279: 40161–40173
- Chelli B, Lena A, Vanacore R, Da Pozzo E, Costa B, Rossi L, Salvetti A, Scatena F, Ceruti S, Abbracchio MP, Gremigni V; Martini C (2004). Peripheral benzodiazepine receptor ligands: mitochondrial transmembrane potential depolarization and apoptosis induction in rat C6 glioma cells. *Journal of Biochemical Pharmacology*. 68: 125–134
- Chien W, Kumagai T, Miller CW, Desmond JC, Frank JM, Said JW, Koeffler HP (2004). Cyr61 Suppresses Growth of Human Endometrial Cancer Cells. *The Journal of Biological Chemistry*. 279:: 53087–53096
- Chinnaiyan AM, O'Rourke K, Lane BR, Dixit VM (1997). Interaction of CED-4 with CED-3 and CED-9: a molecular framework for cell death. *Science* 275: 1122–1126.
- Deng X, Yin F; Lu X, Cai B, Yin W (2006). The Apoptotic Effect of Brucine from the Seed of *Strychnos nux-vomica* on Human Hepatoma Cells is Mediated via Bcl-2 and Ca<sup>2+</sup> Involved Mitochondrial Pathway. *Journal of Toxicological Sciences*. 91: 59–69.

- Dietze EC (2001). Tamoxifen but Not 4-Hydroxytamoxifen Initiates Apoptosis in p53 (-) Normal Human Mammary Epithelial Cells by Inducing Mitochondrial Depolarization. *The Journal of Biological Chemistry*. 276: 5384–5394
- Dirsch VM, Gerbes AL, Vollmar AM (1998). Ajoene, a compound of garlic, induces apoptosis in human promyeloleukemic cells, accompanied by generation of reactive oxygen species and activation of nuclear factor kappa B. *Mol. Pharmacol.* 53: 402–407.
- Dong Z (2003). Molecular mechanism of the chemopreventive effect of resveratrol. *Mutat. Res.* 523–524: 145–150.
- DüBmann H (2003). Outer mitochondrial membrane permeabilization during apoptosis triggers caspase-independent mitochondrial and caspase-dependent plasma membrane potential depolarization: a single-cell analysis. *Journal of Cell Science*. 116: 525–536
- Eloff JN (1998). Which extractant should be used for the screening and isolation of antimicrobial components from plants. *J Ethnopharmacol* 60: 1-8.
- Enari M, Talanian RV, Wong WW, Nagata S (1996). Sequential activation of ICE-like and CPP32-like proteases during Fas-mediated apoptosis. *Nature* 380: 723–726.
- Evan G, Littlewood T (1998). A matter of life, and cell death. *Science* 281: 317-322.
- Fesus L, Szondy Z, Uray I (1995). Probing the molecular program of apoptosis by cancer chemopreventive agents. *J. Cell Biochem. Suppl.* 22: 151–161.
- Fisher DE (1994). Apoptosis in cancer therapy: crossing the threshold. *Cell* 78: 539–542.
- Flescher E (2007). Jasmonates in cancer therapy. *Journal of Cancer Letters*. 245: 1.

- Fleischauer AT, Arab L (2001). Garlic and cancer: a critical review of epidemiological literature. *Journal of Nutrition* 131: 1032S-1040S.
- Fukumori T, Kanayama H, Raz A (2007). The role of galectin-3 in cancer drug resistance. *Journal of Drug Resistance Update*. 10: 101–108.
- Fukumori T (2006). Galectin-3 Regulates Mitochondrial Stability and Antiapoptotic Function in Response to Anticancer Drug in Prostate Cancer. *Journal of Cancer Research*. 66: 3114–3119.
- Gao X (2007). Synthetic triterpenoids inhibit growth and induce apoptosis in human glioblastoma and neuroblastoma cells through inhibition of prosurvival Akt, NF- $\kappa$ B and Notch1 signaling. *Journal of Neurooncology*. 84:147–157
- Garrett MD (2001). Cell cycle control and cancer. *Current Science*. 81: 515 – 522.
- Gescher A (1998). Analogs of staurosporine: potential anticancer drugs. *Gen Pharmacol*. 31: 721 – 728.
- Goto H, Yanagimachi M, Kajiwara R, Kuroki F, Yokota S (2007). Lack of mitochondrial depolarization by oxidative stress is associated with resistance to buthionine sulfoximine in acute lymphoblastic leukemia cells. *Journal of Leukemia Research*. 31: 1293–1301
- Gottlieb E, Van Der Heiden MG, Thompson CB (2000). Bcl-xL Prevents the Initial Decrease in Mitochondrial Membrane Potential and Subsequent Reactive Oxygen Species Production during Tumor Necrosis Factor Alpha-Induced Apoptosis. *Journal of Molecular and Cellular Biology* 20: 5680–5689

- Grimm S, Bauer MKA, Baeuerle PA, Schulze-Osthoff K (1996). Bcl-2 down-regulates the activity of transcription factor NF- $\kappa$ B induced upon apoptosis. *J Cell Biol* 134: 13–23.
- Haenszel W, Kurihara M, Segi M, Lee RK (1972). Stomach cancer among Japanese in Hawaii. *J Natl Cancer Inst* 49: 969–988.
- Ham YM (2006). Ginsenoside-Rh2-Induced Mitochondrial Depolarization and Apoptosis Are Associated with Reactive Oxygen Species and Ca<sup>2+</sup>-Mediated c-Jun NH2-Terminal Kinase 1 Activation in HeLa Cells. *The Journal of Pharmacology and Experimental Therapeutics*. 319: 1276–1285
- Holzman D (1996). Apoptosis provides new targets for chemotherapy. *J. Natl. Cancer Inst.* 88: 1098–1100.
- Hsing AW, Chokkalingam AP, Gao YT, Madigan MP, Deng J, Gridley G, Fraumeni JF (2002). Allium vegetables and risk of prostate cancer: a population-based study. *Journal of National Cancer Institute* 94: 1648-1651.
- Hussain SP, Jannu LN, Rao AR (1990). Chemopreventive action of garlic on methylcholanthrene-induced carcinogenesis in the uterine cervix of mice. *Cancer Lett.* 49: 175–180.
- Hutchings A, Scott AH, Lewis G, Cunningham A (1996). Zulu Medicinal Plants. An Inventory. University of Natal Press, Scottsville.
- Ibrado AM, Huang Y, Fang G, Liu L, Bhalla K (1996). Overexpression of Bcl-2 or Bcl-xL inhibits Ara-C-induced CPP32/Yama protease activity and apoptosis of human acute myelogenous leukemia HL-60 cells. *Cancer Res* 56: 4743–4748.

- Iimuro M, Shibata H, Kawamori T, Matsumoto T, Arakawa M, Sugimura T (2002). Suppressive effects of garlic extract on *Helicobacter pylori*-induced gastritis in Mongolian gerbils, *Cancer Lett* 187: 61–68.
- Jarpe MB, Widmann C, Knall C, Schlesinger TK, Gibson S, Yujiri T, Fanger GR, gelfand EW, Johnson GL (1998). Anti-apoptotic versus pro-apoptotic signal transduction: checkpoints and stop signs along the road to death. *Oncogene* 17: 1475 – 1482.
- Jin YH, Yim H, Park JH, Lee SK (2003). Cdk2 activity is associated with depolarization of mitochondrial membrane potential during apoptosis. *Journal of Biochemical and Biophysical Research Communications*. 305: 974–980
- Knowles LM, Milner JA (2001). Possible mechanism by which allyl sulfides suppress neoplastic cell proliferation. *J Nutr* 131: 1061S–1066S.
- Kothakota S, Azuma T, Reinhard C, Klippel A, Tang J, Chu K, McGarry TJ, Kirschner MW, Kohts K, Kwiatkowski DJ, Williams LT (1997). Caspase-3-generated fragment of gelsolin: effector of morphological change in apoptosis. *Science* 278: 294–298.
- Kroemer G, Zamzami N, Susin SA (1997). Mitochondrial control of apoptosis. *Immun Today* 18: 44-51.
- Krohn AJ, Wahlbrink T, Prehn JHM (1999). Mitochondrial Depolarization Is Not Required for Neuronal Apoptosis. *The Journal of Neuroscience* 19: 7394–7404
- Krzystyniak KL (2002). Current strategies for anticancer chemoprevention and chemoprotection. *Acta Pol. Pharm.* 59: 473–478.

- Kuo ML, Huang TS, Lin JK (1996). Curcumin, an antioxidant and anti-tumor promoter, induces apoptosis in human leukemia cells. *Biochim. Biophys. Acta* 1317: 95–100.
- Kwon KB, Yoo SJ, Ryu DG, Yang JY, Rho HW, Kim JS, Park JW, Kim HR and Park BH (2002). Induction of apoptosis by diallyl disulfide through activation of caspase-3 in human leukemia HL-60 cells. *Biochem. Pharmacol.* 63: 41–47.
- Lazebnik YA, Kaufmann SH, Desnoyers S, Poirier GG, Earnshaw WC (1994). Cleavage of poly(ADP-ribose) polymerase by a proteinase with properties like ICE. *Nature* 371: 346–347.
- Le HV, Minn AJ, Massague J (2005). Cyclin-dependent Kinase Inhibitors Uncouple Cell Cycle Progression from Mitochondrial Apoptotic Functions in DNA-damaged Cancer Cells. *The Journal of Biological Chemistry* 280: 32018–32025
- Ledezma E, Apitz-Castro R, Cardier J (2004). Apoptotic and antiadhesion effect of ajoene, a garlic derived compound, on the murine melanoma B16F10 cells: possible role of caspase-3 and the alpha (4) beta (1) integrin. *Cancer Lett* 206: 35–41.
- Lee HH (2003). Differential effects of natural polyphenols on neuronal survival in primary cultured central neurons against glutamate- and glucose deprivation-induced neuronal death. *Journal of Brain Research* 986: 103–113
- Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES, Wang X (1997). Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* 91: 479–489.
- Lian F, Li Y, Bhuiyan M, Sarkar FH (1999). p53-independent apoptosis induced by genistein in lung cancer cells. *Nutr. Cancer* 33: 125–131.

- Lin HL, Yang JS, Yang JH, Fan SS, Chang WC, Li YC, Chung JG (2006). The Role of Ca<sup>2+</sup> on the DADS-induced Apoptosis in Mouse–Rat Hybrid Retina Ganglion Cells (N18). *Journal of Neurochemical Research* 31:383–393
- Lu D (2007). Protein kinase C-*ε* protects MCF-7 cells from TNF-mediated cell death by inhibiting Bax translocation. *Journal of Apoptosis*. 12: 1893–1900
- Malekova L, Kominkova V, Ferko M, Stefanik P, Krizanova P, Ziegelhöffer A, Szewczyk A, Ondrias K (2007). Bongkrekic acid and atractyloside inhibits chloride channels from mitochondrial membranes of rat heart. 2007. *Journal of Biochimica et Biophysica Acta*. 1767: 31–44
- Mashima T, Naito M, Noguchi K, Miller DK, Nicholson DW, Tsuruo T (1997). Actin cleavage by CPP-32/apopain during the development of apoptosis. *Oncogene* 14: 1007–1012.
- Meyer M, Essack M, Kanyanda S, Rees J (2008). A low-cost flow cytometric assay for the detection and quantification of apoptosis using an anionic halogenated fluorescein dye. *Biotechniques* 45: 317-20.
- Milner JA (1996). Garlic: its anticarcinogenic and antitumorogenic properties. *Nutr. Rev.* 54: S82–S86.
- Milner JA (2001). A historical perspective on garlic and cancer. *J. Nutr.* 131: 1027S–1031S.
- Milner JA (2001). Mechanisms by which garlic and allyl sulphur compounds suppress carcinogen bioactivation. Garlic and carcinogenesis, *Adv Exp Med Biol* 492: 69–81.

- Miyamoto S, Howes AL, Adams JW, Dorn GW, Brown JH (2005).  $\text{Ca}^{2+}$  Dysregulation Induces Mitochondrial Depolarization and Apoptosis: Role of  $\text{Na}^+/\text{Ca}^{2+}$  Exchanger and AKT. *The Journal of Biological Chemistry*. 280: 38505.
- Modrak DE (2004). Synergistic Interaction between Sphingomyelin and Gemcitabine Potentiates Ceramide-Mediated Apoptosis in Pancreatic Cancer. *Journal of Cancer Research*. 64: 8405–8410.
- Murphy AN, Fiskum G, Flint Beal M (1999). Mitochondria in neuro-degeneration: bio-energetic function in cell life and death. *J Cereb Blood Flow Metab* 19: 231-245.
- Natoni F (2005). Sodium butyrate sensitises human pancreatic cancer cells to both the intrinsic and the extrinsic apoptotic pathways. *Journal of Biochimica et Biophysica Acta*. 1745: 318–329.
- Nicotera P, Leist M (1997). Energy supply and the shape of cell death in neurons and lymphoid cells. *Cell Death Differ* 4: 435-442.
- Nigro P, Bloise E, Turco MC, Skhirtladze A, Montoro P, Pizza C, Piacente S, Belisario MA (2007). Antiproliferative and pro-apoptotic activity of novel phenolic derivatives of resveratrol. *Journal of Life Sciences*. 81: 873–883.
- Ohta T, Kinoshita T, Naito M, Nozaki T, Masutani M, Tsuruo T, Miyajima A (1997). Requirement of the caspase-3/CPP32 protease cascade for apoptotic death following cytokine deprivation in hematopoietic cells. *J Biol Chem* 272: 23111–23116.
- Oommen S, Anto RJ, Srinivas G, Karunagaran D (2004). Allicin (from garlic) induces caspase-mediated apoptosis in cancer cells, *Eur J Pharmacol* 485: 97–103.
- Owuor ED, Kong AN (2002). Antioxidants and oxidants regulated signal transduction pathways, *Biochem. Pharmacol*. 64: 765–770.



- Papucci L, Schiavone N, Witort E, Donnini M, Lapucci A, Tempestini A, Formigli L, Zecchi-Orlandini S, Orlandini G, Carella G, Brancato R, Capaccioli S (2003). Coenzyme Q10 Prevents Apoptosis by Inhibiting Mitochondrial Depolarization Independently of Its Free Radical Scavenging Property. *The Journal of Biological Chemistry*. 278: 28220–28228.
- Piedrafita FJ, Pfahl M (1997). Retinoid-induced apoptosis and Sp1 cleavage occur independently of transcription and require caspase activation. *Mol Cell Biol* 17: 6348–6358.
- Pinto JT, Lapsia S, Shah S, Santiago H, Kim G (2001). Antiproliferative effects of garlic-derived and other allium related compounds. *Adv. Exp. Med. Biol.* 492: 83–106.
- Polverino AJ, Patterson SD (1997). Selective activation of caspases during apoptotic induction in HL-60 cells. Effects of a tetrapeptide inhibitor. *J Biol Chem* 272: 7013–7021.
- Qanungo S, Das M, Haldar S, Basu A (2005). Epigallocatechin-3-gallate induces mitochondrial membrane depolarization and caspase-dependent apoptosis in pancreatic cancer cells. *Journal of Carcinogenesis*. 26: 958–967.
- Quadrilatero J, Hoffman-Goetz L (2004). *N*-Acetyl-L-cysteine prevents exercise-induced intestinal lymphocyte apoptosis by maintaining intracellular glutathione levels and reducing mitochondrial membrane depolarization. *Journal of Biochemical and Biophysical Research Communications*. 319: 894–901.

- Ramljak D (2005). Pentameric procyanidin from *Theobroma cacao* selectively inhibits growth of human breast cancer cells. *Journal of Molecular Cancer Therapy*. 4: 537–546.
- Reed JC (1997). Cytochrome c: can't live with it - can't live without it. *Cell* 91: 559-562.
- Reungpatthanaphong P (2003). Rhodamine B as a mitochondrial probe for measurement and monitoring of mitochondrial membrane potential in drug-sensitive and - resistant cells. *Journal of Biochemical Biophysical Methods*. 57: 1–16.
- Robert V, Mouille B, Mayeur C, Michaud M, Blachier F (2001). Effects of the garlic compound diallyl disulfide on the metabolism, adherence and cell cycle of HT-29 colon carcinoma cells: evidence of sensitive and resistant subpopulations. *Carcinogenesis* 22: 1155–1161.
- Rotem R (2005). Jasmonates: Novel Anticancer Agents Acting Directly and Selectively on Human Cancer Cell Mitochondria. *Journal of Cancer Research*. 65: 1984–1993.
- Sakahira H, Enari M, Nagata S (1998). Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis. *Nature* 391: 96–99.
- Salvesen GS, Rønsbo M (2002). Apoptosome: the seven-spoked death machine. *Dev Cell* 2: 256 – 257.
- Schlegel J, Peters I, Orrenius S, Miller DK, Thornberry DA, Yamin TT, and Nicholson DW (1996). CPP32/apopain is a key interleukin 1 $\beta$  converting enzyme-like protease involved in Fas-mediated apoptosis. *J Biol Chem* 271: 1841–1844.

- Sharif-Askari E, Nakhaei P, Oliere S, Tumilasci V, Hernandez E, Wilkinson P, Lin R, Bell J, Hiscott J (2007). Bax-dependent mitochondrial membrane permeabilization enhances IRF3-mediated innate immune response during VSV infection. *Journal of Virology*. 365: 20–33.
- Shibayama-Imazu T (2006). Production of superoxide and dissipation of mitochondrial transmembrane potential by vitamin K2 trigger apoptosis in human ovarian cancer TYK-nu cells. *Journal of Apoptosis*. 11: 1535–1543.
- Shirin H, Pinto JT, Kawabata Y, Soh JW, Delohery T, Moss SF, Murty S, Rivlin RS, Holt PR, Weinstein IB (2001). Antiproliferative effects of *S*-allylmercaptocysteine on colon cancer cells when tested alone or in combination with sulindac sulfide. *Cancer Res*. 61: 725–731.
- Siegel G, Malmsten M, Pietzsch J, Schmidt A, Buddecke E, Michel F (2001). The effect of garlic on arteriosclerotic nanoplaque formation and size, *Phytomedicine* 11: 24–35.
- Sigounas G, Hooker J, Anagnostou A, Steiner M (1997). *S*-allylmercaptocysteine inhibits cell proliferation and reduces the viability of erythroleukemia, breast, and prostate cancer cell lines. *Nutr. Cancer* 27: 186–191.
- Souza-Fagundes EM (2003). Myriadenolide, a labdane diterpene isolated from *Alomia myriadenia* (asteraceae) induces depolarization of mitochondrial membranes and apoptosis associated with activation of caspases-8, -9, and -3 in Jurkat and THP-1 cells. *Journal of Experimental Cell Research*. 290: 420–426

- Steinmetz KA, Kushi LH, Bostick RM, Folsom AR, Potter JD (1994). Vegetables, fruit and colon cancer in the Iowa Women's Health Study. *Am. J. Epidemiol.* 139:1-13.
- Steller H (1995). Mechanisms and genes of cellular suicide. *Science* 267: 1445–1449.
- Stepczynska A, Lauber K, Engels IH, Janssen O, Kabelitz D, Wesselborg S (2001). Staurosporine and conventional anticancer drugs induce overlapping, yet distinct pathways of apoptosis and caspase activation. *Oncogene* 20: 1193 – 202.
- Sun XM, MacFarlane M, Zhuang J, Wolf BB, Green DR, Cohen GM (1999). Distinct caspase cascades are initiated in receptor-mediated and chemical-induced apoptosis. *J. Biol. Chem.* 274: 5053–5060.
- Surh YJ, Hurh YJ, Kang JY, Lee E, Kong G, Lee SJ (1999). Resveratrol, an antioxidant present in red wine, induces apoptosis in human promyelocytic leukemia (HL-60) cells. *Cancer Lett.* 140: 1–10.
- Susin SA, Zamzami N, Castedo M, Hirsch T, Marchetti P, Macho A, Daugas E, Geuskens M, Kroemer G (1996). Bcl-2 inhibits the mitochondrial release of an apoptogenic protease. *J Exp Med* 184: 1331-1342.
- Thornberry NA, Lazebnik Y (1998). Caspases: enemies within. *Science* 281: 1312–1316.
- Tiano L, Fedeli D, Ballarini P, Santoni G, Falcioni G (2001). Mitochondrial membrane potential in density-separated trout erythrocytes exposed to oxidative stress in vitro. *Journal of Biochimica et Biophysica Acta.* 1505: 226–237

- Tsao M, Yin M (2001). In vitro activity of garlic oil and four diallyl sulphides against antibiotic-resistant *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*, *J Antimicrob Chemother* 47: 665–670.
- Tsujimoto Y (1997). Apoptosis and necrosis: intracellular ATP level as a determinant for cell death modes. *Cell Death Differ* 4: 429-434.
- Van Wyk BE, Wink M (2004). Medicinal Plants of the World. Briza Publications, South Africa.
- Vaux DL (1997). CED-4—the third horseman of apoptosis. *Cell* 90: 389–390.
- Wu CC, Sheen LY, Chen HW, Tsai SJ, Lii CK (2001). Effects of organosulfur compounds from garlic oil on the antioxidation system in rat liver and red blood cells, *Food Chem Toxicol* 39: 563–569.
- Wyllie AH, Kerr JF, Currie AR (1980). Cell death: the significance of apoptosis. *Int Rev Cytol* 68: 251–306.
- Wyllie AH (1980). Glucocorticoid induced thymocyte apoptosis is associated with endogenous endonuclease activation. *Nature* 284: 555–6.
- Xiao D, Pinto JT, Soh JW, Deguchi A, Gundersen GG, Palazzo AF, Yoon JT, Shirin H, Weinstein IB (2003). Induction of Apoptosis by the Garlic-Derived Compound S-Allylmercaptocysteine (SAMC) Is Associated with Microtubule Depolymerization and c-Jun NH<sub>2</sub>-Terminal Kinase Activation. *Cancer Research* 63: 6825 – 6837.
- You WC, Blot WJ, Chang YS, Ershow A, Yang ZT, An Q, Henderson BE, Fraumeni JF, Wang TG (1989). Allium vegetables and reduced risk of stomach cancer. *J. Natl. Cancer Inst.* 81: 162-164.

- Zamzami N, Susin SA, Marchetti P, Hirsch T, Gomez-Monterrey I, Castedo M, Kroemer G (1996). Mitochondrial control of nuclear apoptosis. *J Exp Med* 183:1533-1544.
- Zhang XD, Gillespie SK, Hersey P (2004). Staurosporine induces apoptosis of melanoma by both caspase-dependent and - independent apoptotic pathways. *Mol Cancer Ther* 3: 187 – 97.
- Zhao K (2005). Mitochondria-targeted peptide prevents mitochondrial depolarization and apoptosis induced by tert-butyl hydroperoxide in neuronal cell lines. *Journal of Biochemical Pharmacology*. 70: 1796–1806.
- Zhivotovsky B, Burgess DH, Vanags DM, Orrenius S (1997). Involvement of cellular proteolytic machinery in apoptosis. *Biochem Biophys Res Commun* 230: 481–488.
- Zou H, Henzel WJ, Liu X, Lutschg A, Wang X (1997). Apaf-1, a human protein homologous to *C. elegans* CED-4, participates in cytochrome c-dependent activation of caspase-3. *Cell* 90: 405–413.
- Zunino SJ, Storms DH (2006). Resveratrol-induced apoptosis is enhanced in acute lymphoblastic leukemia cells by modulation of the mitochondrial permeability transition pore. *Journal of Cancer Letters*. 240: 123–134.

## **CHAPTER 5**



**An assessment of the toxicity of**  
*Tulbaghia alliacea* **phytotherapy**

UNIVERSITY of the

## 5.1. Abstract

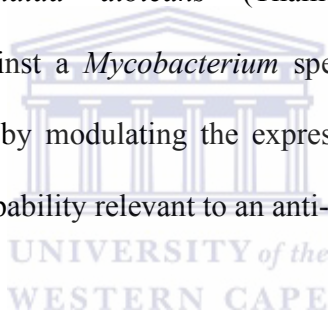
*Tulbaghia alliacea* is used traditionally as a plant medicine for a variety of ailments. Scientific investigations have shown that this indigenous phytotherapy does indeed possess a variety of biological activities *in vitro*; it has significant antifungal activity against the opportunistic pathogen *Candida albicans*, antimycobacterial activity against a *Mycobacterium* species, is able to stimulate the immune system by modulating the expression of interferon-gamma, and has apoptotic-inducing capability relevant to an anti-cancer remedy. There is, however, no scientific information on its *in vivo* safety and toxicity. Establishment of this information is critical, as this phytotherapy has a long history of medicinal use in indigenous communities. In this study, the focus was on investigating the acute and sub-acute toxicity in mice administered different doses of the plant medicine, by analyzing various haematological [red blood cell (RBC), white blood cell (WBC), platelet count, and levels of haemoglobin, haematocrit, mean corpuscular volume, mean corpuscular haemoglobin concentration, lymphocytes, monocytes, eosinophils and neutrophils] and biochemical [glucose, cholesterol, aspartate transaminase (AST) and alanine transaminase(ALT)] markers. None of the animals in this study died, and neither did they exhibit any changes in their general appearance or behavior. In addition, there were no changes in body weight or food and water intake. The haematological analyses showed no significant differences in RBC, WBC and platelet counts in the control and treated groups. Likewise, the plasma biochemistry results showed that AST, ALT, glucose and total cholesterol were similar for the control and treated groups. These values were also well within the normal physiological range, indicating that *Tulbaghia alliacea* phytotherapy at the administered doses is non-toxic in mice.



**Keywords:** *Tulbaghia alliacea*, toxicity, mice, haematology, AST, ALT, glucose, cholesterol.

## 5.2. Introduction

*Tulbaghia alliacea* is used traditionally as a plant medicine for a variety of ailments. Our scientific investigations thus far have shown this indigenous phytotherapy to indeed possess biological activity *in vitro*; it has significant antifungal activity against the opportunistic pathogen *Candida albicans* (Thamburan *et al.*, 2006), and antimycobacterial activity against a *Mycobacterium* species, as well as being able to stimulate the immune system by modulating the expression of interferon-gamma, and showing apoptotic-inducing capability relevant to an anti-cancer remedy.



Even though this indigenous phytotherapy is widely used by many communities, there is no information about its *in vivo* safety and toxicity. This study was thus designed to investigate the safety of the aqueous extract of *Tulbaghia alliacea*, by determining its behavioural and pharmaco-toxicological effects after acute and sub-acute administration in an *in vivo* mouse model.

Investigation of the acute toxicity is the first step in the toxicological analysis of herbal drugs (Deciga-Campos, Rivero-Cruz, Arriaga-Alba, Castaneda-Corral, Angeles-Lopez, Navarrete, Mata, 2007). Acute oral toxicity is the adverse effects occurring within a short period of time after oral administration of a single dose of a substance (OECD, 2001).

In the assessment and evaluation of the toxic characteristics of a substance, determination of acute oral toxicity is usually the initial step. It provides information on health hazards likely to arise from short-term exposure by the oral route. An evaluation of acute toxicity data includes the relationship, if any, between the exposure of animals to the test substance and the incidence and severity of all abnormalities, gross lesions, body weight changes, effects on mortality, and any other toxic effects (USEPA, 1998; Loomis and Hayes, 1996; Timbrell, 2002; Pascoe, 1983).

## **5.3. Materials and Methods**

### **5.3.1. Preparation of plant extract**

An aqueous extract of *T.alliacea* was prepared. Bulbs of *T.alliacea* were washed and chopped into tiny pieces, and then soaked in distilled water overnight, and then vacuum filtered. The crude aqueous extract was then dissolved in saline (0.9% NaCl solution) to make up doses of 7.5, and 10.0 g/body weight, which were administered to the mice by oral gavage.

### **5.3.2. Animals and animal care**

Ethical approval for this study was granted by the Ethics Committee of the University of the Western Cape. Healthy BALB/C mice of both sexes, weighing between 20-30g were obtained from the University of Cape Town Animal Unit, where they had been bred under conventional conditions for research purposes. Prior to dosing and the start of the

experiment, the animals were kept for 5 days at the Animal Room at the School of Pharmacy, UWC, to allow for their acclimatization to the laboratory conditions. The animals were divided into three (3) groups of 6 animals each, matched for weight and size. The animals were housed 3 mice per sex per cage in a well ventilated room with a 12h cycle of day and night conditions, and temperature maintained at 25°C. All animals had free access to tap water and food throughout the experiment, except for the short fasting period before the oral administration of the *Tulbaghia alliacea* phytotherapy. The selected individual animals were weighed and identified via markings made on their tails with permanent ink pens. Additionally, all cages were labeled with details of the animals' sex and the dose administered.

### 5.3.3. Acute toxicity



The acute toxicity of the *Tulbaghia alliacea* phytotherapy was evaluated in mice using the up and down procedure of (OECD, 2001) of the *Acute Oral Toxicity (Guideline 425)* statistical program (AOT425statPgm). The main test was conducted, whereby the first animal was administered a dose and observed for behavioral changes and signs of toxicity. This information was then fed into the computer-based program, after which the next dose was recommended. This method of sequential dosing continued until adverse effects were observed or the test was complete.

The first mouse received *Tulbaghia alliacea* aqueous extract at 1g/kg orally by gavage. The general behavior of the mice were monitored for 1h after dosing, periodically during

the first 24h (with special attention given during the first 4 h) (Hilaly *et al.*, 2004) and then daily thereafter, for a total of 14 days. Changes in the normal activity of mice and their weights were monitored, and the time at which signs of toxicity appeared, were recorded. The next dose to be recommended was 5 g/kg. As this was the limit dose, three animals were sequentially dosed at this level.

### **5.3.4. Sub-acute Toxicity**

#### **5.3.4.1. Experimental animals**

Mice of both sexes (weight: 17-25g, age: 6-8 weeks) were randomly assigned into three groups ( $n = 6$ ), three males and three females in each group. Groups of three mice were housed together in cages (males separated from females). Treatments were administered orally by gavage once a day for 14 days. The first group of animals, serving as control, received normal saline (300 $\mu$ l); the second and third group received the aqueous extract of *Tulbaghia alliacea* at doses of 7.5 and 10.0 g/kg respectively. All mice were observed daily for physiological and behavioral changes.

#### **5.3.4.2. Haematological and biochemical analysis**

On day 15, all surviving animals were then anaesthetized with dry ice and their chests quickly opened via cervical dislocation. Blood was drawn from the most strongly beating position of the heart, and immediately collected into two tubes; heparinized centrifuge tubes and EDTA tubes. The heparinized blood was used for

plasma biochemistry and assayed for glucose, aspartate transaminase (AST), alanine transaminase (ALT), and total cholesterol. Smears made from whole blood with EDTA anticoagulant were used for haematological analysis which included red blood count (RBC), white blood cell count (WBC), platelet count, haemoglobin, haematocrit, mean corpuscular volume, mean corpuscular haemoglobin concentration, as well as levels of lymphocytes, monocytes, eosinophils and neutrophils. Table 1 contains the methods used for individual analyses.

**Table 1.** Analytical methods

<b>Test</b>	<b>Method</b>	<b>Units</b>
Aspartate aminotransferase (AST)	VITROS AST Slide	IU/l
Alanine aminotransferase (ALT)	VITROS ALT Slide	IU/l
Glucose	VITROS GLU Slide	mmol/l
Total Cholesterol	VITROS CHOL Slide	mmol/l
Red blood cells (RBC)	CELL-DYN <sup>®</sup> 3700 System	$\times 10^{12}/l$
White blood cells (WBC)	CELL-DYN <sup>®</sup> 3700 System	$\times 10^9/l$
Haemoglobin (Hb)	CELL-DYN <sup>®</sup> 3700 System	g/dl
Haematocrit (Hct)	CELL-DYN <sup>®</sup> 3700 System	%
Mean cell volume (MCV)	CELL-DYN <sup>®</sup> 3700 System	fl
Mean corpuscular Hb [] (MCHC)	CELL-DYN <sup>®</sup> 3700 System	g/dl
Platelet count	CELL-DYN <sup>®</sup> 3700 System	$\times 10^9/l$
Differentials (neutrophils, lymphocytes, monocytes, eosinophils)	Cytochemical staining	%

## **5.4. Results**

### **5.4.1. Acute toxicity**

No death was recorded in the 14 days of observation period in the animals given the 1 and 5g/kg of the *T.alliacea* phytotherapy (Fig. 1). The animals also did not show any changes in their general appearance or behavior.

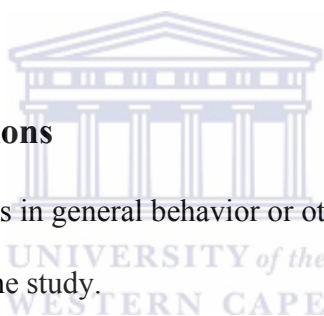
### **5.4.2. Sub-acute toxicity**

#### **5.4.2.1. General observations**

No deaths or significant changes in general behavior or other physiological activities were observed at any point in the study.

#### **5.4.2.2. Body weight, food and water intake**

The body weights of the control and treated groups did not change significantly during the study (Table 2). No differences or changes in food and water intake were observed either.



Test sequence	Animal ID	Dose (mg/kg)	Short-term result	Long-term result
1	1	1000	O	O
2	2	5000	O	O
3	3	5000	O	O
4	4	5000	O	O

(X = Died, O = Survived)

Dose Recommendation: The main test is complete.

Stopping criteria met: 3 at Limit Dose.

#### SUMMARY OF LONG-TERM RESULTS:

Dose (mg/kg)	Survived (O)	Died (X)	Total
1000	1	0	1
5000	3	0	3
All Doses	4	0	4

#### Statistical Estimate based on long term outcomes:

The LD<sub>50</sub> is greater than 5000 mg/kg.

**Fig 1.** Acute toxicity study of *T.alliacea* phytotherapy; results generated by the AOT425statPgm.

**Table 2.** Body weights of mice in the sub-acute toxicity study.

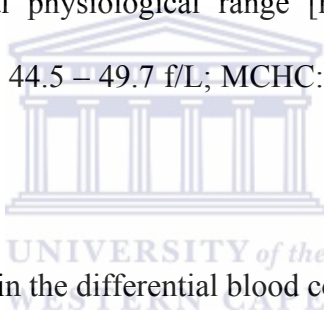
<b>Group #</b> <b>Dose</b>	<b>Animal</b> <b>ID</b>	<b>Bodyweight (g)</b>	
		<b>Day 1</b>	<b>Day 14</b>
<b>Group 1</b> <b>(0 g/kg)</b>	<b>1</b>	24.8	26.0
	<b>2</b>	23.8	25.6
	<b>3</b>	21.7	23.6
	<b>4</b>	19.0	20.4
	<b>5</b>	18.9	21.0
	<b>6</b>	17.1	19.5
	<b>Average ± SEM</b>	20.88 ± 1.24	22.68 ± 1.13
<b>Group 2</b> <b>(7.5 g/kg)</b>	<b>1</b>	24.0	24.4
	<b>2</b>	23.8	24.5
	<b>3</b>	23.1	22.8
	<b>4</b>	19.2	19.1
	<b>5</b>	18.8	19.1
	<b>6</b>	20.3	19.8
	<b>Average ± SEM</b>	21.53 ± 0.97	21.62 ± 1.06
<b>Group 3</b> <b>(10 g/kg)</b>	<b>1</b>	23.5	24.3
	<b>2</b>	24.7	23.8
	<b>3</b>	22.8	24.4
	<b>4</b>	17.2	20.1
	<b>5</b>	18.0	18.7
	<b>6</b>	19.2	19.2
	<b>Average ± SEM</b>	20.9 ± 1.29	21.75 ± 1.10



### 5.4.2.3. Haematological and biochemical analyses

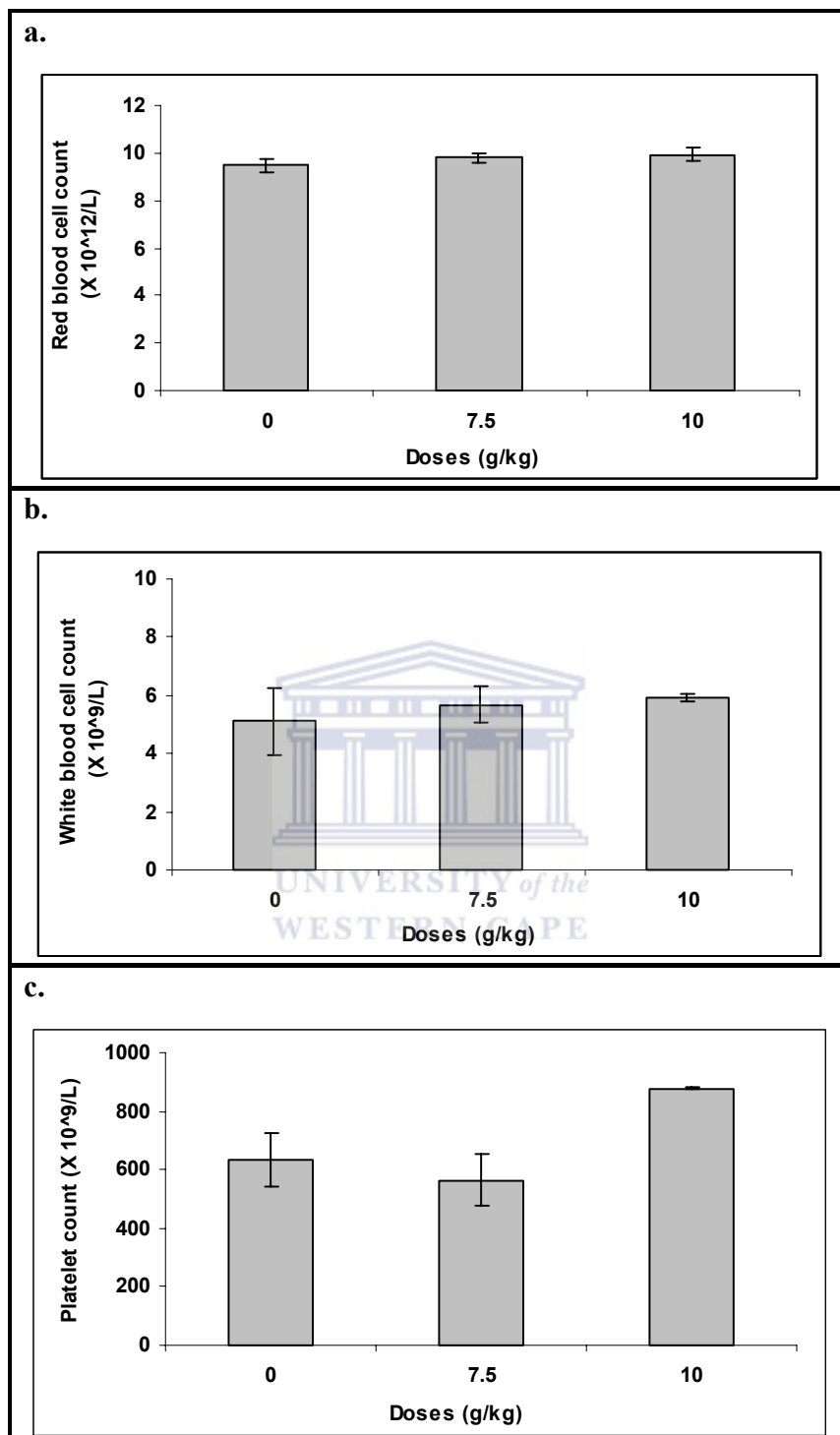
The haematological analyses showed no significant differences in RBC, WBC and platelet counts in the control and treated groups (Fig. 2 a-c). These values were also well within the normal physiological range [RBC:  $7.9 - 10.0 \times 10^{12}/L$ ; platelets:  $860 - 1400 \times 10^9/L$ ] (Wolford *et al.*, 1986).

Figure 3 (a-d) provides the data for the haemoglobin and haematocrit levels as well the MCV and MCHC. No differences between the groups were detected, and these indices were also within the normal physiological range [haemoglobin:  $13.9 - 16.7 \text{ g/dL}$ ; haematocrit:  $37 - 42 \%$ ; MCV:  $44.5 - 49.7 \text{ f/L}$ ; MCHC:  $34.8 - 38.2 \text{ g/dL}$ ] (Wolford *et al.*, 1986).



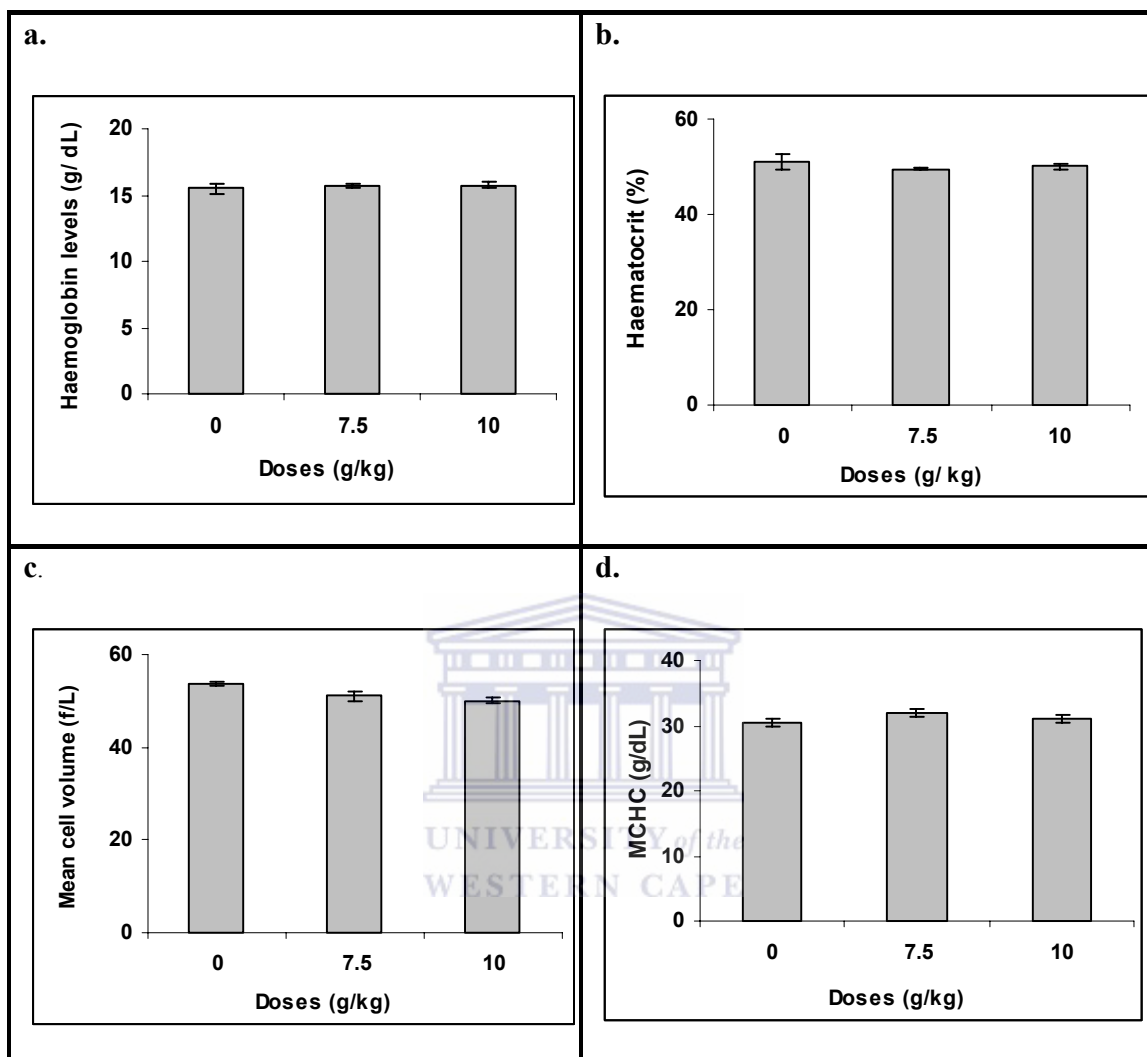
Slight differences were noted in the differential blood counts i.e lymphocytes, monocytes, neutrophils and eosinophils (Fig. 4 a-d). However, these were still within the normal physiological range [lymphocytes:  $63 - 90\%$ ; monocytes:  $0 - 5\%$ , neutrophils:  $8 - 33\%$ ] (Wolford *et al.*, 1986), except for the eosinophil count in the control and  $7.5 \text{ g/kg}$  group which were a little above the normal range [eosinophils:  $0 - 2\%$ ] (Wolford *et al.*, 1986).

The plasma biochemistry results showed that AST, ALT and total cholesterol were similar for the control and treated groups (Fig. 5 a-c). The  $7.5 \text{ g/kg}$  treated group had a lower glucose level than the untreated and  $10 \text{ g/kg}$  group (Fig. 5d); although all this was still within the normal range [cholesterol:  $1.60 - 4.37 \text{ mmol/L}$ ; glucose:  $3.11 - 11.83 \text{ mmol/L}$ ] (Wolford *et al.*, 1986).



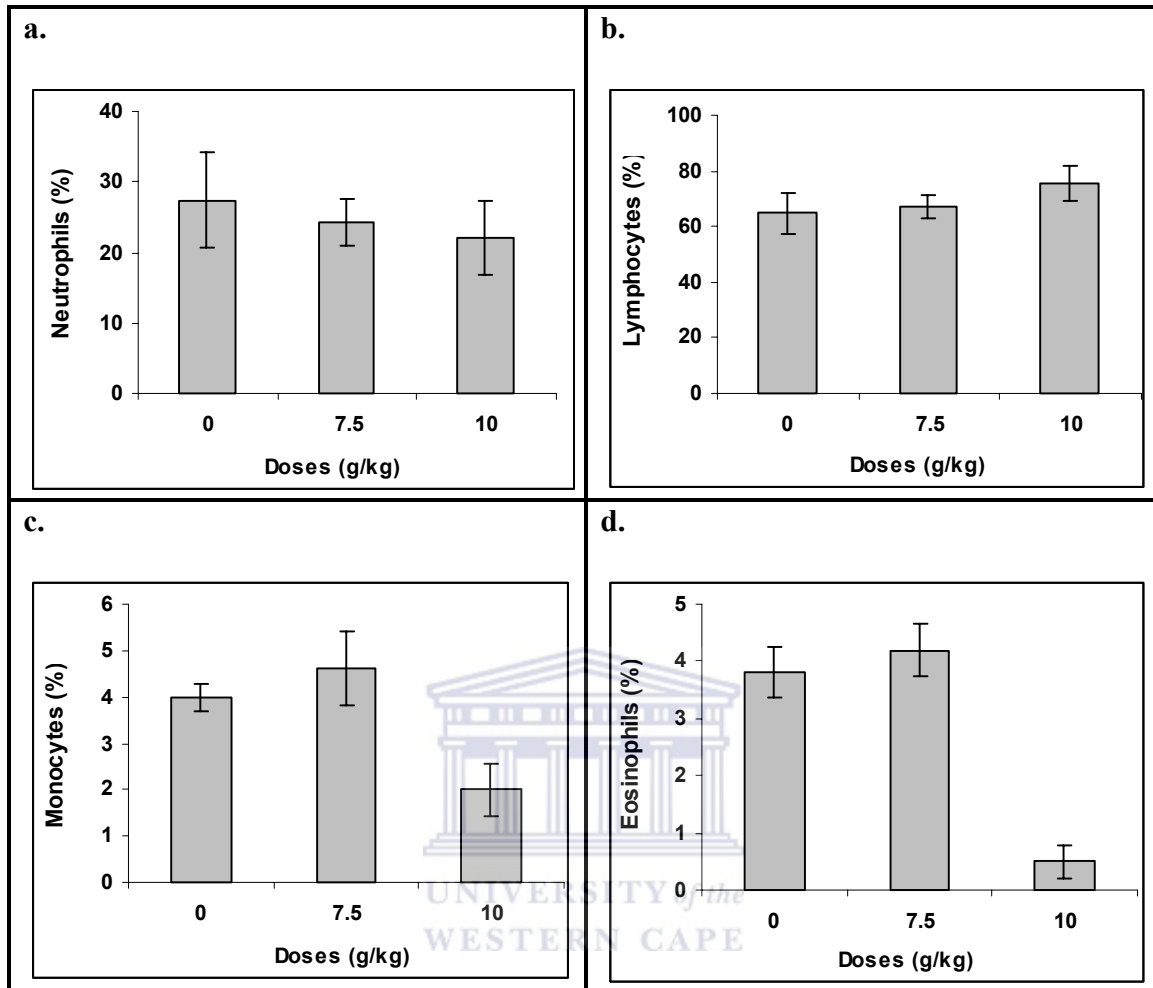
**Fig 2.** Red blood cell (a), white blood cell (b) and platelet (c) counts of mice administered various doses of *T.alliacea* phytotherapy.

All biochemical and haematological data are represented as means  $\pm$  SEM.



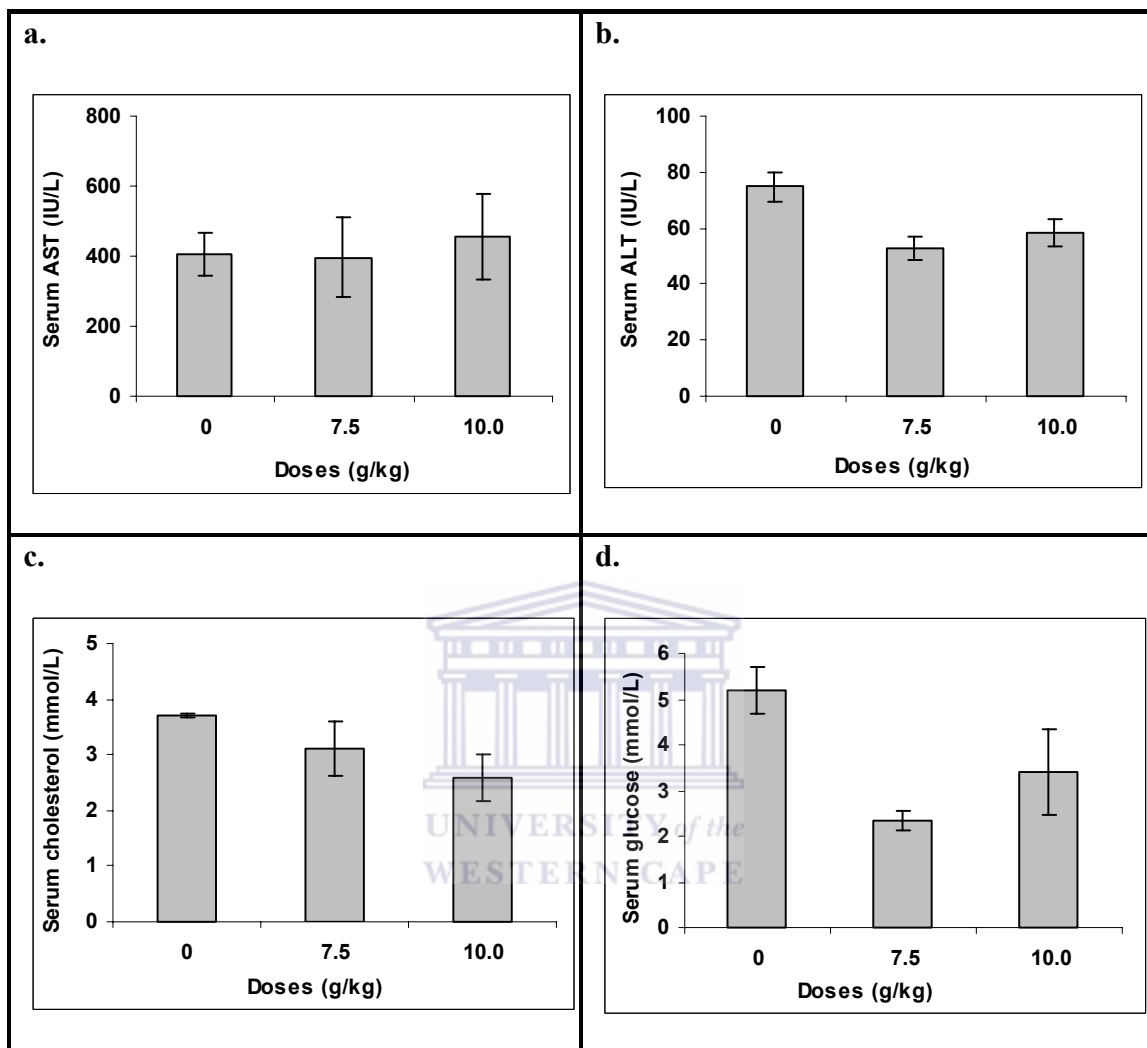
**Fig 3.** The levels of haemoglobin (a), haematocrit, mean cell volume (c) and MCHC (d) of mice administered various doses of *T.alliacea* phytotherapy.

All biochemical and haematological data are represented as means  $\pm$  SEM.



**Fig 4.** The levels of neutrophils (a), lymphocytes (b), monocytes (c) and eosinophils (d) of mice administered different doses of *T. alliacea* phytotherapy.

All biochemical and haematological data are represented as means  $\pm$  SEM.

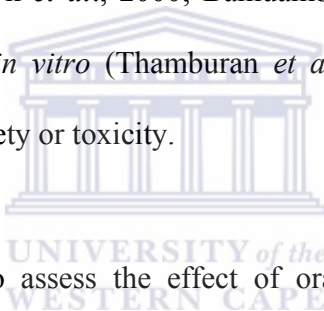


**Fig 5.** Serum levels of total AST (a), total ALT (b), cholesterol (c) and glucose (d) of mice administered different doses of *T.alliacea* phytotherapy.

All biochemical and haematological data are represented as means  $\pm$  SEM.

## 5.5. Discussion

The use of herbal medicines and medicinal plants is on the increase, as consumers seek an alternative to conventional clinical therapy. Such remedies are often believed to be harmless, since these treatments are “natural” and commonly used for self-medication without supervision (Rosidah, Yam, Sadikun, Ahmad, Akowuah, Asmawi, 2009). Experimental screening to ascertain safety, efficacy and toxicity of traditional and herbal products is thus crucial (Veerappan, Miyazaki, Kadarkaraisamy Ranganathan, 2007). *Tulbaghia alliacea*, an indigenous species of garlic is a plant that is used traditionally for a variety of ailments (Van Wyk *et al.*, 2000; Bamuamba *et al.*, 2008) has also shown promising biological activity *in vitro* (Thamburan *et al.*, 2006). To date, there is no information available on its safety or toxicity.



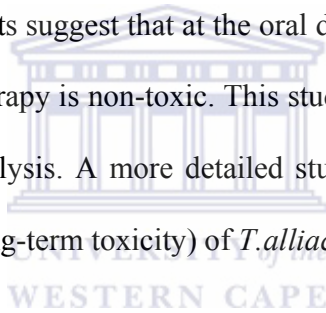
This study therefore aimed to assess the effect of oral administration of *Tulbaghia alliacea* phytotherapy on haematological and biochemical parameters in mice. An acute toxicity study (up-and-down procedure) was carried out to assess the toxicity of *Tulbaghia alliacea* phytotherapy. The concept of the up-and-down testing approach for the determination of acute toxicity of chemicals was first described by Dixon and Mood (Dixon and Mood, 1948; Dixon, 1965; Dixon, 1991a; Dixon, 1991b). The AOT 425 (Acute Oral Toxicity) procedure [which utilizes the up-and-down testing approach] is of value in minimizing the numbers required to estimate the acute oral toxicity of a chemical (OECD, 2001). In addition to the estimation of LD<sub>50</sub> and confidence intervals, the test allows for the observation of signs of toxicity (OECD, 2001).

Using the AOT425 procedure, the first animal received 1 g/kg of *T.alliacea* aqueous extract. After showing no signs of toxicity or mortality after 24 h, the next set of 3 animals received a dose of 5g/ kg. No toxic symptoms, changes in behavior, or mortality, were observed in these animals for the next 14 days after administration of the extract. This was the limit dose per the AOT425statPgm (OECD, 2001), and the LD<sub>50</sub> was thus estimated to be higher than 5 g/kg. Hence, the selection of the higher doses of 7.5 and 10.0 g/kg for the subsequent sub-acute toxicity study.

In the sub-acute toxicity study, it appeared that the aqueous extract of *T.alliacea* did not induce any marked changes in the animals, as evidenced by the absence of toxic symptoms, no changes in water or food ingestion, or abnormal weight loss or gain. The acute toxicity study indicated that *Tulbaghia alliacea* aqueous extract at a dose of 5000 mg/kg caused neither visible signs of toxicity nor mortality. Generally, significant reduction in body weight is a simple and sensitive index of toxicity after exposure to potentially toxic substances (Teo, Stirling, Thomas, Hoberman, Kiorpes, Khetani, 2002). In the present study, *Tulbaghia alliacea* aqueous extract (1000, 5000, 7500 and 10 000 mg/kg, p.o.) did not significantly affect body weight as compared to the control group, which suggests that the extract did not hinder animal growth. This analysis is based on the toxicity classification proposed by Loomis and Hayes (1996), viz., that substances with an LD<sub>50</sub> between 5000 and 15 000 mg/kg bodyweight are regarded as practically non-toxic.

All animals survived until the scheduled euthanasia, and no gross pathological alteration was found in the internal organs (no organ swelling, atrophy or hypertrophy). The haematological and biochemical analyses did not show much variation between the control and treated groups. Furthermore, these parameters were within the normal physiological reference ranges for mice (Wolford, Schroer, Gohs, Gallo, Brodeck, Falk, Ruhren, 1986).

Since there were no changes in animal behavior, body weights, and haematological and biochemical parameters at both doses of the treated animals when compared to the control group, the present results suggest that at the oral doses administered (7.5 g/kg and 10.0 g/kg), *T.alliacea* phytotherapy is non-toxic. This study however, was qualitative and not powered for statistical analysis. A more detailed study is recommended for further analysis of the toxicity (and long-term toxicity) of *T.alliacea* phytotherapy.





## 5.6. References

- Dixon W.J (1991). Design and Analysis of Quantal Dose-Response Experiments (with Emphasis on Staircase Designs). Dixon Statistical Associates, Los Angeles CA, USA.
- Bamuamba K, Gammon DW, Meyers P, Dijoux-Franca M-G, Scott G (2008). Antimycobacterial activity of five plant species used as traditional medicines in the Western Cape Province (South Africa). *Journal of Ethnopharmacology* 117: 385-390.
- Deciga-Campos M, Rivero-Cruz I, Arriaga-Alba M, Castaneda-Corral G, Angeles-Lopez GE, Navarrete A, Mata R, (2007). Acute toxicity and mutagenic activity of Mexican plants used in traditional medicine. *Journal of Ethnopharmacology* 110: 334-342.
- Dixon W.J (1991). Staircase Bioassay: The Up-and-Down Method. *Neurosci. Biobehav. Rev.*15: 47-50.
- Dixon WJ, Mood AM (1948). A Method for Obtaining and Analyzing Sensitivity Data. *J. Amer. Statist. Assoc.*43: 109-126.
- Dixon WJ (1965). The Up-and-Down Method for Small Samples. *J. Amer. Statist. Assoc.* 60: 967-978.
- Hilaly EJ, Israili ZH, Lyoussi B (2004). Acute and chronic toxicological studies of *Ajuga Iva* in experimental animals, *Journal of Ethnopharmacology* 91: 43–50.
- Loomis, T. A. and Hayes, A.W (1996). Loomis's essentials of toxicology. 4<sup>th</sup> ed., California, *Academic press*: 208- 245.

- Organization for Economic Cooperation and Development (2001). OECD Guidelines for testing of chemicals. No. 425. Acute Oral Toxicity: Up-and-Down Procedure. URL: <http://www.epa.gov/oppfead1/harmonization/docs/E425guideline.pdf> [Accessed: 03 November 2008].
- Pascoe D (1983). Toxicology. England, London, *Edward Arnold limited*. 1-60.
- Rosidah, Yam MF, Sadikun A, Ahmad M, Akowuah GA, Asmawi MZ (2009). Toxicology evaluation of standardized methanol extract of *Gynura procumbens*. *Journal of Ethnopharmacology* 123: 244-249.
- Teo S, Stirling D, Thomas S, Hoberman A, Kiorpes A, Khetani V (2002). A 90-day oral gavage toxicity study of D-methylphenidate and D,L- methylphenidate in Sprague-Dawley rats. *Toxicology* 179: 183-196.
- Thamburan S, Klaasen J, Mabusela WT, Cannon JF, Folk W, Johnson Q (2006). *Tulbaghia alliacea* Phytotherapy: A Potential Anti-infective Remedy for Candidiasis. *Phytother Res* 20: 844-850.
- Timbrell, J (2002). Introduction to toxicology. 3<sup>rd</sup> ed., London, *Taylor & Francis*: 163 – 179.
- United States Environmental Protection Agency Prevention (1998). Pesticides and Toxic Substances (7101) EPA 712-C-98-190. Health Effects Test Guidelines OPPTS 870.1100 Acute Oral Toxicity.
- Van Wyk B, Van Oudtshoorn B, Gericke N (2000). *Medicinal Plants of South Africa*. Briza Publications: South Africa.

- Veerappan A, Miyazaki S, Kadarkaraisamy M, Ranganathan D (2007). Acute and subacute toxicity studies of *Aegle marmelos* Corr., an Indian medicinal plant. *Phytomedicine* 14: 209-215.
- Wolford ST, Schroer RA, Gohs FX, Gallo PP, Brodeck M, Falk HB, Ruhren R (1986). Reference range data base for serum chemistry and hematology values in laboratory animals. *J Toxic Environ Health* 18: 161 – 188.



## **CHAPTER 6**



UNIVERSITY *of the*

**General Summary**  
WESTERN CAPE

## 6.1. Introduction

Medicinal plants have been used, since times immemorial in virtually all cultures as a source of medicine. Herbal remedies and plant-based healthcare preparations obtained from traditionally used plants have been traced to the occurrence of natural products with medicinal properties. It is estimated that more than 80% of the world's population utilise plants as their primary source of medicinal agents (Cordell, 1995) and that moreover, traditional medicine is still the only health resource available to about 60% of the world population (Le Grand and Wondergem, 1989).

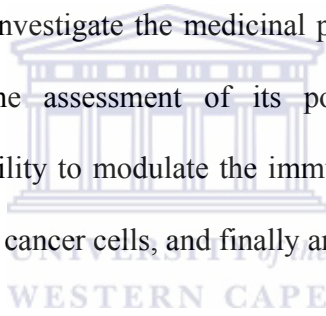
The majority of the population in urban South Africa as well as in smaller rural communities, are reliant on herbal medicines for their health care needs. Apart from their cultural significance, this is because herbal medicines are generally more accessible and affordable (Fennel *et al.*, 2004). As a consequence, there is an increasing trend, worldwide, to integrate traditional medicine with primary health care.

Commercial garlic (*Allium sativum*) is one of the edible plants, which has generated a lot of interest throughout human history as a medicinal panacea. A variety of biological activities have been reported for garlic including effects on tumorigenesis, atherosclerosis, microbial growth, blood sugar modulation and immuno-stimulatory properties (Milner, 2001; Siegel, 2004; Tsao, 2001; Iimuro, 2002). The anti-tumorigenic effects of garlic have been related to induction of apoptosis in tumor cells (Ledezma, 2004; Knowles, 2001; Oommen, 2004; Robert, 2001; Wu, 2001). Garlic also has diverse

antimicrobial activity; a wide range of microorganisms, including bacteria, fungi, protozoa and viruses, have been shown to be sensitive to crushed garlic preparations (Ankri and Mirelman, 1999).

*Tulbaghia alliacea* is an indigenous garlic species that occurs mainly in the Eastern Cape and southern KwaZulu-Natal (Van Wyk *et al.*, 2000). It is used traditionally for fever, colds, asthma, tuberculosis and stomach problems. In Cape Dutch tradition, it is used as a purgative, and for fits, rheumatism and paralysis (Van Wyk *et al.*, 2000).

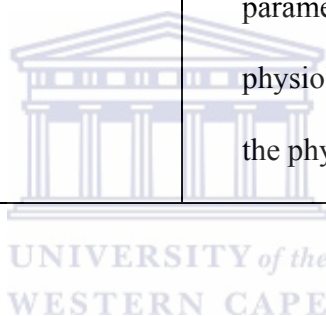
The aim of this study was to investigate the medicinal properties of *Tulbaghia alliacea* extracts with a focus on the assessment of its potential as an antifungal and antimycobacterial agent; its ability to modulate the immune system during infection, its potential to induce apoptosis in cancer cells, and finally an assessment of its *in vivo* safety and toxicity.



**Table 1. Summary of the key findings of this investigation.**

AIMS	OUTCOMES
<b>1. Antifungal Evaluation</b>	
<ul style="list-style-type: none"> <li>▪ To establish if <i>T.alliacea</i> extracts have anti-infective activity against opportunistic pathogenic fungi.</li> <li>▪ To determine its mechanism of antifungal activity.</li> <li>▪ To identify the active compound/s.</li> </ul>	<ul style="list-style-type: none"> <li>▪ The chloroform extract of <i>T.alliacea</i> was most potent in inhibiting the growth of <i>Candidal</i> species.</li> <li>▪ <i>T.alliacea</i> extracts are fungicidal (kills yeast cells).</li> <li>▪ Methyl and methylene groups of <i>Marasmicin</i> were indentified in the <i>T.alliacea</i> inhibitory fraction.</li> </ul>
<b>2. Antimycobacterial Evaluation</b>	
<ul style="list-style-type: none"> <li>▪ To determine if <i>T.alliacea</i> extracts have antimycobacterial activity.</li> </ul>	<ul style="list-style-type: none"> <li>▪ The chloroform extract of <i>T.alliacea</i> was most potent in inhibiting the growth of <i>M.smegmatis</i>.</li> </ul>
<b>3. Immune Modulation Assessment</b>	
<ul style="list-style-type: none"> <li>▪ To assess the potential of <i>T.alliacea</i> extracts to modulate the immune system response during infection.</li> </ul>	<ul style="list-style-type: none"> <li>▪ Aqueous extracts of <i>T.alliacea</i> modulated the immune system by inducing increased expression of IFN-<math>\gamma</math> during infection.</li> </ul>

<b>4. Investigation of anti-cancer properties</b>	
<ul style="list-style-type: none"> <li>▪ To evaluate the apoptosis-inducing potential of <i>T.alliacea</i> extracts in human cancer cell lines.</li> </ul>	<ul style="list-style-type: none"> <li>▪ Aqueous and chloroform extracts of <i>T.alliacea</i> induced apoptosis in human cancer cell lines differentially.</li> </ul>
<b>5. Assessment of <i>in vivo</i> safety and toxicity</b>	
<ul style="list-style-type: none"> <li>▪ To assess the effects of oral consumption of <i>T.alliacea</i> extracts on physiological parameters <i>in vivo</i>.</li> </ul>	<ul style="list-style-type: none"> <li>▪ No toxic symptoms were observed in mice treated with the extracts.</li> <li>▪ Haematological and biochemical parameters were within the normal physiological ranges, indicating that the phytotherapy is safe.</li> </ul>





## 6.2. Antifungal Evaluation

Oral candidiasis, caused by *Candida albicans*, is the most prevalent oral complication in HIV/AIDS patients. A comparative study of the antifungal effects of the indigenous South African garlic plants *T.alliacea* and *T.violacea*, to the commercial variety *A.sativum*, was conducted. This comparison was made because of the reputed antimicrobial effects of the commercial garlic species (Uchida *et al.* 1975) and traditional use of the indigenous varieties (van Wyk *et al.*, 2000; Motsei, *et al.*, 2003). Aqueous, methanol and chloroform extracts of the garlic plants were evaluated for antifungal activity against a variety of pathogenic fungi (*Candida albicans*, *Candida glabrata*, *Candida krusei*, *Cryptococcus neoformans*).

All extracts of *T.alliacea* were more potent than the other two garlic species, in inhibiting the growth of *C.albicans*, and in most cases the difference was statistically significant i.e.  $P < 0.05$  (Table 2). A preliminary viability assay revealed the *T.alliacea* extract to be fungicidal (kills yeast cells rather than merely inhibiting growth).

TLC-bioautography separation of the chloroform extract separated into three major fractions, with one predominant inhibitory fraction (Fig 1). NMR spectral analysis of this inhibitory fraction indicated the presence of the methyl and methylene groups of Marasmicin. These chemical shift values were in agreement with those reported for the same compound, previously isolated from the related *Tulbaghia violacea* (Kubec *et al.* 2002).

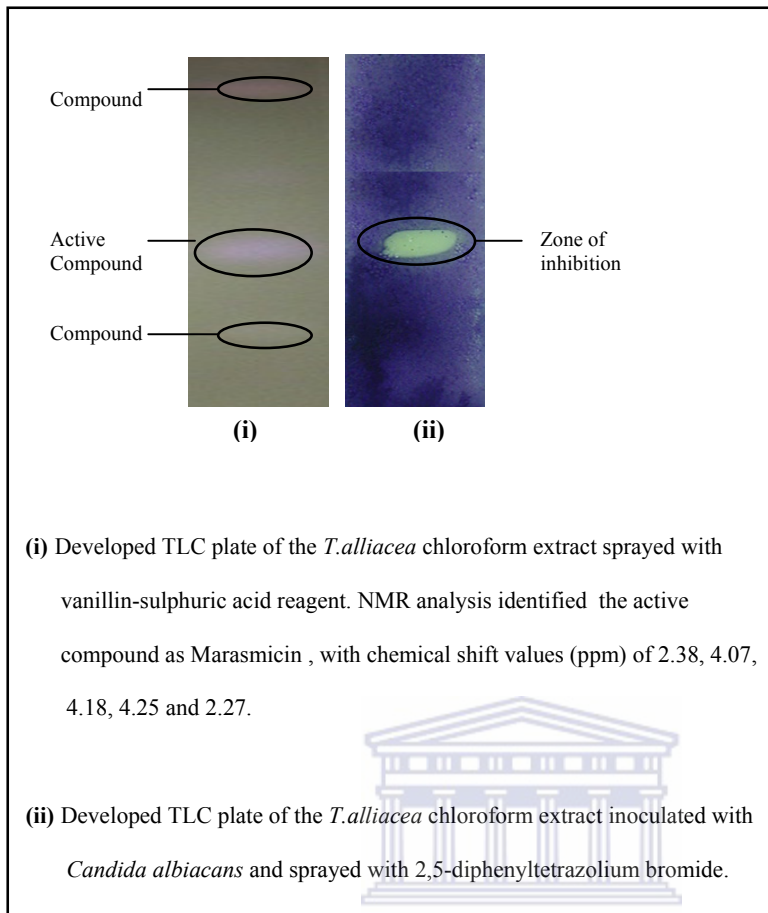
**Table 2.** The anti-infective activity of *Tulbaghia alliacea*, *Tulbaghia violacea* and *Allium sativum* extracts against *Candida albicans*\*

Extract	Concentration (%)	Zone of Inhibition (mm ± SEM)			Statistical significance*		
		<i>A. sativum</i> (a)	<i>T. alliacea</i> (b)	<i>T. violacea</i> (c)	ab	ac	bc
Aqueous (H <sub>2</sub> O)	0.06	3.33 ± 0.33	4.33 ± 0.33	0	0.1012	0.0006	0.0002
	0.15	4.00 ± 0.00	6.33 ± 0.33	0	0.0022	0.0002	0.0001
	0.30	5.33 ± 0.33	8.33 ± 0.33	1.00 ± 0.00	0.0031	0.0002	0.0001
Methanol (MeOH)	0.06	1.67 ± 0.33	4.00 ± 0.00	0	0.0023	0.0075	0.0002
	0.15	3.67 ± 0.33	5.33 ± 0.33	0.66 ± 0.33	0.0241	0.0031	0.0006
	0.30	5.33 ± 0.33	7.33 ± 0.33	3.33 ± 0.33	0.0132	0.0132	0.0011
Chloroform (CHCl <sub>3</sub> )	0.06	4.00 ± 0.00	5.33 ± 0.33	0	0.1012	0.0006	0.0002
	0.15	4.33 ± 0.33	7.33 ± 0.33	0	0.0031	0.0002	0.0001
	0.30	5.33 ± 0.33	10.67 ± 0.33	2.33 ± 0.33	0.0004	0.0031	0.0001

\* *P* values for comparing main effects: ab = *A. sativum* and *T. alliacea*, ac = *A. sativum* and *T. violacea*, bc = *T. alliacea* and *T. violacea*.

# Fluconazole (2%) inhibited *C. albicans* with a 7mm zone of inhibition.

Negative controls (water, methanol and chloroform) did not inhibit *C. albicans*, hence no zone of inhibition was observed.



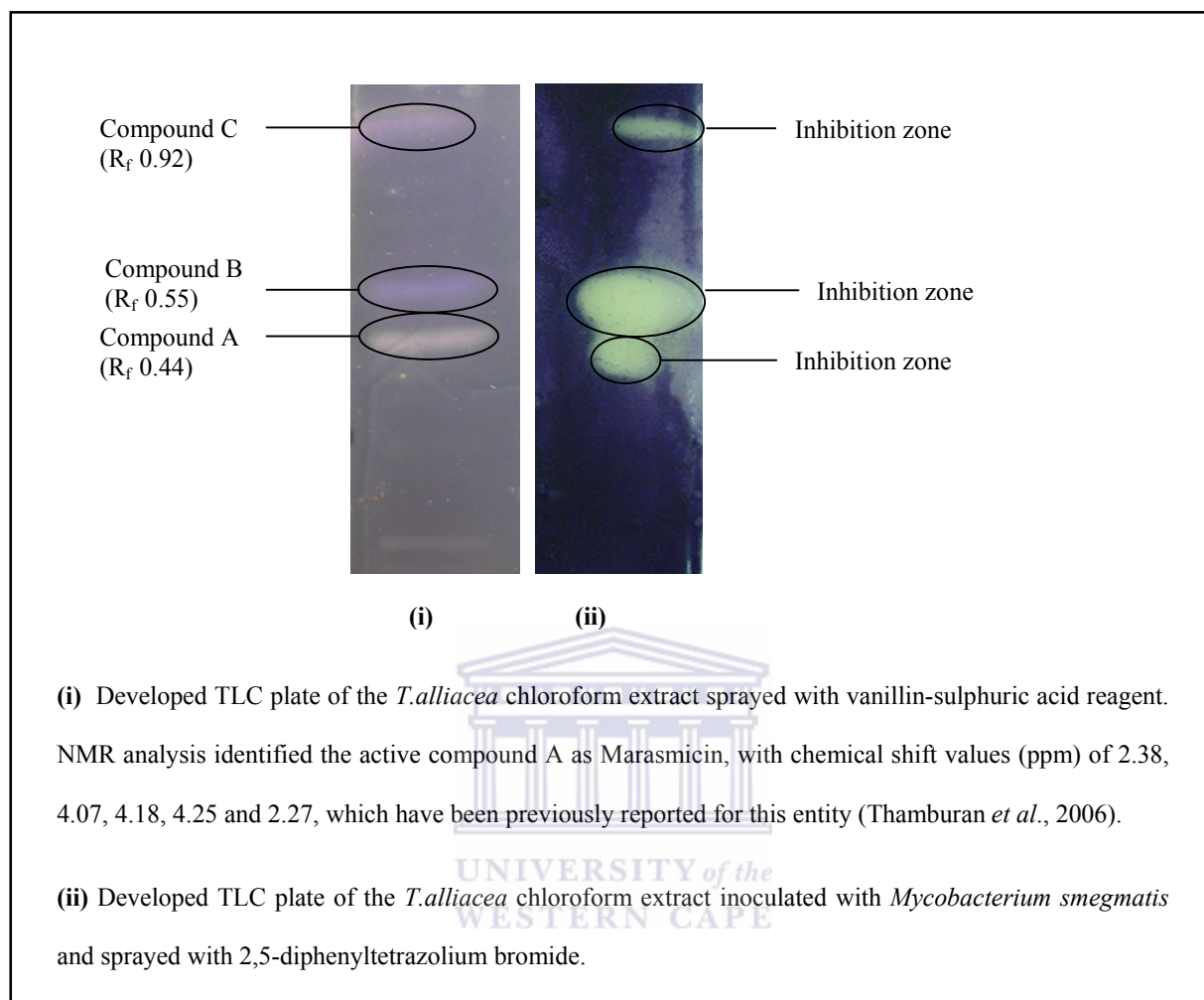
**Fig 1.** TLC-bioautography of *T.alliacea* phytotherapy showing antifungal activity.

### 6.3. Antimycobacterial Evaluation

It is believed that indigenous garlic, *Tulbaghia alliacea* might have some antimycobacterial activity since it is traditionally used for fever, colds, asthma and tuberculosis (TB) more specifically (Van Wyk *et al.*, 2000; Bamuamba *et al.*, 2008).

For the antimycobacterial screening of *T.alliacea* extracts, *Mycobacterium smegmatis* was used as a test model organism due to its genetic similarity to *Mycobacterium tuberculosis*. In comparison to *A.sativum*, the chloroform extract of *T.alliacea* was more potent in inhibiting the growth of *M.smegmatis* ( $P<0.05$ ). Further phytochemical analyses showed three major inhibitory compounds present in the *T.alliacea* chloroform extract. One of the compounds could tentatively be identified as Marasmicin as it had the same  $R_f$  value (Fig 2). This is indeed a very important finding as it indicates that in addition to possessing antifungal activity (Thamburan *et al.*, 2006), Marasmicin also has antimycobacterial activity.

A further two compounds that exhibit strong antimycobacterial activity have also been located in this extract. The structural analysis of these moieties presents the basis for a more advanced phytochemical study of *T.alliacea*. Consequently, Marasmicin may act singularly or in concert with synergistic compounds in *Tulbaghia alliacea*, as a very significant natural antibiotic. The results of this study prove that *Tulbaghia alliacea* does indeed have antimycobacterial activity and thereby validates the traditional use of this plant remedy for TB.



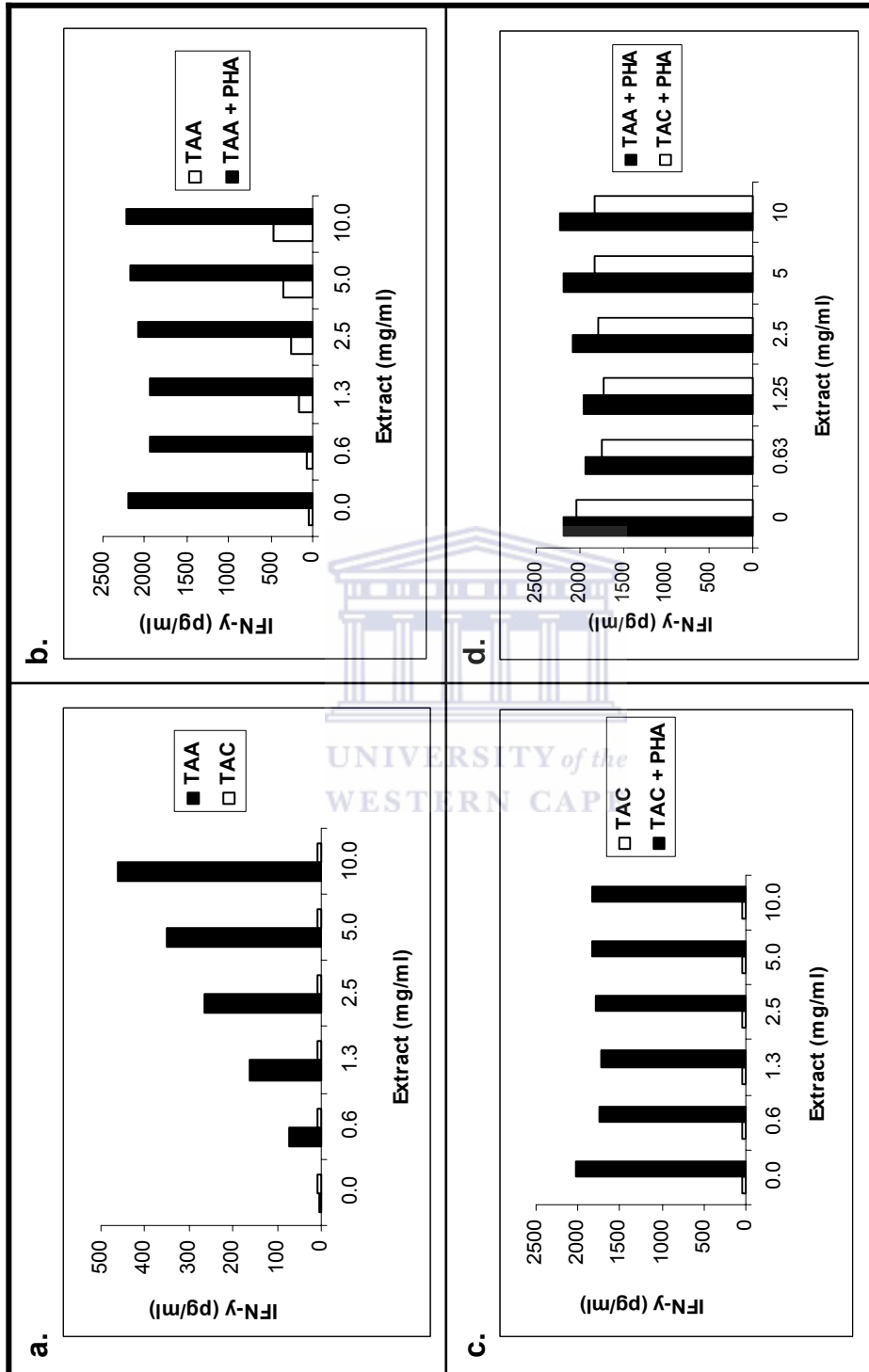
**Fig 2.** TLC-bioautography of *T.alliacea* phytotherapy showing antimycobacterial activity.

## 6.4. Immune Modulation Assessment

IFN- $\gamma$  is a multipotent cytokine and its biological functions are complex. Deficiency of IFN- $\gamma$  has been associated with the pathogenesis of many diseases (Chen and Liu, 2009). IFN- $\gamma$  plays important roles both in the innate and adaptive immune responses. Its deficiency will therefore lead to susceptibility to microbial infections and tumor development (Dunn, Old and Schreiber, 2004). Mutation in the IFN- $\gamma$  gene has been shown to lower the body's ability to resist mycobacterial infection (Patel, Doffinger, Barcenas-Morales, Kumararatne, 2008).

To assess the immune-modulatory effects of *Tulbaghia alliacea* extracts on IFN- $\gamma$  expression, diluted whole blood was stimulated with the non-specific mitogen PHA and the TB-specific antigen PPD, and exposed to varying concentrations of *Tulbaghia alliacea* aqueous and chloroform extracts.

A noteworthy finding in this study is that the aqueous extract of *Tulbaghia alliacea* was more active than the chloroform extract, in stimulating the expression of IFN- $\gamma$  (Fig 3). This is significant because an aqueous extract is the form in which this phytotherapy is used in indigenous cultures, as a remedy for various ailments including TB and TB-related conditions (Van Wyk *et al.*, 2000; Bamuamba *et al.*, 2008). The aqueous extract also showed considerable antimycobacterial activity against *Mycobacterium smegmatis in vitro*, indicating that administration of the phytotherapy during infection could work either by eradicating the pathogen, or by stimulating the host's immune system to mount a protective response via expression of IFN- $\gamma$ .



**Fig 3.** The modulation of IFN- $\gamma$  by *Tulbaghia alliacea* extracts in unstimulated (a) and PHA-stimulated (b-d) whole blood cell cultures.

## 6.5. Investigation of anti-cancer properties

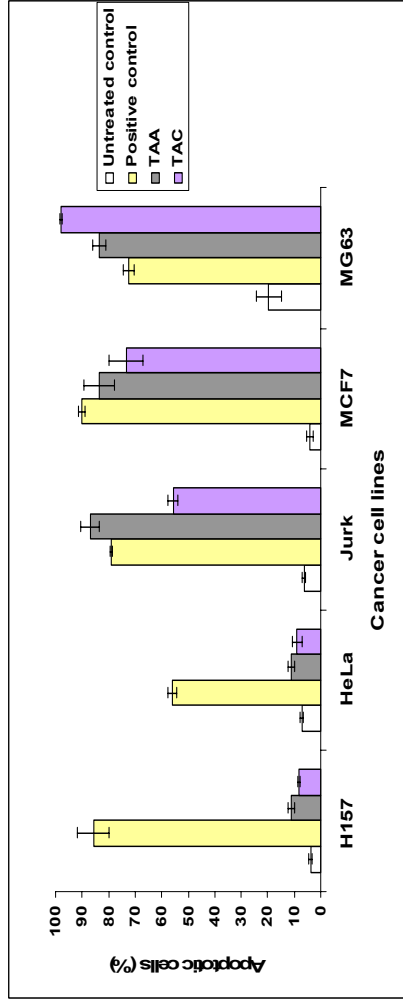
Considerable attention has been given to the manipulation of apoptosis as a novel and promising strategy for cancer chemoprevention and therapy (Cal *et al.*, 2003; Dong, 2003; Fesus *et al.*, 1995; Holzman, 1996; Krzystyniak, 2002). Several anti-cancer drugs have been shown to exert their effects through the activation of apoptosis and a substantial number of these drugs have been isolated from medicinal plants. To evaluate the anti-cancer potential of *T. alliacea*, the pro-apoptotic activity of the aqueous (TAA) and chloroform (TAC) extracts were assessed using several bioassays, which detect biochemical and morphological changes that are associated with apoptosis.

Using the APOPercentage™ assay, CHO cells treated with the extracts showed morphological changes (cell shrinkage and cell detachment); that were indicative of cells undergoing apoptosis. A panel of human cancer cell lines, which included H157 (small lung cell carcinoma), HeLa (cervical carcinoma), Jurkat (leukaemia T cells), MCF7 (breast adenocarcinoma), and MG63 (osteosarcoma) were tested for susceptibility to these extracts. Three of these cell lines, which included Jurkat, MCF7 and MG63 cells, were highly susceptible to both the extracts, while HeLa and H157 cells were resistant (Fig 4).

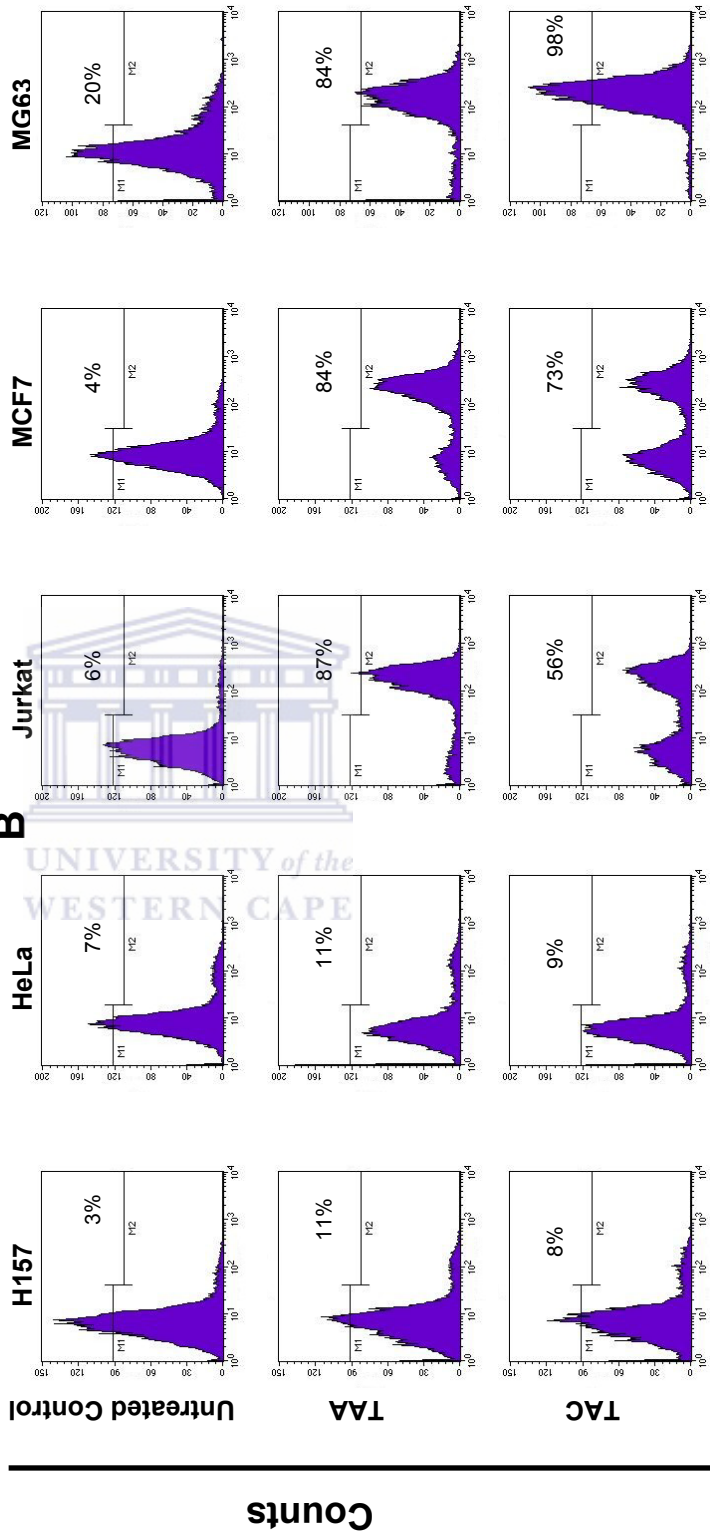
A comparison of the effects of the two extracts shows that the MCF7 cells were equally susceptible to both the TAA and TAC extract, while Jurkat cells were more susceptible ( $P < 0.05$ ) to the TAA extract. The MG63 cells were more susceptible ( $P < 0.05$ ) to the



**A**



**B**

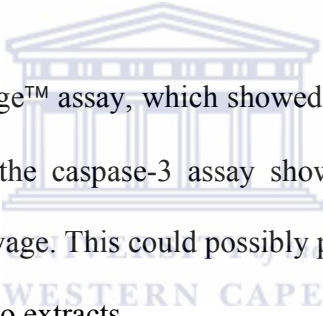


**APOPercentage**

**Fig 4. Apoptosis induction of *Tulbaghia alliacea* extracts in different cancer cell lines**

TAC extract. This suggests that the plant extracts show selectivity towards particular human cancer cell lines. The difference in the bioactivity may also be as a result of genetic variance between the different cell lines.

Jurkat is one of the two cell lines that displayed differential susceptibility to the two extracts (TAA and TAC), and was hence selected to further characterise the pro-apoptotic activity of these extracts. Three other markers of apoptosis (mitochondrial depolarisation, caspase-3 cleavage, and DNA fragmentation) were used to assess the bioactivity of the plant extracts.



In contrast to the APOPercentage™ assay, which showed that the TAA extract was more active than the TAC extract, the caspase-3 assay showed that both extracts induced similar levels of caspase-3 cleavage. This could possibly point to different mechanisms of apoptosis induction for these two extracts.

Since the caspase-3 assay confirmed that both the TAA and TAC extracts induce the activation of caspase-3, the TUNEL assay was used to evaluate DNA fragmentation in Jurkat cells treated with TAA and TAC extracts. Both extracts demonstrated a time-dependant increase in DNA fragmentation (Fig 5), but the TAA extract was significantly more active ( $P < 0.05$ ). This is in agreement with the APOPercentage™ assay and the TMRE assay, which showed that TAA is more active than the TAC extract.

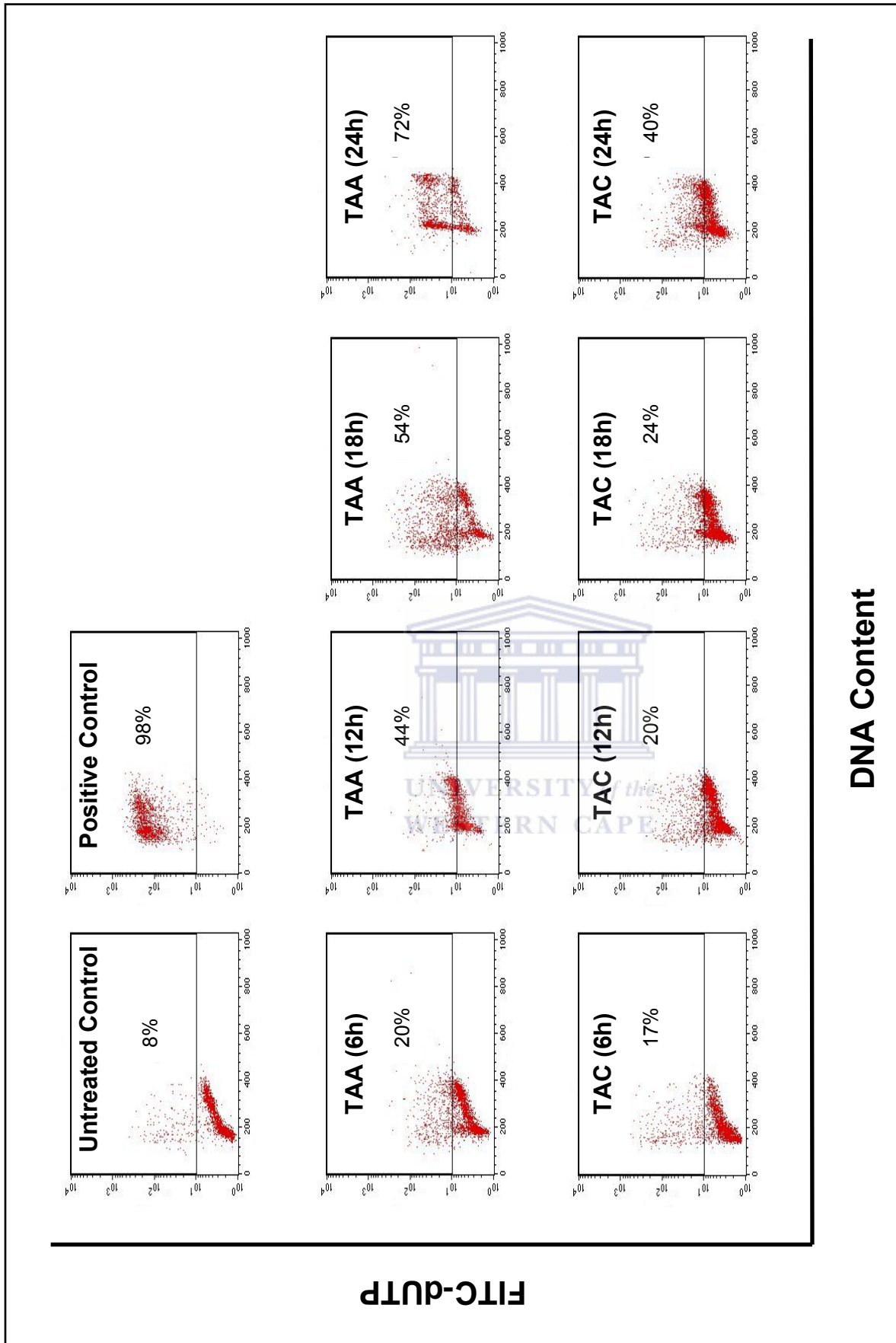
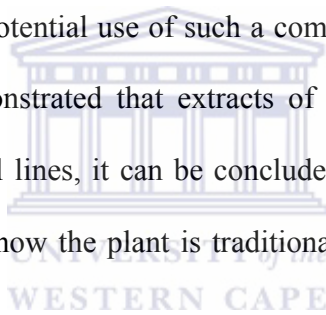
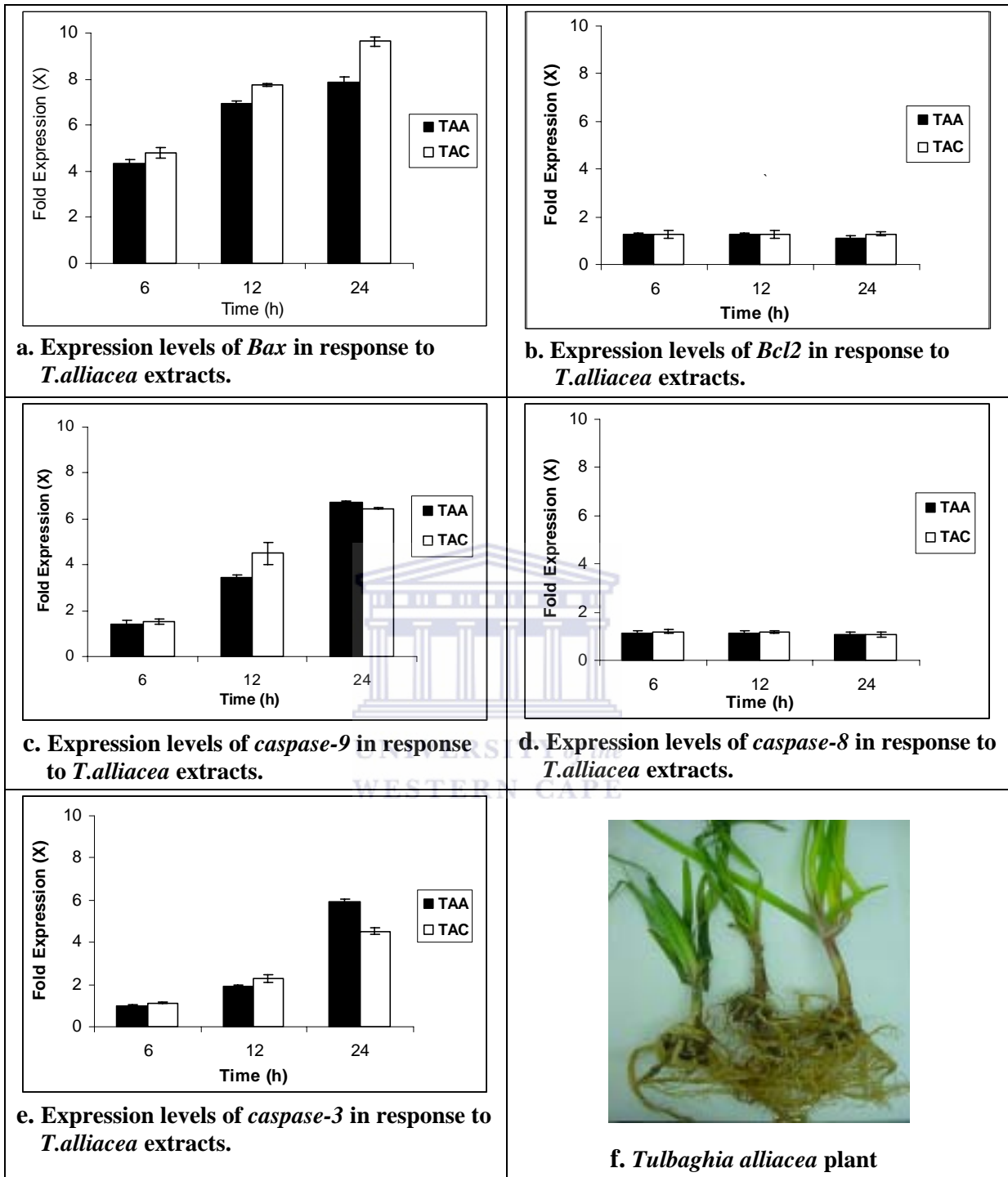


Fig 5. DNA fragmentation induced by *Tulbaghia alliacea* extracts in Jurkat cells.

In an effort to understand and further characterise the pro-apoptotic activity of the plant extracts, the expression levels of five genes (*Bax*, *Bcl2*, *caspase-3*, *caspase-8* and *caspase-9*) that play key roles in the regulation and execution of apoptosis were evaluated by real time PCR. The expression levels of three of these genes (*Bax*, *caspase-3*, and *caspase-9*) were significantly up-regulated over a 24 h period in Jurkat cells treated with the extracts ( $P<0.05$ ), while the other two genes, *caspase-8* and the anti-apoptotic gene, *Bcl2* were only moderately (1-fold increase) increased in the treated cells (Fig 6).

The ability of a compound or a plant extract to induce apoptosis in cancer cells can be used as a test to evaluate the potential use of such a compound or a plant extract to treat cancer. Since this study demonstrated that extracts of *T. alliacea* selectively induced apoptosis in human cancer cell lines, it can be concluded that extracts and in particular the aqueous extract (which is how the plant is traditionally used) shows potential as an anti-cancer remedy.





**Fig 6. Quantitative PCR analysis of apoptosis-related genes (a-e) in response to treatment with *T.alliacea* (f) extracts during a time-course experiment.**

## 6.6. Assessment of *in vivo* safety and toxicity

Even though *Tulbaghia alliacea*, an indigenous phytotherapy, is widely used by many communities, there is no information about its *in vivo* safety and toxicity. This study was thus designed to investigate the safety of the aqueous extract of *Tulbaghia alliacea*, by determining its behavioural and pharmaco-toxicological effects after acute and sub-acute administration in an *in vivo* mouse model.

In the sub-acute toxicity study, it appeared that the aqueous extract of *T.alliacea* did not induce any marked changes in the animals, as evidenced by the absence of toxic symptoms, no changes in water or food ingestion, or abnormal weight loss or gain.

The haematological and biochemical analyses did not show much variation between the control and treated groups. Furthermore, these parameters were within the normal physiological reference ranges for mice (Wolford *et al.*, 1986).

The results of this study suggest that at the oral doses administered (7.5 g/kg and 10.0 g/kg), *T.alliacea* phytotherapy is non-toxic. This study however, was qualitative and not powered for statistical analysis. A more detailed study is recommended for further analysis of the toxicity of *T.alliacea* phytotherapy.

## 6.7. References

- Bamuamba K, Gammon DW, Meyers P, Dijoux-Franca M-G, Scott G. 2008. Antimycobacterial activity of five plant species used as traditional medicines in the Western Cape Province (South Africa). *Journal of Ethnopharmacology* 117: 385-390.
- Cal C, Garban H, Jazirehi A, Yeh C, Mizutani Y, Bonavida B. 2003. Resveratrol and cancer: chemoprevention, apoptosis, and chemo-immunosensitizing activities. *Curr. Med. Chem. Anti-cancer Agents* 3: 77-93.
- Dong Z. 2003. Molecular mechanism of the chemopreventive effect of resveratrol. *Mutat. Res.* 523-524: 145-150.
- Dunn GP, Old LJ, Schreiber RD. 2004. The immunobiology of cancer immunosurveillance and immunoediting. *Immunity* 21: 137-148.
- Fesus L, Szondy Z, Uray I. 1995. Probing the molecular program of apoptosis by cancer chemopreventive agents. *J. Cell Biochem. Suppl.* 22: 151-161.
- Holzman D. 1996. Apoptosis provides new targets for chemotherapy. *J. Natl. Cancer Inst.* 88: 1098-1100.
- Krzystyniak KL. 2002. Current strategies for anticancer chemoprevention and chemoprotection. *Acta Pol. Pharm.* 59: 473-478.
- Kubec R., Velisek J. and Musah R.A. 2002. The amino acid precursors and odor formation in society garlic (*Tulbaghia violacea*). *Phytochemistry* 60: 21-25.

- Motsei ML, Lindsey KL, van Staden J, Jager AK. 2003. Screening of traditionally used South African plants for antifungal activity against *Candida albicans*. *Journal of Ethnopharmacology* 86: 235-241.
- Patel SY, Doffinger R, Barcenas-Morales G, Kumararatne D. 2008. Genetically determined susceptibility to mycobacterial infection. *J. Clin. Pathol.* 61: 1006-1012.
- Uchida Y, Takahashi T, Sato N. 1975. The characteristics of the antibacterial activity of garlic. *Jpn J Antibiot* 28:638-642.
- Van Wyk B, Van Oudtshoorn B, Gericke N. 2000. *Medicinal Plants of South Africa*. Briza Publications: South Africa.
- Wolford ST, Schroer RA, Gohs FX, Gallo PP, Brodeck M, Falk HB, Ruhren R. 1986. Reference range data base for serum chemistry and hematology values in laboratory animals. *J Toxic Environ Health* 18: 161 – 188.



# APPENDICES



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## *Tulbaghia alliacea* Phytotherapy: A Potential Anti-infective Remedy for Candidiasis

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The reproductive health of individuals is severely compromised by HIV infection, with candidiasis being the most prevalent oral complication in patients. Although not usually associated with severe morbidity, oropharyngeal candidiasis can be clinically significant, as it can interfere with the administration of medications and adequate nutritional intake, and may spread to the esophagus. Azole antifungal agents are commonly prescribed for the treatment and prophylaxis of candidal infections, however, the emergence of drug resistant strains and dose limiting toxic effects has complicated the treatment of candidiasis. Consequently, safe and effective and affordable medicine is required to combat this fungus. Commercial garlic (*Allium sativum*) has been used since time immemorial as a natural antibiotic, however, very little is known about the antifungal properties of two indigenous South African species of garlic, namely *Tulbaghia alliacea* and *Tulbaghia violacea*, used as folk medicines for a variety of infections. This study compares the *in vitro* anticandidal activity of *Tulbaghia alliacea*, *Tulbaghia violacea* and *Allium sativum* extracts. It was found that the greatest concentrations of inhibitory components were extracted by chloroform or water. The IC<sub>50</sub> concentrations of *Tulbaghia alliacea* were 0.007–0.038% (w/v). Assays using *S. cerevisiae* revealed that the *T. alliacea* extract was fungicidal, with a killing half-life of approximately 2 h. This inhibitory effect of the *T. alliacea* extracts was observed via TLC, and may be due to an active compound called marasmicin, that was identified using NMR. This investigation confirms that extracts of *T. alliacea* exhibit anti-infective activity against candida species *in vitro*. Copyright © 2006 John Wiley & Sons, Ltd.

**Keywords:** reproductive health; candidiasis; HIV; *Tulbaghia alliacea*; *Tulbaghia violacea*; *Allium sativum*; indigenous; antifungal and anti-infective.

### INTRODUCTION

The reproductive health of individuals is severely compromised by HIV infection, with candidiasis being the most prevalent oral complication in patients. HIV/AIDS is a public health emergency in Africa and according to the UN, South Africa has the fastest growing epidemic in the world, with more than 4.5 million HIV-positive people, thousands of AIDS deaths, 2500 new infections daily and 100 000–150 000 AIDS orphans annually (Stegmann, 2001). Studies of AIDS all over the world show that 58%–81% of all patients contract a fungal infection at some time during the primordial stage or after developing AIDS, and 10%–20% have died as a direct consequence of fungal infections (Drouhent and Dupont, 1989).

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Candidiasis is the most common oral fungal infection in man and manifests in a variety of clinical guises ranging from pseudomembraneous (thrush), erythematous and hyperplastic variants to linear gingival erythema associated with HIV infection (Samaranayake and Scully, 1989; Ellepola and Samaranayake, 2000). Oropharyngeal candidiasis (OPC) is the most prevalent oral complication in HIV/AIDS patients (Samaranayake, 1992; Glick *et al.*, 1994; Blignaut *et al.*, 2002), and it is generally accepted that *Candida albicans* is the most commonly isolated species from clinical specimens (Pfaller, 1994). *Candida* species are normal inhabitants of the human gastrointestinal tract and may be recovered from up to one third of the mouths of normal individuals and two thirds of those with advanced HIV disease (Fichtenbaum *et al.*, 2000). Oral colonization with inherently drug-resistant organisms is more common in advanced HIV infection (CD4-lymphocyte counts <50 cells/mm<sup>3</sup>) (Fichtenbaum *et al.*, 2000).

Although not usually associated with severe morbidity, OPC can be clinically significant as it can interfere with the administration of medications and adequate nutritional intake, and may spread to the esophagus (Tavitian *et al.*, 1986).

The increasing prevalence of other compromised patient groups in the community, common endocrine

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disorders such as diabetes mellitus and nutritional deficiencies have also contributed to the resurgence of oral candidiasis as a relatively common affliction (Ellepola and Samaranayake, 2000). In addition, the use of oral antibiotics, broad-spectrum antibiotics (Samaranayake *et al.*, 2002), corticosteroids, or immunosuppressives may predispose individuals to infection (Soysa *et al.*, 2004). These factors disrupt the balance between the healthy host and the *Candida*, which under normal circumstances, may be present in the gastrointestinal flora but is not disease producing (Bruce and Rogers, 2004).

Since its introduction, fluconazole has been used to prevent and treat *Candida* infections in HIV-1-infected patients because of its efficacy and good tolerability (Koks *et al.*, 2002). However, after the use of fluconazole for the treatment and prophylaxis of fungal infections became widespread, reports began to appear which suggested reduced effectiveness of the standard fluconazole therapy and also side effects (Ng and Denning, 1993). The side effects of many azole antifungals such as ketoconazole, itraconazole, fluconazole, posaconazole and voriconazole are similar; the more common being headache, dyspepsia, diarrhea, nausea, vomiting, hepatitis and skin rash (Munoz *et al.*, 1991).

Emergence of drug resistant strains and dose limiting toxic effects has further complicated the treatment of infectious diseases. These complications have necessitated the search for new antimicrobial substances from various sources. Extracts of plants and phytochemicals have been shown to possess activity against many infectious diseases, and may thus be a good source of new active agents (Mekki *et al.*, 1995; Vlietinck *et al.*, 1998; Matsuse *et al.*, 1999). One of the most widely used medicinal plants is garlic (*Allium sativum*), which is reported to be effective against a wide range of microorganisms, including bacteria, fungi, protozoa and viruses (Ankri and Mirelman, 1999). *Tulbaghia alliacea* and *Tulbaghia violacea* are two indigenous South African garlic species, which are traditionally used as remedies for a variety of infections and ailments (Van Wyk *et al.*, 2000). This investigation therefore aimed to assess the antifungal properties of *T. alliacea*, with a view to using this indigenous phytotherapy as a potential anticandidal remedy.

## MATERIALS AND METHODS

**Plant material.** Bulbs of *Tulbaghia alliacea* were obtained from Rastafarian herbal traders in Cape Town, South Africa; *Tulbaghia violacea* was obtained from the Educational and Environmental Resources Unit (EERU) at the University of the Western Cape, while a commercial garlic brand of *Allium sativum* was purchased from a retail store. All three species were verified by a botanist at the University of the Western Cape Herbarium where voucher specimens (Thamburan001 for *T. alliacea*, Thamburan002 for *T. violacea* and Thamburan003 for *A. sativum*) were deposited.

**Preparation of plant extracts.** Bulbs of *T. alliacea* and *T. violacea* and cloves of *A. sativum* were washed with distilled water, peeled and chopped into tiny pieces (Eloff, 1998). Solvent extractions of different concentrations (0.06%, 0.15% and 0.30%) of the bulbs and

cloves were prepared using solvents of differing polarity (water, methanol and chloroform). Extracts were prepared by soaking the chopped plant material in the solvents overnight, and then vacuum filtering it.

**Microorganisms and growth media.** For the preliminary antifungal screening, a culture of *Candida albicans* was obtained from the Department of Medical Biosciences at the University of the Western Cape, South Africa. *C. albicans* was maintained on Sabouraud dextrose agar. Subcultures of *C. albicans* were inoculated in nutrient broth and spread onto prepared agar plates using sterile swab sticks under aseptic conditions. Plated cultures were incubated at 37 °C for 24 h. The agar plate method for plant extract susceptibility testing is well standardized and widely used (Vanden Bergh and Vlietinck, 1991).

For more detailed antifungal analyses, the broth dilution antifungal susceptibility testing method (Agarwal *et al.*, 2003) was used to assess the growth inhibitory activity of *T. alliacea* extracts on a variety of pathogenic fungi.

The yeast strains: *Saccharomyces cerevisiae* (JCT746-9D), *Candida albicans* (ATCC 90028), *Candida glabrata* (ATCC 90030), *Candida krusei* (ATCC 6258) and *Cryptococcus neoformans* (ATCC 90113) were obtained from the National Center for Natural Products Research, University of Mississippi, Oxford.

YPD medium contained 10 g yeast extract, 20 g peptone and 20 g glucose per liter. Minimal medium contained 7 g yeast nitrogen base, 20 g glucose, 10 g succinic acid, 4.5 g NaOH per liter and was supplemented with amino acids (Guthrie and Fink, 1991). Solid media contained 20 g agar per liter.

**Antifungal screening: Disk diffusion assays.** All solvent (aqueous, methanol and chloroform) extracts of *T. alliacea*, *T. violacea* and *A. sativum* were tested for *in vitro* antifungal activity against *C. albicans* using the agar plate disk diffusion method. The pharmaceutical drug, fluconazole (40 µg/disk) was used as a positive control, as it is a broad-spectrum antibiotic. 9 mm filter paper disks (Schleider and Schuell) were autoclaved at 120 °C in a Speedy Autoclave (Lasec) for 20 min, and then incubated at 37 °C overnight. Each disk was impregnated with 50 µL of a particular extract and was allowed to dry overnight. Fluconazole served as a positive control, and the extraction solvents (water, methanol and chloroform) served as a negative control. The dried impregnated disks were placed on the surface of agar plates inoculated with the candidal culture. Each plate consisted of the following disks: a positive control (fluconazole), a negative control (particular solvent) and a different extraction of each solvent (e.g. 0.30% water). Each extract was tested in triplicate. After being accurately labeled, the agar plates were inverted and incubated at 37 °C for 24 h. After incubation, inhibition zones were recorded as the diameter of the growth-free zones around the disk.

**Minimum inhibitory concentrations (MIC).** The MIC for each extraction was calculated by preparing serial dilutions of the original extractions. The extract concentration exhibiting a 1 mm zone of inhibition was taken to be the MIC value for that solvent extraction, as a 1 mm zone is the smallest zone that can be

detected as growth inhibition of microorganisms *in vitro* using the agar plate disk diffusion method.

**Broth dilution antifungal susceptibility testing.** After establishing the potent antifungal growth inhibitory effect of *T. alliacea* using the disk diffusion assay method, further analyses using the more sensitive optical density growth inhibition method, which closely approximates the dynamics of the fungus in biological fluid, was used.

The fungal strains [*Saccharomyces cerevisiae* (JC746-9D), *Candida albicans* (ATCC 90028), *Candida glabrata* (ATCC 90030), *Candida krusei* (ATCC 6258) and *Cryptococcus neoformans* (ATCC 90113)] were grown overnight in YPD medium. The YPD medium was diluted to  $3 \times 10^6$  cells/mL in yeast minimal medium and 175  $\mu$ L of diluted cells was placed in each of the wells of a 96-well plate (Nunc) with the extract dilution.

Absorbance at 630 nm was measured at zero time and after 24 h of growth using a Dynatech MR5000 microplate reader. The plate wells were sealed with Parafilm and the plate was shaken at 250 rpm at 30 °C. Dilutions of chloroform extracts were made in a final volume of 50  $\mu$ L chloroform in 1.5 mL polypropylene tubes (Sarstedt).

Chloroform was evaporated from the uncapped tubes in a chemical hood for 3 h at 22 °C. Tubes were filled with 400  $\mu$ L of diluted cells from above, capped and shaken at 250 rpm at 30 °C. Cell densities were measured from 200  $\mu$ L aliquots placed into a 96-well plate as above. All assays were done in triplicate and the average data were plotted.

**Viability assay.** A viability assay to determine the mechanism of action (fungistatic or fungicidal) of *T. alliacea* extracts was carried out. An *S. cerevisiae* culture was diluted to  $3 \times 10^6$  cells/mL in 400  $\mu$ L yeast minimal medium in a 1.5 mL polypropylene tube. Aqueous *T. alliacea* extract was added to a final 0.075% (w/v) (four-fold dilution of aqueous extract stock). The capped tube was incubated at 30 °C with 250 rpm shaking. Samples of cells removed before extract addition and after 5 and 24 h of growth were diluted and plated on YPD plates. Viable cell numbers were calculated from the colony forming units after three days of growth.

**Thin-layer chromatography (TLC) – bioautographic analyses.** For direct bioassay on TLC plates, a 30  $\mu$ L aliquot of each extract was applied to 5  $\times$  20 cm silica F254 (Merck) glass backed plates in duplicate for fingerprinting. All plates were developed in hexane: ethyl acetate (1:2 v/v) (Wagner and Bladt, 1996). The developed TLC plates were dried overnight and then viewed under ultraviolet (UV) light (366 nm).

Thereafter, one set of plates (of each extract) was sprayed with vanillin–sulphuric acid reagent, made up by dissolving 3 g vanillin in 30 mL ethanol, to which 5 mL concentrated sulphuric acid was added. Some compounds that are not visible under UV light become visible upon spraying and heating (for approximately 20 min) of the developed plates.

The duplicate plates were used for the bioautographic assay (inoculated TLC), whereby Sabouraud dextrose agar inoculated with *C. albicans* was poured over the plates under aseptic conditions. These plates

were placed on damp paper towels in a plastic tray, covered with cling wrap and incubated at 37 °C for 24 h. To detect the antimicrobial activity on the plates, they were sprayed with a 0.2 mg/mL solution of 2,5-diphenyltetrazolium bromide (MTT) (Sigma Chemicals Co.). Clear zones on the chromatogram indicated inhibition of growth (Beugue and Kline, 1972).

**Nuclear magnetic resonance (NMR) analyses.** The active compound from the chloroform extract was isolated by preparative TLC using a mixture of hexane and ethyl acetate (4:1) as mobile phase. The <sup>1</sup>H NMR spectrum was recorded on a Gemini Varian 200 instrument at 200 MHz, in deuteriochloroform as solvent and tetramethylsilane as internal standard; the chemical shifts were reported in ( $\alpha$ ) ppm.

**Statistical analyses.** Inhibition zones were statistically compared by conducting an unpaired *t*-test using the MedCalc (version 7.1, 2000) statistical programme. The mean  $\pm$  SEM (standard error of the mean) was considered significant at  $p < 0.05$ .

## RESULTS

### Disk diffusion assays

Fluconazole (2%) served as a positive control and inhibited the growth of *C. albicans* with a zone of 7 mm. The negative controls (extraction solvents) did not have any effect on the fungus and thus no zones of inhibition were observed around these disks. Inhibition zones caused by extracts of *A. sativum*, *T. alliacea* and *T. violacea* were compared using an unpaired *t*-test (Table 1).

At the 0.06% concentration, the aqueous extracts of *T. violacea* had no effect on *C. albicans*, whereas *A. sativum* and *T. alliacea* had similar inhibitory effects ( $p = 0.1012$ ), however, at both 0.15% and 0.30% *T. alliacea* exhibited a statistically bigger zone of inhibition than *A. sativum* ( $p < 0.05$ ) and *T. violacea* ( $p < 0.0001$ ).

*T. alliacea* methanol extracts at all concentrations exhibited statistically bigger zones of inhibition than *A. sativum* and *T. violacea* ( $p < 0.05$ ).

Chloroform extracts of *T. violacea* only became active at the highest concentration (0.30%), whereas *A. sativum* and *T. alliacea* inhibited *Candida* at all concentrations, with *T. alliacea* exhibiting the biggest zone of inhibition (10.67 mm) at 0.30%. In general, extracts of *T. violacea* were poorest in inhibiting the growth of the fungus; they were inactive at the lowest concentration of 0.06% and only exhibited small zones of inhibition at the higher concentrations. In contrast, all extracts of *T. alliacea* at all concentrations exhibited antifungal activity.

An MIC value is defined as the lowest concentration of an extract, which visibly inhibits the growth of the microorganism after incubation. *T. alliacea* extracts were shown to have lower MIC values than both *A. sativum* and *T. violacea* (Table 2). The lowest MIC value against *C. albicans* was obtained with the 0.024% chloroform extract of *T. alliacea*.

**Table 1.** The antiinfective activity of *Tulbaghia alliacea*, *Tulbaghia violacea* and *Allium sativum* extracts against *Candida albicans*

Extract	Concentration (%)	Zone of inhibition (mm ± SEM)			Statistical significance*		
		<i>A. sativum</i> (a)	<i>T. alliacea</i> (b)	<i>T. violacea</i> (c)	ab	ac	bc
Aqueous (H <sub>2</sub> O)	0.06	3.33 ± 0.33	4.33 ± 0.33	0	0.1012	0.0006	0.0002
	0.15	4.00 ± 0.00	6.33 ± 0.33	0	0.0022	0.0002	0.0001
	0.30	5.33 ± 0.33	8.33 ± 0.33	1.00 ± 0.00	0.0031	0.0002	0.0001
Methanol (MeOH)	0.06	1.67 ± 0.33	4.00 ± 0.00	0	0.0023	0.0075	0.0002
	0.15	3.67 ± 0.33	5.33 ± 0.33	0.66 ± 0.33	0.0241	0.0031	0.0006
	0.30	5.33 ± 0.33	7.33 ± 0.33	3.33 ± 0.33	0.0132	0.0132	0.0011
Chloroform (CHCl <sub>3</sub> )	0.06	4.00 ± 0.00	5.33 ± 0.33	0	0.1012	0.0006	0.0002
	0.15	4.33 ± 0.33	7.33 ± 0.33	0	0.0031	0.0002	0.0001
	0.30	5.33 ± 0.33	10.67 ± 0.33	2.33 ± 0.33	0.0004	0.0031	0.0001

\* Values of *p* for comparing main effects: ab = *A. sativum* and *T. alliacea*, ac = *A. sativum* and *T. violacea*, bc = *T. alliacea* and *T. violacea*.

# Fluconazole (2%) inhibited *C. albicans* with a 7 mm zone of inhibition.

Negative controls (water, methanol and chloroform) did not inhibit *C. albicans*, hence no zone of inhibition was observed.

**Table 2.** The minimum inhibitory concentrations (MIC) for *Tulbaghia alliacea*, *Tulbaghia violacea* and *Allium sativum* extracts against *Candida albicans*\*

Solvent	Extract concentration (%)			Zone of inhibition (mm)
	<i>A. sativum</i>	<i>T. alliacea</i>	<i>T. violacea</i>	
Aqueous (H <sub>2</sub> O)	0.132	0.036	0.300	1
Methanol (MeOH)	0.048	0.048	0.150	1
Chloroform (CHCl <sub>3</sub> )	0.048	0.024	0.060	1

\* The MIC for fluconazole was 0.25% (1 mm zone of inhibition).

### Broth dilution antifungal susceptibility testing

All five yeast species grew well in YPD and minimal medium normally used for *S. cerevisiae* growth. Therefore, these media were used for growth inhibition assays following a described method (Agarwal *et al.*, 2003). Chloroform extracts required a significant alteration in protocol because chloroform dissolved the 96-well plates.

Growth inhibition by chloroform extracts was assayed by growth in polypropylene tubes after the chloroform was evaporated. The methanol extraction solvent and evaporated chloroform residue did not inhibit yeast growth.

Cell densities after 24 h growth in the presence of extracts were estimated by 630 nm light scattering. According to the estimated IC<sub>50</sub> concentrations (Table 3), *C. neoformans* was the most sensitive strain to the *T. alliacea* extracts because its growth was inhibited at the lowest concentrations. In contrast, *C. glabrata* was slightly more resistant. In all cases, the methanol extracts were the poorest in inhibiting microbial growth. On the other hand, the chloroform and aqueous extracts appeared to be similarly potent. With all but the exception of the *C. glabrata* strain, the chloroform extract was slightly more potent than the aqueous extract.

Figure 1 (A–E) provides the data for the effect of the various aqueous, methanol and chloroform concentrations of *Tulbaghia alliacea* extracts, on the growth of *Saccharomyces cerevisiae*, *Candida albicans*, *Candida glabrata*, *Candida krusei* and *Cryptococcus neoformans*.

**Table 3.** IC<sub>50</sub> concentrations of *Tulbaghia alliacea* extracts for five fungal species

Fungal species	<i>T. alliacea</i> extracts		
	Aqueous (H <sub>2</sub> O)	Methanol (MeOH)	Chloroform (CHCl <sub>3</sub> )
<i>Saccharomyces cerevisiae</i>	0.038	0.15	0.038
<i>Candida albicans</i>	0.075	0.15	0.028
<i>Candida glabrata</i>	0.056	0.23	0.110
<i>Candida krusei</i>	0.056	0.23	0.038
<i>Cryptococcus neoformans</i>	0.019	0.056	0.007

These data indicate that the IC<sub>50</sub> concentrations of *Tulbaghia alliacea* extracts were between 0.007% and 0.038% (w/v).

### Mechanism of antifungal action of *T. alliacea* extract

Preliminary experiments were performed to analyse the killing mechanism of the *T. alliacea* extract. An *S. cerevisiae* culture was prepared with minimum concentration of aqueous extract (0.075%, w/v) that allowed no detectable growth.

Cell viability was measured by comparing the total cells (by hemocytometer) and viable cells (colony forming units). It was found that after 5 h of exposure, the cell viability had decreased to about one-half. After

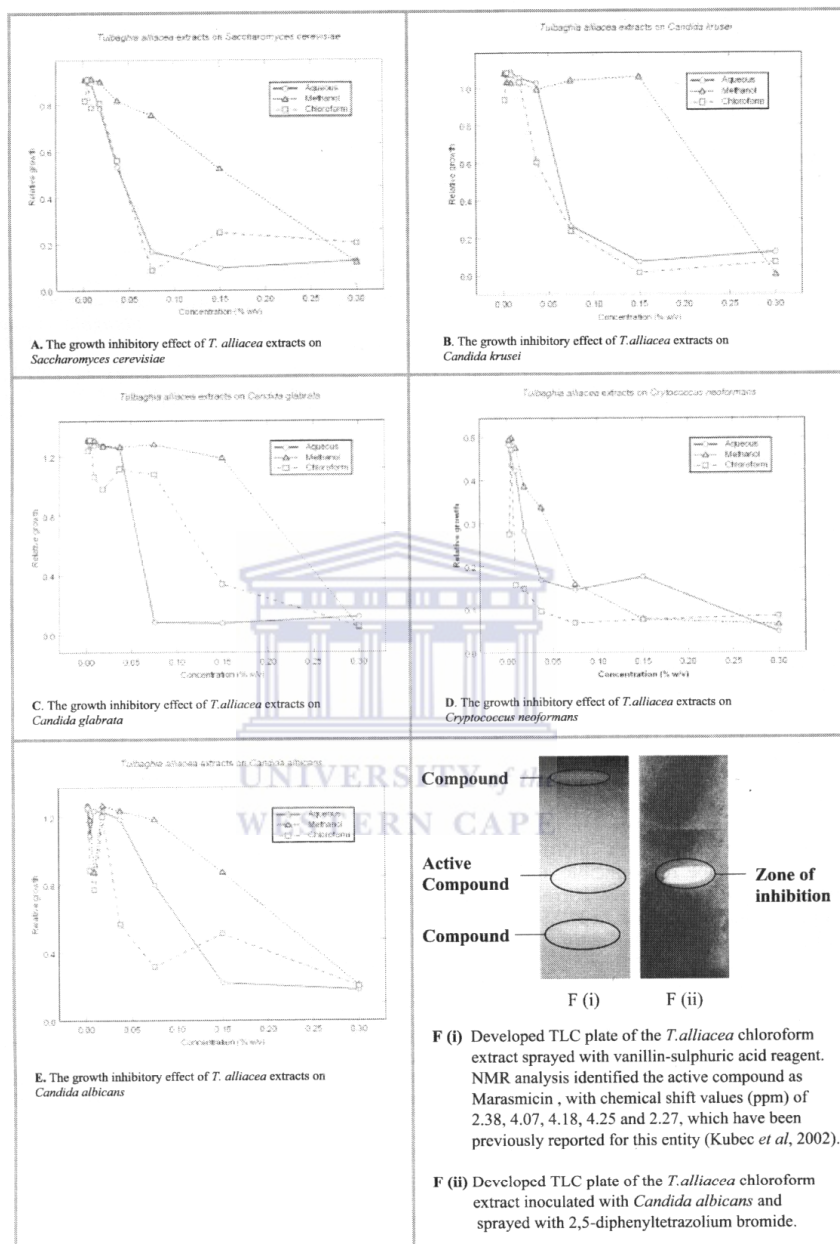


Figure 1. The antifungal activity of *T. alliacea* extracts.

24 h, there was a greater than 1000-fold drop in viable cells. Had the action of the extract been fungistatic, no reduction in the number of viable cells would have been observed. Therefore, *T. alliacea* extracts kill yeast cells rather than merely inhibiting growth. Furthermore, the death rate that would reduce the viable cells by 1000-fold in 24 h, gave a half-life of about 2 h. These outcomes are stated in orders of magnitude, since these experiments were not quantitative.

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#### Antifungal activity of *T. alliacea* extracts on TLC

The aqueous, methanol and chloroform extracts of *T. alliacea* separated with one major inhibitory fraction, which was indicated as the zone of growth inhibition on the bioautograms.

TLC separations showed the compounds present in the extracts under UV-366 nm are the same as the antifungal compounds visible on the bioautograms.

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based on the distance they traveled on the TLC plates (Fig. 1F).

#### Nuclear magnetic resonance (NMR) analysis

NMR spectral analysis of the *T. alliacea* inhibitory fraction (Fig. 1F) showed prominent signals at 2.38, 4.07, 4.18, 4.25 and 2.27, which were due to the methyl and methylene groups of marasmin. These chemical shift values are in agreement with those reported for the same compound, previously isolated from the related *Tulbaghia violacea* (Kubec *et al.*, 2002).

#### DISCUSSION

This investigation aimed at comparing the antifungal effect of the indigenous South African garlic plants, *T. alliacea* and *T. violacea*, with the commercial variety *A. sativum*. This comparison was made because of the reputed antimicrobial effects of the commercial garlic species (Uchida *et al.*, 1975) and the traditional use of the indigenous varieties. *Allium sativum* is quite expensive while the indigenous forms (*Tulbaghia alliacea* and *Tulbaghia violacea*), are significantly more affordable to the poorer communities afflicted by opportunistic fungal infections such as candidiasis.

The disk diffusion method was well suited for preliminary screening and has the advantage of allowing for the use of small sample sizes (Rios *et al.*, 1988).

Chloroform extracts of *T. alliacea* were more potent than *A. sativum* and *T. violacea*, in inhibiting the growth of *C. albicans*. Further analyses of fungal growth inhibition were carried out using the more sensitive broth dilution susceptibility test. The broth micro-dilution method has been shown to be reproducible and clinically useful, with a good *in vitro*-*in vivo* correlation in the setting of oropharyngeal candidiasis in HIV-infected patients (Revankar *et al.*, 1998). The chloroform extract of *T. alliacea* was more potent than the aqueous and methanol extracts in inhibiting the growth of the yeast strains (*Saccharomyces cerevisiae*, *Candida albicans*, *Candida glabrata*, *Candida krusei* and *Cryptococcus neoformans*).

Bioautography is a method that facilitates the localization of antimicrobial activity on a chromatogram, and provides qualitative information about the active components of plants. By separating extracts on TLC plates,

it is possible to secure information on the bioactive compounds present in the mixture.

Aqueous, methanol and chloroform extracts of *T. alliacea* showed one major inhibiting fraction, indicated as a zone of inhibition on the bioautograms. TLC separations showed that the fraction present in the extract under UV-366 nm was the same as the compound exhibiting antifungal activity, which was visible on the bioautograms. NMR spectral analysis of the *T. alliacea* inhibitory fraction indicated the presence of the methyl and methylene groups of marasmin. These chemical shift values are in agreement with those reported for the same compound, previously isolated from the related *Tulbaghia violacea* (Kubec *et al.*, 2002).

A preliminary viability assay revealed *T. alliacea* extract to be fungicidal (kills yeast cells rather than merely inhibiting growth). Future experiments that address the mechanism of *T. alliacea* fungicidal activity, will require the comprehensive assessment of mutants resistant to the extracts. Initial experiments showed that the assay plates that were incubated for up to 3 days did not show any increase in cell density. This observation is consistent with the fungicidal action of the extracts. It also shows that spontaneous mutants resistant to the extract occur at a frequency less than  $10^{-9}$ . These observations are indeed very important in the development of a mutant isolation scheme, and the creation of a scientifically proven phytotherapeutic remedy for candidiasis.

This investigation has indicated that *T. alliacea* might be of value in the continuing struggle to control opportunistic fungal infections, which are especially important in HIV and AIDS patients. Furthermore, it also verifies the traditional use of indigenous garlic as an anti-infective herbal medicine. This study provides useful leads for the development of novel pharmaceuticals, in the ongoing search for effective and affordable antifungal remedies.

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#### REFERENCES

- Agarwal AK, Rogers PD, Baerson SR *et al.* 2003. Genome-wide expression profiling of the response to polyene, pyrimidine, azole, and echinocandin antifungal agents in *Saccharomyces cerevisiae*. *Biol Chem* **278**: 34998–35015.
- Ankri S, Mirelman D. 1999. Antimicrobial properties of allicin from garlic. *Microbes Infect* **1**: 125–129.
- Beugue WJ, Kline RM. 1972. The use of tetrazolium salts in bioautographic procedures. *J Chromatogr* **64**: 182–184.
- Blignaut E, Messer S, Hollis RJ, Pfaller MA. 2002. Antifungal susceptibility of South African oral yeast isolates from HIV/AIDS patients and healthy individuals. *Diagn Microbiol Infect Dis* **44**: 169–174.
- Bruce AJ, Rogers RS. 2004. Oral manifestations of sexually transmitted diseases. *Clin Dermatol* **22**: 520–527.
- Drouhent E, Dupont B. 1989. Fluconazole for the treatment of fungal diseases in immunosuppressed patients. *Ann N Y Acad Sci* **544**: 564–570.
- Ellepola ANB, Samaranyake LP. 2000. Antimycotic agents in oral candidosis: an overview. 1. Clinical variants. *Dent Update* **27**: 111–116.
- Eloff JN. 1998. Which extractant should be used for the screening and isolation of antimicrobial components from plants. *J Ethnopharmacol* **60**: 1–8.
- Fichtenbaum CJ, Koletar S, Yiannoutsos C *et al.* 2000. Refractory mucosal candidiasis in advanced human immunodeficiency virus infection. *Clin Infect Dis* **30**: 749–756.
- Glick M, Muzyka BC, Lurie D, Salkin LM. 1994. Oral manifestations associated with HIV-related disease as markers for

- immune suppression and AIDS. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* **77**: 344–345.
- Guthrie C, Fink GR. 1991. *Guide to Yeast Genetics and Molecular Biology* (Methods in Enzymology, Vol. 194). Academic Press: San Diego.
- Koks CHW, Crommentuyn KML, Mathot RAA, Mulder JW, Meenhorst PL, Beijnen JH. 2002. Prognostic factors for the clinical effectiveness of fluconazole in the treatment of oral candidiasis in HIV-1-infected individuals. *Pharmacol Res* **46**: 89–94.
- Kubec R, Velisek J, Musah RA. 2002. The amino acid precursors and odor formation in society garlic (*Tulbaghia violacea*). *Phytochemistry* **60**: 21–25.
- Matsuse IT, Lim YA, Hattori M, Correa M, Gupta MP. 1999. A search for antiviral properties in Panamanian medicinal plants – the effect on HIV and its essential enzymes. *J Ethnopharmacol* **64**: 15–22.
- Mekkawy SE, Meselhy MR, Kusumoto IT, Kadota S, Hattori M, Namba T. 1995. Inhibitory effects of Egyptian folk medicines on human immunodeficiency virus (HIV) reverse transcriptase. *Chem Pharm Bull* **43**: 641–648.
- Munoz P, Moreno S, Berenuer J, Bernaldo de Quiros JC, Bouza E. 1991. Fluconazole-related hepatotoxicity in patients with acquired immunodeficiency syndrome. *Arch Intern Med* **5**: 1020–1021.
- Ng TT, Denning DW. 1993. Fluconazole resistance in *Candida* in patients with AIDS – a therapeutic approach. *J Infect* **26**: 117–125.
- Pfaller MA. 1994. Epidemiology and control of fungal infections. *Clin Infect Dis* **19**: S8–S13.
- Revankar SG, Kirkpatrick WR, McAtee RK *et al.* 1998. Interpretation of trailing endpoints in antifungal susceptibility testing by the National Committee for Clinical Laboratory Standards method. *J Clin Microbiol* **36**: 153–156.
- Rios JL, Recio MC, Villar A. 1988. Screening methods for natural products with antimicrobial activity: a review of the literature. *J Ethnopharmacol* **23**: 127–149.
- Samaranayake LP. 1992. Oral mycoses in human immunodeficiency virus infection: a review. *Oral Surg Oral Med Oral Path Oral Radiol Endod* **73**: 171–180.
- Samaranayake LP, Cheung LK, Samaranayake YH. 2002. Candidiasis and other fungal diseases of the mouth. *Dermatol Ther* **15**: 252–270.
- Samaranayake LP, Scully C. 1989. Oral candidosis in HIV infection. *Lancet* **ii**: 1491–1492.
- Soysa NS, Samaranayake LP, Ellepola ANB. 2004. Cytotoxic drugs, radiotherapy and oral candidiasis. *Oral Oncol* **40**: 970–978.
- Stegmann J. 2001. The new South Africa – challenge to the church. *Grace and Truth* **18**: 22–27.
- Tavitian A, Raufman JP, Rosenthal LE. 1986. Oral candidiasis as a marker for esophageal candidiasis in the acquired immunodeficiency syndrome. *Ann Intern Med* **104**: 54–55.
- Uchida Y, Takahashi T, Sato N. 1975. The characteristics of the antibacterial activity of garlic. *Jpn J Antibiot* **28**: 638–642.
- Vanden Berghe DA, Vlietinck AJ. 1991. Screening methods for antibacterial and antiviral agents from higher plants. In *Methods in Plant Biochemistry* (Vol. 6), Dey PM, Harborne JB (eds). Academic Press: London.
- Van Wyk B, Van Oudtshoorn B, Gericke N. 2000. *Medicinal Plants of South Africa*. Briza Publications: South Africa.
- Vlietinck AJ, Bruyne TD, Apers S, Pieters LA. 1998. Plant derived leading compounds for chemotherapy of human immunodeficiency virus infection. *J Ethnopharmacol* **64**: 97–109.
- Wagner H, Bladt S. 1996. *Plant Drug Analysis – A Thin Layer Chromatography Atlas*, 2nd edn. Springer-Verlag: Berlin Heidelberg.





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### ***Tulbaghia alliacea*: A potential anti-tuberculosis phytotherapy**

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*Tulbaghia alliacea* is used in traditional medicine to combat infections. Extracts of *T.alliacea* (0-10mg/ml), were comparatively assessed for *in vitro* activity against *Mycobacterium smegmatis* using a disk diffusion assay, and IFN- $\gamma$  in human cells using ELISA technology. *T.alliacea* aqueous ( $P<0.002$ ) and ethanolic ( $P<0.003$ ) extracts inhibit the pathogen in a dose-dependent fashion compared to controls. More specifically, the 10 mg/ml chloroform extract of *T. alliacea* most potently inhibited the growth of the pathogen ( $P<0.0001$ ).

Developed TLC plates of the *T.alliacea* chloroform extract inoculated with *Mycobacterium smegmatis* were sprayed with 2,5-diphenyltetrazolium bromide. Comparatively, developed TLC plates of the *T.alliacea* chloroform extract were sprayed with vanillin-sulphuric acid reagent. NMR analysis identified the active compound A as Marasmicin, with chemical shift values (ppm) of 2.38, 4.07, 4.18, 4.25 and 2.27, which have been previously reported for this entity<sup>1</sup>.

The inhibitory effect of *Tulbaghia alliacea* against *Mycobacterium smegmatis*, is due to three active compounds, observed using TLC. Through NMR, one of these compounds was identified as Marasmicin ( $R_f$  0.44), a potent anti-infective compound previously identified in *T. alliacea*<sup>1</sup>. In addition, the aqueous extracts of *Tulbaghia alliacea* showed greater potency in stimulating the expression of IFN- $\gamma$ , when compared with the chloroform extract ( $P<0.05$ ). *Tulbaghia alliacea* phytotherapy is antimycobacterial and modulates IFN- $\gamma$ , which is vital in fighting TB infection.

1. Thamburan S., Klaasen J., Mabusela WT., Cannon JF., Folk W. and Johnson Q. (2006). *Tulbaghia alliacea* phytotherapy: a potential anti- infective remedy for candidiasis. *Phytother. Res.* 20 (10): 844-50

### ***Tulbaghia alliacea*: A potential anti-cancer phytotherapy**

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*Tulbaghia alliacea* is an indigenous garlic plant used in traditional medicine as an anti-cancer remedy. To evaluate this claim, five human cancer cell lines were treated with *Tulbaghia alliacea* aqueous (TAA) and chloroform extract (TAC), for their potential to induce apoptosis (0-10mg/ml over 24 hours) *in vitro*. Using phosphatidyl serine externalisation, Caspase-3 cleavage, mitochondrial depolarisation and DNA fragmentation as markers, this study showed that both extracts induced apoptosis in three of these cell lines (Jurkat, MCF7 and MG63) while the other two cell lines (HeLa and H157) were completely resistant.

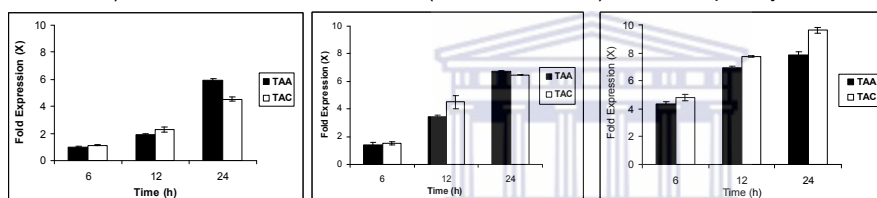


Figure 1: FLTR-Effects of TAA and TAC (6mg/ml) on Bax, Caspase 3 and Caspase 9

Gene product studies through real time PCR revealed that TAA and TAC significantly induced the expression of *Caspase-3*, *Caspase-9* and *Bax*, over time ( $P < 0.001$ ). Whilst a previous study showed that *Tulbaghia violacea* extracts induced apoptosis<sup>1</sup>, this is the first report on the apoptotic effects of *T. alliacea* in Jurkat, MCF7 and MG63 cancer cells during *in vitro* conditions.

1. Bungu L, Frost CL, Brauns SC, Van de Venter M (2006). *Tulbaghia violacea* inhibits growth and induces apoptosis in cancer cells in vitro. Afr. J. Biotech 5, 1936-1943.

**Table 1. The modulation of IFN- $\gamma$  by *Tulbaghia alliacea* aqueous extract in unstimulated whole blood cell cultures (3-day incubation).**

Extract (mg/ml)	IFN- $\gamma$ expression of donors (pg/ml)								Average	Median	STDev
	1	2	3	4	5	6	7	8			
<b>0.00</b>	3.61	3.62	2.9585	2.00	4.96	0.00	0.00	0.00	2.14	2.48	1.95
<b>0.63</b>	127.27	63.24	70.26	54.01	68.52	79.72	78.00	112.23	81.65	74.13	25.18
<b>1.25</b>	278.67	184.89	143.89	98.03	127.12	180.00	201.00	129.07	167.83	161.95	56.60
<b>2.50</b>	383.38	298.92	232.00	264.76	235.70	264.23	278.41	245.84	275.41	264.49	48.98
<b>5.00</b>	476.65	458.85	311.00	301.36	342.12	322.72	357.09	370.89	367.59	349.60	66.11
<b>10.00</b>	669.70	552.49	444.53	468.97	448.54	452.81	439.21	493.72	496.25	460.89	79.36

**Table 2. The modulation of IFN- $\gamma$  by *Tulbaghia alliacea* chloroform extract in unstimulated whole blood cell cultures. (3-day incubation).**

Extract (mg/ml)	IFN- $\gamma$ expression of donors (pg/ml)								Average	Median	STDev	
	1	2	3	4	5	6	7	8				
<b>0.00</b>	0.00	100.49	0.69	0.00	0.00	14.21	0.00	0.00	0.00	14.42	0.00	35.12
<b>0.63</b>	0.00	98.92	0.00	0.00	0.00	9.90	0.00	0.00	0.00	13.60	0.00	34.65
<b>1.25</b>	0.00	75.58	0.00	0.00	0.00	18.52	0.00	0.00	0.00	11.76	0.00	26.59
<b>2.50</b>	0.00	144.75	175.87	0.00	0.00	22.82	0.00	0.00	0.00	42.93	0.00	73.35
<b>5.00</b>	0.00	232.54	56.29	0.00	0.00	39.98	0.00	0.00	0.00	41.10	0.00	80.47
<b>10.00</b>	0.00	244.16	0.00	0.00	0.00	44.26	0.00	0.00	0.00	36.05	0.00	85.50

**Table 3. The modulation of IFN- $\gamma$  by *Tulbaghia alliacea* aqueous extract in PHA-stimulated whole blood cell cultures.**

Extract (mg/ml)	IFN- $\gamma$ expression of donors (pg/ml)								STDev		
	1	2	3	4	5	6	7	8			
<b>0.00</b>	3402.89	2186.54	5299.91	1124.34	2243.62	1956.74	1950.28	2199.46	2545.47	2193.00	1275.16
<b>0.63</b>	3574.41	6745.92	4099.10	1413.71	1209.33	1806.66	822.10	2075.24	2718.31	1940.95	1988.55
<b>1.25</b>	3544.07	7764.79	4668.90	1787.29	1656.04	1727.27	1682.15	2106.59	3117.14	1946.94	2174.74
<b>2.50</b>	3660.99	8236.79	5431.71	1711.24	2043.90	1656.04	1939.08	2118.38	3349.77	2081.14	2362.79
<b>5.00</b>	3705.29	8735.03	4139.63	1718.13	2182.61	1394.28	1948.42	2170.32	3249.21	2176.46	2416.42
<b>10.00</b>	3521.29	7999.24	5302.59	1806.66	2245.22	1078.35	1981.24	2204.92	3267.44	2225.07	2309.93

**Table 4. The modulation of IFN- $\gamma$  by *Tulbaghia alliacea* chloroform extract in PHA-stimulated whole blood cell cultures.**

Extract (mg/ml)	IFN- $\gamma$ expression of donors (pg/ml)								Average	Median	STDev
	1	2	3	4	5	6	7	8			
<b>0.00</b>	1626.98	2144.42	5896.79	1311.76	2332.99	1913.38	1922.03	2199.46	2418.47	2033.22	1443.31
<b>0.63</b>	1767.32	1929.38	6734.32	1528.84	816.84	1820.46	1729.54	797.51	2140.53	1748.43	1908.18
<b>1.25</b>	1786.47	2053.40	5601.69	1630.68	1526.24	1654.85	1772.00	858.62	2110.49	1713.42	1451.98
<b>2.50</b>	1763.49	2018.98	5643.41	1635.55	1962.25	1727.27	1792.70	932.06	2184.46	1778.10	1436.79
<b>5.00</b>	1785.78	2041.65	5577.92	1651.25	2208.52	1715.84	1865.93	1002.06	2231.12	1825.85	1398.04
<b>10.00</b>	1697.22	2102.17	5221.42	1749.83	2224.83	1578.68	1902.70	1071.94	2193.60	1826.27	1272.86

**Table 5. The modulation of IFN- $\gamma$  by *Allium sativum* aqueous extract in unstimulated whole blood cell cultures (3-day incubation).**

Extract (mg/ml)	IFN- $\gamma$ expression of donors (pg/ml)								Average	Median	STDev	
	1	2	3	4	5	6	7	8				
<b>0.00</b>	0.00	100.49	0.00	0.00	20.67	0.00	0.00	0.00	0.00	15.09	0.00	35.26
<b>0.63</b>	0.00	98.92	11.58	0.00	12.06	0.00	0.00	0.00	0.00	15.32	0.00	34.20
<b>1.25</b>	0.00	75.58	460.16	0.00	24.97	0.00	0.00	0.00	5.59	70.79	2.80	159.48
<b>2.50</b>	4.97	144.75	798.07	0.00	441.01	35.70	0.00	0.00	97.45	190.24	66.57	286.26
<b>5.00</b>	65.92	232.54	675.08	0.00	143.75	103.79	3.43	118.56	167.88	167.88	111.17	218.49
<b>10.00</b>	50.69	244.16	403.40	0.00	243.13	0.00	22.82	46.40	126.32	126.32	48.55	150.73

**Table 6. The modulation of IFN- $\gamma$  by *Allium sativum* chloroform extract in unstimulated whole blood cell cultures. (3-day incubation).**

Extract (mg/ml)	IFN- $\gamma$ expression of donors (pg/ml)								Average	Median	STDev
	1	2	3	4	5	6	7	8			
<b>0.00</b>	0.00	103.07	4.87	0.00	22.82	0.00	0.00	0.00	16.34	0.00	35.92
<b>0.63</b>	0.00	132.78	4.87	0.00	7.75	0.00	0.00	0.00	18.17	0.00	46.40
<b>1.25</b>	0.00	165.54	0.00	0.00	27.12	0.00	0.00	0.00	24.08	0.00	57.94
<b>2.50</b>	0.00	165.54	11.20	0.00	44.26	0.00	0.00	0.00	27.63	0.00	57.80
<b>5.00</b>	0.00	152.47	4.87	0.00	50.68	0.00	0.00	0.00	26.00	0.00	54.02
<b>10.00</b>	0.00	210.95	0.00	0.00	57.08	0.00	0.00	0.00	33.50	0.00	74.43



**Table 7. The modulation of IFN- $\gamma$  by *Allium sativum* aqueous extract in PHA-stimulated whole blood cell cultures.**

Extract (mg/ml)	IFN- $\gamma$ expression of donors (pg/ml)								Average	Median	STDev
	1	2	3	4	5	6	7	8			
<b>0.00</b>	3666.40	2240.39	6611.10	0.00	2260.77	1753.18	1928.70	2165.70	2578.28	2203.05	1911.73
<b>0.63</b>	3361.53	5097.26	6290.51	0.00	2208.52	1872.99	1927.75	2074.45	2854.13	2141.49	2003.19
<b>1.25</b>	3680.45	4933.46	5882.77	0.00	2212.67	1846.52	1912.41	2096.83	2820.64	2154.75	1897.49
<b>2.50</b>	3699.89	5293.81	6227.12	0.00	2221.40	1895.85	1962.25	2114.72	2926.88	2168.06	2028.68
<b>5.00</b>	3735.52	5279.03	6057.94	103.79	2257.73	1890.92	1863.90	2132.06	2915.11	2194.90	1972.72
<b>10.00</b>	3899.22	5665.20	5249.38	310.15	2268.71	1704.31	1784.03	2137.01	2877.25	2202.86	1871.72

**Table 8. The modulation of IFN- $\gamma$  by *Allium sativum* chloroform extract in PHA-stimulated whole blood cell cultures.**

Extract (mg/ml)	IFN- $\gamma$ expression of donors (pg/ml)								Average	Median	STDev
	1	2	3	4	5	6	7	8			
<b>0.00</b>	1626.98	2144.42	5896.79	1311.76	2332.99	1913.38	1922.03	2199.46	2418.47	2033.22	1443.31
<b>0.63</b>	1767.32	1929.38	6734.32	1528.84	816.84	1820.46	1729.54	797.51	2140.53	1748.43	1908.18
<b>1.25</b>	1786.47	2053.40	5601.69	1630.68	1526.24	1654.85	1772.00	858.62	2110.49	1713.42	1451.98
<b>2.50</b>	1763.49	2018.98	5643.41	1635.55	1962.25	1727.27	1792.70	932.06	2184.46	1778.10	1436.79
<b>5.00</b>	1785.78	2041.65	5577.92	1651.25	2208.52	1715.84	1865.93	1002.06	2231.12	1825.85	1398.04
<b>10.00</b>	1697.22	2102.17	5221.42	1749.83	2224.83	1578.68	1902.70	1071.94	2193.60	1826.27	1272.86

**Table 9. The modulation of IFN- $\gamma$  by *Tubbaghia alliacea* aqueous extract in unstimulated whole blood cell cultures (5-day incubation).**

Extract (mg/ml)	IFN- $\gamma$ expression of donors (pg/ml)								Average	Median	STDev	
	1	2	3	4	5	6	7	8				
<b>0.00</b>	10.35	253.56	0.00	0.00	0.00	0.00	0.00	0.00	0.00	32.99	0.00	89.20
<b>0.63</b>	0.00	393.32	0.00	0.00	0.00	0.00	0.00	0.00	7.75	50.13	0.00	138.69
<b>1.25</b>	0.00	407.45	0.00	0.00	0.00	0.00	0.00	0.00	50.68	57.27	0.00	142.60
<b>2.50</b>	5.65	484.70	0.00	0.00	0.00	0.00	0.00	0.00	57.08	68.43	0.00	169.35
<b>5.00</b>	33.79	466.33	68.74	0.00	1.28	0.00	0.00	0.00	82.62	81.59	17.53	158.97
<b>10.00</b>	15.04	604.25	97.59	0.00	18.52	33.55	0.00	93.22	107.77	26.04	204.28	

**Table 10. The modulation of IFN- $\gamma$  by *Tulbaghia alliacea* chloroform extract in unstimulated whole blood cell cultures. (5-day incubation).**

Extract (mg/ml)	IFN- $\gamma$ expression of donors (pg/ml)								Average	Median	STDev	
	1	2	3	4	5	6	7	8				
<b>0.00</b>	0.00	245.93	0.00	0.00	0.00	0.00	0.00	0.00	0.00	30.74	0.00	86.95
<b>0.63</b>	0.00	292.76	0.00	0.00	0.00	0.00	0.00	0.00	0.00	36.60	0.00	103.51
<b>1.25</b>	0.00	371.06	0.00	0.00	0.00	0.00	0.00	0.00	0.00	46.38	0.00	131.19
<b>2.50</b>	0.00	311.63	0.00	0.00	0.00	0.00	0.00	0.00	0.00	38.95	0.00	110.18
<b>5.00</b>	0.00	288.06	0.00	0.00	3.43	0.00	0.00	0.00	9.90	37.67	0.00	101.23
<b>10.00</b>	0.00	276.32	0.00	0.00	9.90	0.00	0.00	0.00	18.52	38.09	0.00	96.50

**Table 11. The modulation of IFN- $\gamma$  by *Tulbaghia alliacea* aqueous extract in PPD-stimulated whole blood cell cultures.**

Extract (mg/ml)	IFN- $\gamma$ expression of donors (pg/ml)								Average	Median	STDev	
	1	2	3	4	5	6	7	8				
<b>0.00</b>	0.95	2931.27	0.00	0.00	0.00	0.00	0.00	175.05	684.49	473.97	0.48	1020.81
<b>0.63</b>	41.98	845.05	0.00	0.00	0.00	3.43	101.68	135.37	868.98	193.55	22.71	324.25
<b>1.25</b>	67.67	2665.54	68.13	0.00	1.28	5.59	135.37	139.56	1023.38	476.57	67.90	931.27
<b>2.50</b>	136.22	1187.05	24.54	0.00	7.75	18.52	139.56	154.21	1094.30	317.13	80.38	491.48
<b>5.00</b>	24.42	1873.25	128.28	0.00	35.70	42.12	154.21	168.81	1154.03	419.03	85.20	691.35
<b>10.00</b>	54.84	1180.34	50.94	16.37	120.66	69.87	168.81	351.98	505.35	351.98	95.27	505.35

**Table 12. The modulation of IFN- $\gamma$  by *Tulbaghia alliacea* chloroform extract in PPD-stimulated whole blood cell cultures.**

Extract (mg/ml)	IFN- $\gamma$ expression of donors (pg/ml)								Average	Median	STDev
	1	2	3	4	5	6	7	8			
<b>0.00</b>	0.00	2959.66	0.00	0.00	0.00	0.00	78.37	629.32	458.42	0.00	1033.76
<b>0.63</b>	0.00	842.61	0.00	0.00	0.00	7.75	139.56	170.89	145.10	3.87	290.39
<b>1.25</b>	6.35	777.47	0.00	0.00	0.00	44.26	80.50	175.05	135.45	25.31	266.40
<b>2.50</b>	0.00	652.82	0.00	0.00	3.43	61.35	112.23	187.51	127.17	32.39	223.07
<b>5.00</b>	14.02	1142.09	5.11	0.00	9.90	72.00	118.56	191.65	194.17	43.01	388.96
<b>10.00</b>	0.00	1133.90	0.00	0.00	29.26	88.98	156.30	197.86	200.79	59.12	384.63

**Table 13. The modulation of IFN- $\gamma$  by *Allium sativum* aqueous extract in unstimulated whole blood cell cultures (5-day incubation).**

Extract (mg/ml)	IFN- $\gamma$ expression of donors (pg/ml)								Average	Median	STDev	
	1	2	3	4	5	6	7	8				
<b>0.00</b>	6.83	249.60	0.00	0.00	0.00	0.00	0.00	0.00	0.00	32.05	0.00	87.94
<b>0.63</b>	0.00	309.11	0.00	0.00	0.00	0.00	0.00	0.00	0.00	38.64	0.00	109.29
<b>1.25</b>	0.00	329.07	68.13	0.00	7.75	0.00	0.00	0.00	0.00	50.62	0.00	114.95
<b>2.50</b>	3.30	359.11	226.43	33.55	29.26	84.74	16.37	5.59	94.79	156.84	31.41	129.73
<b>5.00</b>	26.76	494.93	259.54	110.13	86.86	168.81	72.00	35.70	247.14	131.14	98.49	156.24
<b>10.00</b>	10.35	564.87	542.57	147.94	114.34	395.89	86.86	114.34	247.14	131.14	98.49	156.24

**Table 14. The modulation of IFN- $\gamma$  by *Allium sativum* chloroform extract in unstimulated whole blood cell cultures. (5-day incubation).**

Extract (mg/ml)	IFN- $\gamma$ expression of donors (pg/ml)								Average	Median	STDev
	1	2	3	4	5	6	7	8			
<b>0.00</b>	6.83	249.60	0.00	0.00	0.00	0.00	0.00	0.00	32.05	0.00	87.94
<b>0.63</b>	0.00	309.11	0.00	0.00	0.00	0.00	0.00	0.00	38.64	0.00	109.29
<b>1.25</b>	0.00	329.07	68.13	0.00	7.75	0.00	0.00	0.00	50.62	0.00	114.95
<b>2.50</b>	3.30	359.11	226.43	33.55	29.26	84.74	16.37	5.59	94.79	31.41	129.73
<b>5.00</b>	26.76	494.93	259.54	110.13	86.86	168.81	72.00	35.70	156.84	98.49	156.24
<b>10.00</b>	10.35	564.87	542.57	147.94	114.34	395.89	86.86	114.34	247.14	131.14	219.49



**Table 15. The modulation of IFN- $\gamma$  by *Allium sativum* aqueous extract in PPD-stimulated whole blood cell cultures.**

Extract (mg/ml)	IFN- $\gamma$ expression of donors (pg/ml)								Average	Median	STDev
	1	2	3	4	5	6	7	8			
<b>0.00</b>	0.95	2929.49	0.00	0.00	5.59	0.00	405.74	625.61	495.92	3.27	1012.10
<b>0.63</b>	41.98	2199.71	48.17	0.00	9.90	0.00	558.17	935.44	474.17	45.08	777.43
<b>1.25</b>	67.67	3552.41	282.50	0.00	16.37	417.53	535.41	1044.53	739.55	350.02	1188.09
<b>2.50</b>	136.22	4568.90	348.59	42.12	137.47	448.80	631.17	1086.34	924.95	398.70	1510.50
<b>5.00</b>	24.42	4695.42	519.69	172.97	308.14	460.46	735.20	1252.94	1021.16	490.08	1531.70
<b>10.00</b>	54.84	3849.64	299.02	195.79	535.41	493.31	801.03	1273.71	937.84	514.36	1236.82

**Table 16. The modulation of IFN- $\gamma$  by *Allium sativum* chloroform extract in PPD-stimulated whole blood cell cultures.**

Extract (mg/ml)	IFN- $\gamma$ expression of donors (pg/ml)								Average	Median	STDev	
	1	2	3	4	5	6	7	8				
<b>0.00</b>	0.00	2973.72	0.00	0.00	42.12	0.00	0.00	48.54	680.84	468.15	21.06	1039.05
<b>0.63</b>	0.00	896.94	0.00	0.00	0.00	0.00	0.00	0.00	456.58	169.19	0.00	334.66
<b>1.25</b>	6.35	869.77	0.00	0.00	1.28	0.00	0.00	0.00	535.41	176.60	0.64	336.73
<b>2.50</b>	0.00	1022.11	11.61	0.00	9.90	0.00	0.00	0.00	724.40	221.00	4.95	410.39
<b>5.00</b>	14.02	791.03	0.00	0.00	22.82	0.00	0.00	0.00	740.59	196.06	7.01	352.02
<b>10.00</b>	0.00	1517.21	0.00	0.00	27.12	0.00	0.00	76.25	887.87	313.56	13.56	574.49