Chapter 1 Introduction

1.1 Problem statement

Global overexploitation of indigenous medicinal plants has become a serious challenge especially in the developing countries where healthcare systems and livelihoods depend largely on herbal medicines. South Africa is no exception to this challenge as its natural populations of medicinal plants dwindle. This has led to local extinction of some highly sought after medicinal plant species. Local extinction is commonest outside protected areas in South Africa. The species under severe pressure include *Warburgia salutaris*, *Cassine transvalensis*, *Alepidea amatyambica* and *Erythrophleum lasianthum* (Fennel et al., 2004).

Cultivation is one of the interventions that could reduce the pressure on the natural populations. However, some conservative traditional practitioners do not accept cultivated plants, as they perceive them to lack the “power” possessed by wild plants (Cunningham, 1994). Scientific studies also suggest that plants form secondary metabolites when under stress conditions and competition (Schippmann et al., 2002). This have been proven in cases where there is pathogenic infection, browsing by herbivores or allelopathy. Monoculture conditions may not trigger the production of secondary metabolites due to lack of stress conditions usually experienced in natural environments.

There is limited understanding of how the environmental stress conditions affect the biological activity of plants. Although much work has been done on the influence of environmental factors on the content of certain chemicals the influence of environmental factors on biological activity of plan extracts that

depends on a multiplicity of chemical compounds has not been widely studied. This limited understanding has major implications for sustainable harvesting and species biodiversity. In a certain sense, cultivating medicinal plants is equivalent to the production of chemical compounds instead of fruit or seed. If only one compound is responsible for the biological activity, chemical analysis of the plants would have been rational and sufficient. In many, if not most cases however, the biological activity is a result of interaction between different compounds in a plant. It therefore makes more sense to investigate the effects of cultivation under limited stress conditions on biological activity rather than the concentration of a single compound.

1.2 General literature survey

Herbal medicines, in several developing countries, continue to be the mainstay of the local healthcare systems (Hoareau & DaSilva, 1999). In the developing world, at least 75% of all medical drugs is based on natural products and their derivatives (Principe, 1991). According to Huxley (1984), approximately 95% of traditional drugs, many rituals and modern medicines have their origins in indigenous medicinal plants. However, extreme overexploitation puts medicinal plants under immense pressure (Canter et al., 2005). Between 35,000 and 50,000 plant species are used globally for medicinal purposes (GTZ, 2001, Schippmann et al., 2002), which is about 13% of all flowering plants worldwide. Two-thirds of the 50,000 medicinal plant species in use in developing countries are collected from the wild populations (Canter et al., 2005).

The increased dependence on natural medicines is relevant given the escalating occurrence of microbial resistance to conventional antibiotics as well the increase in occurrence of bacterial, viral and fungal diseases that affect humans, livestock, other animals and plants. The ever-increasing African

population has the world's highest burden of infectious and neglected diseases while there is no automatic guarantee for access to modern medicine (COHRED & NEPAD, 2009). This has led to an increased dependence on herbal medicines by a large number of Africans. The challenge is that consumers usually meet their extreme demand for herbal medicines largely through harvesting of dwindling natural populations (Guo et al., 2009). Wild plants with high economic value are the ones always under increasing pressure of over-exploitation (Cunningham, 1989; Schipmann et al., 2002). This leads to loss of genetic diversity and habitat destruction (Azaizeh et al., 2005).

Some reasons why developing countries are too dependent on indigenous medicinal plants are high cost of and limited access to western healthcare services (World Bank Group, 1997) as well as the perceived dual healing powers (physical and spiritual), lack of side effects, cultural acceptability and widespread availability exacerbate exploitation (Aumeeruddy-Thomas, 1998). Black communities' reliance on dual healthcare system that employs both western and indigenous practices further enhances overexploitation (Mander, 1997). The demand for herbal medicines will undoubtedly increase further given a discovery of some biological activities from *Warburgia salutaris* and other species that have potential to treat HIV/AIDS related illnesses in KwaZulu-Natal (Hutchings, 2002).

The use of herbal medicines is also widespread in some South American countries such as Columbia and Asian countries that include China, India, Pakistan, Japan, Sri Lanka and Thailand (Hoareau & DaSilva, 1999; Rojas et al., 2006). About 90% and 40% of China's rural and urban patients, respectively, use indigenous medicines for primary healthcare with a demand that exceeds 700,000 tonnes of material per year (Dubey et al., 2004). India uses more than 6,000 plant species for medicinal purposes and about 7,000 firms manufacture traditional medicines with or without standardization there

(Dubey et al., 2004). India is the largest producer of medicinal herbs and therefore regarded by some as the botanical garden of the world (Dubey et al., 2004).

Developed countries such as Belgium, France, Germany, Netherlands and the United Kingdom are also showing an increased interest in herbal medicines with about 25% of their populations taking herbal medicines regularly (Hoareau & DaSilva, 1999). The global market, for example uses between 600 and 700 metric tons of *Harpagophytum procumbens* (Devil's claw) per year to treat European ageing population with escalating number of cases of arthritis (Schneider et al., 2006).

Of the total of 670 market-based sellers of indigenous medicines during 1989 and early 1990s in the Southern African Developing Countries (SADC) and Côte d'Ivoire, South Africa represents the majority (58.5%) (People & Plants, 2002). Overall, there are up to 100 million consumers of herbal medicines in southern Africa and as many as 500,000 traditional healers (Wiersum et al., 2006). Therefore, up to 700,000 tonnes of plant material are consumed annually with an estimated value of as much as US$150 million per annum.

It is estimated that 20,000 tonnes of material from more than 700 plant species are traded nationwide in South Africa with a value of approximately R270 million (Wiersum et al., 2006). According to Keirungi & Fabricius (2005), approximately 27 million South Africans (or 72% of national population) use indigenous medicines with a value ranging between R2.9 billion and R4 billion per year, representing between 5.6% and 7.7% of the National Health budget, respectively.

Trading in traditional medicines has become a large and growing industry that forms a big part of a multimillion-rand "hidden economy" (Cunningham, 1989). It has provided employment to at least

133,000 people in South Africa alone (Mander et al., 2007). There are 63,000 and 68,000 plant harvesters and herbalists respectively in South Africa (Mander et al., 2007). The use and trade of plants for medicine has entered both the informal and formal entrepreneurial sectors of the South African economy, resulting in an increase in the number of herbal gatherers and traders (Cocks et al., 2004). Trading of approximately 120 medicinal plants takes place in Mpumalanga with over 500 tonnes of medicinal products harvested and traded in Bushbuckridge (Mander, 1997). In Durban and Johannesburg, harvesters sell about 30 to 40 tonnes of material from 400 indigenous medicinal plant species as medicines (Mander, 1997). In Kwazulu-Natal province, harvesters trade more than 4,000 tonnes of plant material at a value of approximately R 60 million (Mander, 1999). The province also has about six million consumers of indigenous medicines.

The overwhelming harvesting intensity has led to increased distance to existing plant populations, acute shortages, and imports from neighbouring countries, price increases and declining plant size in market outlets (Wiersum et al., 2006; Bodeker et al., 1997). The interest in and demand for herbal medicines in the international pharmaceutical industry have led to changes in the traditional patterns of medicinal plant harvesting (Cunningham, 2001). As a result, massive volumes of indigenous medicinal plants are being destructively extracted everyday resulting in the decline of stocks (Keirungi & Fabricius, 2005). The increased demand for medicines has even led to local extinction of *Siphononchilus aethiopicus* and *Warburgia salutatris* outside the protected areas in KwaZulu-Natal (Mander, 1997) and *Harpagophytum procumbens* in some parts of North-West Province. Several studies link increasing harvesting pressures on supply areas and a growing shortage in supply of popular medicinal plant species (Wiersum et al., 2006). The overwhelming market and public demand poses a great risk to medicinal plants of extinction and (or) loss of genetic diversity (Hoareau & DaSilva, 1999).

Unsustainable harvesting methods, destruction of habitat due to demand for farming and development land and unmonitored trade of medicinal plants further threaten medicinal species and therefore supplies (Hoareau & DaSilva, 1999). Loss of large populations of important medicinal plants in the Eastern Cape and KwaZulu-Natal provinces of South Africa happened because of the introduction of pineapple plantations and sugarcane industries, respectively (Osborne et al., 1994). This has led to scarcity of further indigenous medicinal species such as *Cassine aethiopica*, *Curtisia dentata* and *Podocarpus falcatus* at the Tootabie Nature Reserve in the Eastern Cape (La Cock & Brier, 1992).

The eThekwini Medicinal Plant Sector Support Programme (TMPSSP) of South Africa is now integrating policy, skills and market development, plant production, beneficiation and conservation in the business of herbal medicines (Planting, 2009). The reason for TMPSSP to be involved in the sector is the potential international market opportunity such as Hong Kong and Germany that import 77 250 tonnes and 42 800 tonnes of pharmaceutical plants per year, respectively (Planting, 2009). With the increasing demand for and price of herbal medicines as well as opportunity to access markets, trading of these products provides business and job opportunities for local people. For instance in Asia, the Bhotiya community in the western Himalaya earns a decent living from selling cultivated medicinal plants (Silori & Bado, 2000). In addition, communities of Uttaranchal, one of the poorest regions in India, depend on non-farm income that includes collection and sale of medicinal plants (Alam, 2003).

Keirungi & Fabricius (2005) indicate that some traditional healers still consider certain medicinal plants unsuitable for *ex situ* cultivation due to perceived loss of healing powers when taken out of their natural habitats. There are also doubts about whether cultivation will fulfil ritual or traditional requirements. The perception is such that cultivated plants can lose their potency when touched by ‘polluted’ people or witches (Wiersum et al., 2006). Therefore medicinal plants that are cultivated through the assistance of

modern farming methods and in contact with 'polluted' people are "unnatural" and "impure" (Canter et al., 2005). A person is 'polluted' during menstruation, after sexual intercourse and childbirth, or if involved in witchcraft and when there is death in the family.

Kuipers (1995) pointed out that wild plants are more potent and fetch higher prices than the cultivated ones. Nevertheless, a recent study in the Eastern Cape Province indicates that up to 82% of urban-based healers and 69% of clinic patients are willing to make use of the cultivated plant material (Keirungi & Fabricius, 2005). However, some years earlier traditional healers still preferred to use herbal medicines harvested from the wild populations (Mander, 1997). Some local users of herbal medicines at Amatola region in South Africa strongly feel that plant species collected following ritual practice and species indicated by the ancestors in dreams should be strictly collected from the wild to make them remain effective (Wiersum et al., 2006). It is therefore important to ensure that cultivation embraces the cultural values in order to create a positive attitude towards conservation in general (Wiersum et al., 2006). Restricting people from accessing and using wild natural resources for their healthcare and livelihoods is probably a cause for negative attitude (Silori, 2007).

The challenge in bringing medicinal plants into cultivation could be the difficulty of predicting the extracts that will remain marketable and the likely market preference for what is naturally sourced extracts (Canter et al., 2005). This is because the quality and quantity of secondary metabolites such as antioxidant, flavonoids or alkaloids change according to fluctuating environmental conditions such as temperature and light (Canter et al., 2005). The following are two examples showing how abiotic factors change the contents of some secondary compounds (McChesney, 1999):

- Shade-grown *Mentha piperata* has lower essential oil (1.09% v 1.43%) and menthol contents within the oil (57.5% v 61.8%) compared with light grown *Mentha piperata*.

- Cool-grown *Papaver somniferum* (poppy) contains more morphine with lower alkaloid content than the warm-grown *P. somniferum*.

Changes of environmental conditions as well as the effect of pathogens (Fluck, 1955), allelopathy (competition) and herbivory (Gershenzon, 1984), on plants may trigger the production of high levels of secondary metabolites (Vickery & Vickery, 1981). Water availability, exposure to soil pathogens and variations of soil pH and nutrients affect the accumulation of secondary metabolites (Economakis et al., 2002). The environmental factors such as temperature, rainfall, day length and edaphic factors, affect the efficacy of the medicinal properties (Dubey et al., 2004). In order for a plant to survive growing under different climatic and stressful conditions, different genes are expressed leading to production of different concentrations of biological activities (Dubey et al., 2004). Irrigation increases anthelmintic activity in areas of low rainfall (Fennel et al., 2004). In addition, reduced watering and nutrient levels also increase the concentration of pentaynene (a compound for defence) (Almeida-Cortez et al., 2003) and antioxidant activity (McCune & Johns, 2007).

Sometimes the biological activity of cultivated plants and those growing naturally in the wild can be the same (Rowson & Hans 1973). In another study, there was no correlation between artemisinin content and a season long water stress (Charles et al., 1993). However, extreme soil water stress has led to reduced leaf artemisinin content. Post-harvest handling also affects the biological activities. Artemisinin content is retained to a considerable extent when dried under ambient conditions (Charles et al. 1993). Similarly, harpagoside retention of Devil's claw plant (*Harpagophytum procumbens*) is significantly lower when sun-dried than tunnel-dried (Joubert et al., 2005).

Variability of biological activity in plants due to genetic factors may also influence the use of medicinal plants. Sunderland & Tako (1999) point out that there is a possibility that isolated medicinal plant populations from their natural habitats may have considerable genetic and chemical differences. This variability in genetic potential may favour the *ex situ* production of medicinal plants. If a chemotype with a higher concentration is stable, plant breeders' rights may be obtained. Furthermore, delivery of a plant product with a high and stable biological activity will have a strong competitive advantage if handled properly.

1.3 Aim and objectives

The overall aim of this study was to evaluate to what degree the biological activities of plants cultivated outside their natural environment are influenced by important environmental factors.

This aim was addressed by investigating the following objectives:

- Select the plant species to work on and the biological activities to be determined
- Evaluate the antimicrobial activities of three long-lived plant species in populations subjected to natural water stress conditions
- Evaluate the antimicrobial and antioxidant activity of short-lived herbaceous species with limited genetic diversity subjected to different water stress levels *ex situ*
- Evaluate the antimicrobial and antioxidant activity of short-lived herbaceous species with limited genetic diversity subjected to different temperature stresses *ex situ*

Some notes:

Chapter 1 has demonstrated greatly the importance of and demand for herbal medicines in the developing countries. It is evident that the overwhelming dependence on the wild populations for herbal medicines has already led to local extinctions. The study objectives are set out to address this challenge as outlined in the next **Chapters 2 – 6**. **Chapters 2 and 3** are exploratory studies intended to evaluate the effects of different rates of rainfall and induced water stress conditions on the antibacterial activity of three tree and two herbaceous plant species growing in the field environment and glasshouse, respectively. **Chapters 4 – 6** are in depth studies that evaluate the effects of induced water and temperature stress conditions on qualitative and quantitative antibacterial, antifungal and antioxidant activity of three herbaceous plants growing in the glasshouse and growth chambers. **Chapters 2 – 6** are in a form of publications and therefore based on the requirements of different journals, to which they have been or will be submitted. **Chapter 7** provides conclusions and recommendations based on the overall study findings.

Chapter 2 Antibacterial activity of acetone leaf extracts of three tree species from areas receiving different rates of annual rainfall

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Abstract

Scientists and conservative traditional practitioners believe that stress triggers production of a strong activity. If the stress is from a pathogen, herbivore or allelopathy there are defence responses that would influence biological activity, but not clear with other types of stresses. The study evaluated the effects of different rates of annual rainfall on antibacterial activity of leaf extracts of *Terminalia sericea* Burch. ex DC. (Combretaceae), *Combretum collinum* Fresen. (Combretaceae) and *Sclerocarya birrea* (A. Rich.) Hochst. (Anacardiaceae). Leaves were harvested from trees growing in high (≥870 mm/year), medium (651 mm/year)) and low (<484 mm/year) rates of annual rainfall. Air-dried leaves were finely ground and extracted with acetone. Minimum inhibitory concentration (MIC) was determined by using microplate serial dilution technique with four bacterial strains. Thin layer chromatography (TLC) and bioautography determined chemical constituents and bacterial growth inhibition, respectively. The majority of leaf extracts had low MIC values (<250 µg/ml) depicting good antibacterial activity. Leaf extracts of *C. collinum* and *S. birrea* against *Staphylococcus aureus* (range of 390 – 100 µg/ml), *Escherichia coli* (310 -70 µg/ml) and *Pseudomonas aeruginosa* (520 - 70 µg/ml) had antibacterial activity increased significantly with low rate of annual rainfall. Extracts of *T. sericea* against *P. aeruginosa* (240 - 100 µg/ml) and *Enterococcus faecalis* (150 - 820 µg/ml) had antibacterial activity significantly increased and decreased, respectively. Extracts of *C. collinum* and *S. birrea* against *E. faecalis* as well as *T. sericea* against *S. aureus* and *E. coli* did not show any pattern. Inconsistent results suggest that different rates of annual rainfall may not solely affect antibacterial activity of extracts.

Keywords: Bacterial strains, chemical constituents, bioautography, minimum inhibitory concentration, total activity, trees

2.1 Introduction

Herbal medicines continue to be the mainstay of the local healthcare systems (Hoareau & DaSilva, 1999) especially those of developing countries (Keirungi & Fabricius, 2005). It is therefore evident that plant derived medicines are making large contributions to human health and wellbeing. About 27 million South Africans use indigenous biomedicines valued at about R4 billion (Keirungi & Fabricius, 2005). The increased dependence on herbal medicines is relevant due to several reasons. These include microbial resistance to conventional antibiotics and prevalence of pathogens and inflammation diseases that affect human, livestock and other animals.

Dependence on natural populations of medicinal plants leads to local extinctions. Cultivation of medicinal plants is seen as a long-term solution that can reduce pressure of wild populations. However, conservative traditional practitioners do not accept cultivated plants, as they perceive them to lack the "power" possessed by wild plants (Cunningham, 1994). Scientific studies also suggest that plants form secondary metabolites when under stress conditions and competition (Schippmann et al., 2002).

There is uncertainty around the extent to which abiotic and biotic stresses may affect the antimicrobial activity in the field environment. It was against this backdrop that the study evaluated the effects of different rates of annual rainfall implying different water stress conditions on qualitative and quantitative antibacterial activity of the tree species. Brief descriptions of the study trees are provided below.

Trees selected for this study were *Terminalia sericea* Burch. ex DC. (Combretaceae), *Combretum collinum* Fresen.) (Combretaceae) and *Sclerocarya birrea* (A. Rich.) Hochst. (Anacardiaceae). Several members of the Combretaceae and Anacardiaceae continue to be widely used in South Africa for medicinal purposes. They possess anti-inflammatory, antioxidant and antimicrobial activities (Masoko &

Eloff, 2007; Masoko et al., 2008; Eloff, 1999, 2001; Eloff et al., 2001). The following criteria contributed in the decision to select the abovementioned tree species:

- Easy to identify by a non-taxonomist
- Accessible and obtainable
- Abundance and wide distribution
- Scientifically proven antimicrobial activity

S. birrea is commonly known by local people as *mufula* (Tshivenda), *nkanyi* (Shangaan, isiNdebele), *umganu* (isiZulu, isiSwati), *marula* (English), maroela (Afrikaans) and morula (seSotho). It is one of the highly valued indigenous trees for a wide range of reasons (Shackleton et al., 2002). This tree has both nutritional and medicinal properties (van Wyk & Gericke, 2000). Its decoction of the bark has prophylactic activity against malaria and it treats dysentery, ulcer, stomach ailments, fever, haemorrhoids, diabetes, rheumatism and diarrhoea (Van Wyk et al., 1997). Local people use root and bark products as laxatives. A drink made from leaves is a treatment for gonorrhoea. The marula fruit is also a potent insecticide (Watt & Breyer-Brandwijk, 1962). Wild animals and livestock browsed its leaves (Muok et al., 2007). The tree contains secondary compounds such as gallotannins, flavonoids and catechins (Van Wyk et al., 1997). Leaf extracts possess antifungal (average MIC of 0.3 mg/ml) (Masoko et al., 2008) and antibacterial activities (average MIC of 1.4 mg/ml) (Eloff, 2001).

The common names for *T. sericea* are *mususu* (Shona, Tshivenda), *amangwe amhlope* (isiZulu), mangwe or *ivikani* (isiNdebele) (Watt & Breyer-Brandwijk, 1962), *mogonono* (seTswana), *moxonono* (Northern Sotho), vaalboom (Afrikaans) and silver cluster-leaf (English) (Van Wyk et al., 1997). It is also widely used for medicinal purposes by traditional healers. It has some antifungal activity (Masoko et al., 2005). The tree species is also included in the list of the African Herbal Pharmacopoeia as one of the

most important medicinal plants in Africa (Brendler et al., 2010). Its extracts treat sore throats, diarrhoea, venereal diseases, syphilis, toothache, diabetes (van Wyk et al., 1997) and cough (Dery et al., 1999). The tree also treats bilharzias, pneumonia, stomach pain and wounds (Palgrave, 1985; Hines & Eckman, 1993). People use the decoction as eye lotions (Van Wyk et al., 1997). The decoction also treats a cow suffering from a retained placenta (Watt & Breyer-Brandwijk, 1962). The tree possesses compounds such as glucoside and nerifolin (Palgrave, 1985). Leaf extracts of *T. sericea* has good antibacterial (Eloff, 1999; Fyhrquist et al., 2002) and antioxidant activities (Masoko & Eloff, 2007). *Terminalia* species in general produce pentacyclic triterpenoids of which sericic acid and ester thereof (known as sericoside) are the main compounds in the roots (Van Wyk et al., 1997).

C. collinum root decoction is drunk for the treatment of diarrhoea, sterility and pyomyositis (Odda et al., 2008). The infusion of roots is given to pregnant women to enhance labour. Epilectic patients mix root powder with hot water to use as “tea” or pour the powder into bathing water to treat their condition. Leaf extracts of *C. collinum* possess antifungal (Masoko & Eloff, 2006) and antioxidant activities (Masoko & Eloff, 2007). *Combretum* species in general possess moluscicidal and antimicrobial activities (Eloff et al. 2001). *C. collinum* in particular also has some larvicidal activity against *Aedes aegypti* (vector responsible for yellow fever) (Odda et al., 2008).

2.2 Materials and methods

2.2.1 Localities

Leaf samples of *Terminalia sericea*, *Combretum collinum* and *Sclerocarya birrea* were collected from three localities subjected to different rates of annual rainfall in the Lowveld region of Limpopo Province where the trees are abundant and widely distributed. The chosen localities were Hazyview, Wits Rural Facility in Acornhoek and Manyeleti Game Reserve with rates of annual rainfall of ≥ 870 mm, c. 651 mm and ≤484 mm, respectively, and about 550 m above sea level (Shackleton, 1999). Other factors such as age, herbivores, pathogens, topography, metal pollutants and microclimate were not accounted for in the study. Voucher specimens of *T. sericea* (117134), *C. collinum* (117133), and *S. birrea* (117135) were collected. Mrs Elsa van Wyk, the curator, verified and kept specimens in the H.G.W.J. Schweickerdt Herbarium situated at the University of Pretoria.

2.2.2 Collection of leaf samples

Leaf samples from twelve trees (4 per species) per each annual rainfall level were collected. Only trees situated within a radius of about 50 metres per site were considered in order to minimise genetic variability. Fresh leaves were collected from the lowest branches of 36 individual trees during spring (between October and November) in 2004 after leaves had fully developed. Time for collecting leaves ranged between 9 a.m. and 4 p.m. as study sites were situated far apart. After collection, leaves were separated from the stems, dried in a ventilated storeroom at room temperature of 25°C, and ground into a fine powder in a Jankel and Kunkel Model A10 mill. The powder was stored in the airtight

containers and kept in the dark cupboards at room temperature until required. Keeping leaf samples in the dark ensures stable biological activity.

2.2.3 Extraction procedure

One gram of the finely ground air-dried leaves of each tree was extracted with 10 ml acetone in 50 ml centrifuge tubes. Acetone is a good extractant (Eloff 1998a) and least toxic to organisms in bioassays (Masoko & Eloff, 2007). Centrifuge tubes were vigorously shaken in a Labotec model 20.2 machine for 3-5 minutes at high speed to ensure uniform samples (Eloff, 1998a). The extracts were centrifuged at 3000 x g for 10 minutes and the supernatant was filtered through Whatman No. 1 filter paper into a pre-weighed glass vials. The same process was repeated twice in order to exhaustively extract the plant material and the extracts were combined. Exactly 5 ml of the filtrate was removed and placed into a pre-weighed vial under a stream of air at room temperature in a fume cupboard. That was done to remove the acetone and to determine the concentration of the combined extract. The required quantity of acetone in the combined extract was removed to yield a concentration of 10 ml/mg. This process limits problems experienced in redissolving dried extracts (Eloff, 2004).

2.2.4 Test bacterial strains

Gram-positive [*Staphylococcus aureus* (ATCC 29213) and *Enterococcus faecalis* (ATCC 29212)] and Gram-negative [*Pseudomonas aeruginosa* (ATCC 25922) and *Escherichia coli* (ATCC 27853)] bacterial strains were used to evaluate the antibacterial activity of both plant and tree species. These are the four most important nosocomial bacteria. The strains were obtained from the Central Microbiology

Laboratory, Faculty of Veterinary Science at the University of Pretoria. The strains were grown at 37°C in Mueller-Hinton broth (Merck chemicals) (Eloff, 1998b).

2.2.5 Phytochemical analysis

Chemical constituents of the extracts were analysed by thin layer chromatography (TLC) using aluminium-backed plates (Merck, silica gel 60 F_{24}). The TLC plates were developed in the three mobile systems of differing polarity that gave excellent separation of many different compounds in acetone leaf extracts (Kotze & Eloff, 2002). The mobile systems used were a) chloroform/ethyl acetate/formic acid (CEF: intermediate) (5:4:1), b) benzene:ethyl acetate:ammonia (BEA: non-polar) (9:1:0.1) and c) ethyl acetate:methanol:water (EMW: polar) (40:5.4:5). The TLC plates were visualized under UV light (250 and 360 nm, Camac Universal lamp TL-600) to detect UV active absorbing sports or plant constituents. The plates were then sprayed with vanillin reagent (0.1% vanillin dissolved in 28 ml methanol and 1 ml sulphuric acid) and heated at 100°C to optimal colour development. The position of the visible compounds on the TLC plate was established by calculating the retardation factor (R_f), which is the distance compound travelled divided by the distance the solvent had travelled from the origin.

2.2.6 Bioautography assay

The developed TLC plates or chromatograms (not sprayed with vanillin spray reagent) were air-dried overnight and sprayed with a concentrated suspension of actively growing cells of test bacteria. This method relies on the direct growth inhibition or killing of pathogens on contact with the active band (Begue & Kline, 1972). The chromatograms were incubated overnight at 38°C in a chamber at 100% relative humidity. The incubation allowed pathogens to grow on the chromatograms. After the incubation, bioautograms were sprayed with an aqueous solution of 2 mg/ml p-iodonitrotetrazolium

violet (INT) (Sigma) before being incubated for 30 minutes (Begue & Kline, 1972). The observation was done to check clear zones on the plates indicating growth inhibition of pathogens by bioactive compounds in the extracts. The TLC plates sprayed with vanillin were used as reference chromatograms for bioautography plates displaying areas of inhibition. The R_f values of active zones were aligned with those bands of compounds on the reference chromatograms.

2.2.7 Minimum inhibitory concentration

The Minimum inhibitory concentration (MIC) values (mg/ml) were determined by two-fold serial dilution (e.g. 10, 5, 2.5, 1.3, 0.65, 0.32, 0.16, 0.08 mg/ml, etc) of extracts beyond where no inhibition of growth of test bacteria was observed (Eloff, 2001). This method was used to evaluate the antibacterial activity of extracts (Eloff, 1998a). Sufficient acetone was diluted to a concentration of 10 mg/ml. Plant extracts (100 µl) in triplicate for each experiment were serially diluted two-fold in a 96-well microlitre plates. A similar volume 100 µl of the actively growing test organism cultures was added to each well and the cultures were incubated overnight at 37°C under 100% relative humidity. As an indicator of bacterial growth, 40 µl of 0.2 mg/ml of ρ-iodonitrotetrazolium violet (INT) dissolved in water was added to each microplate well before being incubated for an hour or two (Eloff, 1998b). The MIC value was recorded as the lowest concentration that inhibited growth of bacteria. The colourless tetrazolium salt acts as an electron acceptor and is reduced to a red-coloured formazan product by biologically active pathogens (Eloff, 1998b). Clear zones on the chromatogram indicated inhibition of the growth of bacteria after incubation with INT. The experiment was repeated twice to confirm the results, and three replicates were included in each experiment.

2.2.8 Total activity

Total activity was used as a parameter that would be applied to measure the effects of different rates of annual rainfall on plant activity. It indicates the degree to which the active compounds in one gram of plant material can be diluted and still inhibit growth of pathogens. Total activity value (ml) measures the total antibacterial activity present in the plant by dividing the quantity (mg) extracted from 1 gram of plant material with the MIC value in mg/ml (Eloff, 2000). The result in ml indicates the volume to which the compounds present in 1 g of plant material can be diluted and still inhibit the growth of the microorganism. If different rates of annual rainfall affected the quantity present in the plant without affecting the potency of the bioactive compounds the quantity extracted should also be taken into account.

2.2.9 Statistical analysis

Results for antibacterial activity of all tree species were reported as means ± standard error (SE). Significant differences for comparisons were determined by a one-way analysis of variance (ANOVA) procedure. The results with 5% level of confidence ($P \leq 0.05$) were regarded as statistically significant. Data were statistically analysed using GenStat ® for Windows ® (2003) and SA® PROC GLM.

2.3 Results

2.3.1 Minimum inhibitory concentrations and total activity

The majority of acetone leaf extracts of all tree species in general had good antibacterial activity against test bacteria (240 µg/ml - 60 µg/ml) (Table 2.1). The lowest MIC value was 60 µg/ml (highest antibacterial activity) while the highest was 1460 µg/ml (lowest antibacterial activity). Leaf extracts of *C. collinum* and *Sclerocarya birrea* against *Staphylococcus aureus* (390 – 130 µg/ml; 340 - 100 µg/ml), *E. coli* (270 – 70 µg/ml; 310 -110 µg/ml) and *P. aeruginosa* (240 - 80 µg/ml; 520 - 70 µg/ml) had significantly increased antibacterial activity towards low rate of annual rainfall with clear trends. Leaf extracts of *T. sericea* against *P. aeruginosa* (240 - 100 µg/ml) and *E. faecalis* (150 - 820 µg/ml) with significantly increased and decreased antibacterial activity towards low rate of annual rainfall also showed clear trends, respectively. Leaf extracts of *C. collinum* and *S. birrea* against *E. faecalis* as well as those of *T. sericea* against *S. aureus* and *E. coli* did not show any correlation between antibacterial activity and rates of annual rainfall.

Table 2.1. Minimum inhibitory concentration of leaf extracts of medicinal tree species subjected to different rates of annual rainfalls. Values (means ± standard error; n=4) showing the same superscripts in the same column are not significantly different at the 5% confidence level (P≤0.05).

Tree species	**Rates of annual rainfall**	**Minimum inhibitory concentration ± standard error (µg/ml)**			
		Staphyloccocus aureus	*Escherichia coli*	*Pseudomonas aeruginosa*	*Enteroccocus faecalis*
Terminalia sericea	High	130 ± 12^{a}	180 ± 16^{a}	240 ± 95^{a}	150 ± 21^{a}
	Medium	240 ± 14^{b}	310 ± 14^{b}	120 ± 44^{b}	310 ± 60^{b}
	Low	60 ± 12^{c}	80 ± 14^{c}	100 ± 55^{b}	820 ± 72^{c}
Combretum collinum	High	390 ± 43^{a}	270 ± 14^{a}	240 ± 35^{a}	270 ± 70^{a}
	Medium	150 ± 46^{b}	240 ± 12^{a}	190 ± 31^{a}	100 ± 42^{b}
	Low	130 ± 48^{b}	70 ± 14^{b}	80 ± 31^{b}	820 ± 67^{c}
Sclerocarya birrea	High	340 ± 77^{a}	310 ± 25^{a}	520 ± 63^{a}	230 ± 77^{a}
	Medium	110 ± 77^{b}	240 ± 25^{a}	130 ± 61^{b}	170 ± 77^{a}
	Low	100 ± 77^{b}	110 ± 25^{b}	70 ± 12^{b}	1460 ± 77^{b}

Rates of annual rainfalls: High (c. ≥870 mm annual rainfall), medium (c. 651 mm) and low (c. <484 mm).

As shown in Figure 2.1 (adapted from Table 2.2), there was poor or no consistent pattern between rates of rainfall and total activity. With the *T. sericea* extracts, low rate of annual rainfall significantly decreased the total activity against *E. faecalis*, but there was no clear pattern with the other three pathogens. In the case of the *C. collinum* extracts, low rate of rainfall led to significantly increased total activity against *S. aureus, E. coli,* and *P. aeruginosa*, but there was no clear pattern with *E, faecalis.* With the *S. birrea*, reduced rate of rainfall led to significantly increased total activity against *E. coli* and *P. aeruginosa*, but there was no clear pattern with remaining bacteria. It does appear as if there are

different responses between the different bacteria. Reduced rate of rainfall generally led to increased activity against *P. aeruginosa* and decreased activity against *E. faecalis*.

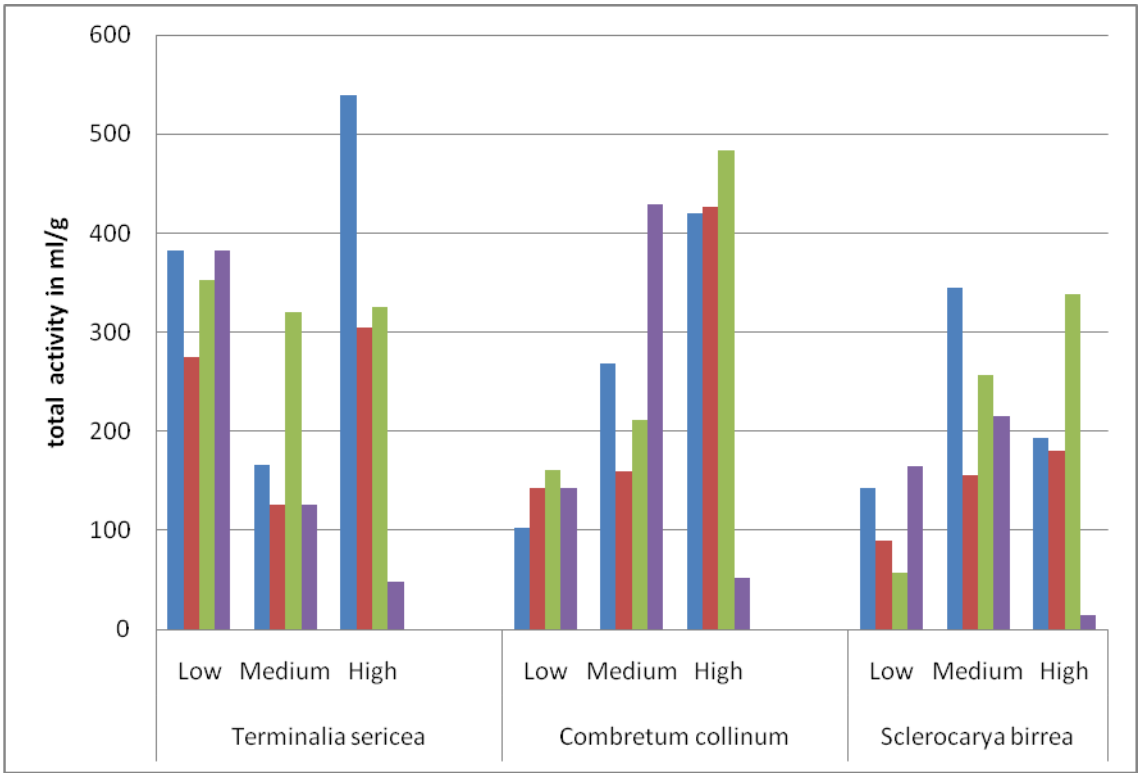

Figure 2.1. Influence of different levels of annual rainfalls on the total activity in ml/g of acetone leaf extracts of three tree species (adapted from data in Table 2.2). Bars from left to right represent activity against *S. aureus, E. coli, P. aeruginosa* and *E. faecalis*. Rates of annual rainfalls: Low stress (≥870 mm mean annual rainfall) denotes high rate of annual rainfall, medium (*ca*. 651 mm) and high stress (< 484 mm) denotes low rate of annual rainfall.

Table 2.2. Total activity of leaf extracts of medicinal tree species subjected to different rates of annual rainfalls. Values (means ± standard error; n=4) showing the same superscripts in the same column are not significantly different at the 5% confidence level (P≤0.05).

Tree species	Rates of annual rainfall	Mean total activity ± Standard Error (ml/g)			
		Staphyloccocus aureus	*Escherichia coli*	*Pseudomonas aeruginosa*	*Enteroccocus faecalis*
Terminalia sericea	High	383 ± 67^{a}	275 ± 53^{a}	353 ± 160^{a}	383 ± 66^{a}
	Medium	166 ± 72^{b}	126 ± 61^{b}	320 ± 127^{a}	126 ± 76^{b}
	Low	539 ± 167^{a}	304 ± 53^{a}	325 ± 147^{a}	48 ± 27^{c}
Combretum collinum	High	102 ± 73^{a}	142 ± 31^{a}	161± 74^{a}	142 ± 36^{a}
	Medium	268 ± 73^{b}	160 ± 31^{a}	211 ± 74^{a}	429 ± 36^{b}
	Low	420 ± 73^{b}	427 ± 31^{b}	483 ± 74^{b}	52 ± 36^{c}
Sclerocarya birrea	High	142 ± 71^{a}	90 ± 22^{a}	57 ± 53^{a}	164 ± 48^{a}
	Medium	345 ± 71^{b}	155 ± 22^{b}	257 ± 48^{b}	215 ± 48^{a}
	Low	193 ± 71^{ab}	180 ± 22^{bc}	338 ± 58^{b}	14 ± 7^{b}

Rates of annual rainfalls: Low (≥870 mm mean annual rainfall), medium (c. 651 mm) and high (< 484 mm).

2.3.2 Active compounds of tree species

Biautography was used to determine the number of active compounds present under different levels of rainfall against *P. aeruginosa* (Table 2.3). There were at least two active compounds present in each extract. Several numbers of those compounds had inhibition ranging from highest to lowest levels. Because no separation was obtained with the EMW system, the R_f values were relatively high indicating that the antibacterial compounds were probably medium polarity compounds.

Table 2.3. Antibacterial activity separated zones at different R_f values of extracts of tree species against *Pseudomonas aeruginosa* (other three pathogens were not tested).

Tree species	R_f value	Antibacterial activity		
		High rate ofannual rainfall	**Medium rate of annual rainfall**	**Low rate of annual rainfall**
BEA mobile system				
S. birrea	0.84	X	-	-
	0.42	XXX	XXXX	XXXX
C. collinum	0.84	XX	-	-
	0.53	-	-	XX
T. sericea	0.84	X	-	-
	0.42	-	XXX	-
CEF mobile system				
S. birrea	0.83	-	-	XXX
	0.80	-	XXXX	-
	0.47	X	-	-
C. collinum	0.83	-	-	XXX
	0.47	X	-	-
T. sericea	0.80	-	XX	-
	0.53	X	-	-
EMW mobile system				
S. birrea	0.94	XXX	XX	XX
C. collinum	0.94	XXX	-	XX
T. sericea	0.94	XXX	XX	XX

Rates of annual rainfalls: High (≥870 mm), medium (c. 651 mm) and low (< 484 mm). Degree of inhibition: XXXX – highest activity, X – least activity.

In general, there was no clear pattern between the degree of inhibition and low rate of annual rainfall. However, an increased activity with higher stress of the *S. birrea* extract against *P. aeruginosa* was

observed. That may be explained by a change of concentration of the compound with an R_f of 0.83 in the CEF system.

2.4 Discussion and conclusion

The majority of leaf extracts of tree species showed low MIC (<250 µg/ml) or total activity values demonstrating presence of good antibacterial activity. The MIC values were comparable to or even smaller than the published data using the same techniques in Eloff (1999, 2001, 2004). The extracts could be a good alternative of expensive modern medicines.

A clear trend with significantly increased antibacterial activity of extracts of *C. collinum* and *S. birrea* against all bacteria except *E. faecalis* towards low rate of annual rainfall suggests positive influence of water stress (Table 2.1). Water stress has also led to increased phytoalexins (Plumbe & Willmer, 1985) and terpenes (Turtola et al., 2003) of certain plants. Water stress may lead to reallocation of the assimilated carbon that triggers increased production of secondary metabolites in plants (de Abreu & Mazzafera, 2007). The other investigation by Kirakosyan et al. (2004) has shown that water deficit stress may enhance the levels of desired polyphenolics in the leaves of *Crataegus laevigata* and *C. monogyna*.

There has been a significant increase and decrease in antibacterial activity of extracts of *T. sericea* against *P. aeruginosa* and *E. faecalis* towards low annual rainfall, respectively. That was partly attributable to varying sensitivity of different bacteria. The relative less sensitivity of *Pseudomonas aeruginosa* to plant extracts could be due to a double membrane, characterising Gram-negative bacterial strains. Other studies show that water stress affects the efficacy of medicinal plants by reducing enzymatic activity responsible for the production of biological activity (Osuagwu et al., 2010; Schneider et al., 2006). Water stress has also reduced the activity of a certain compound by inducing high rate of its transformation into another form of compound (Aziz et al., 2008).

The correlation between rates of annual rainfall and antibacterial activity of the extracts of *C. collinum* and *S. birrea* against *E. faecalis* as well as of *T. sericea* against *S. aereus* and *E. coli* was very poor. The results were also in agreement with poor correlation between active compounds (separated by mobile systems) and low rate of annual rainfall in Table 2.3. The results suggest that the annual rainfall regime did not affect the antibacterial activity of the extracts. Synergistic or antagonistic effects between secondary compounds may also alter biological activity (Dorman & Deans, 2000) or chemical composition (Burt, 2004). Pathogens, herbivore (Banchio et al., 2007) or allelochemical attacks (Jahangir et al., 2009; Blanco, 2009) can also affect the plant activity. Metabolites accumulated in plants have clear ecological roles such as protection against pathogens (Rios et al., 1988). Age (Dunford & Vazquez, 2005), soil nutrient (Almeida-Cortez et al., 2003) and soil characteristics () are other important factors.

To conclude, extracts of the study tree species had good antibacterial activity characterized by majority of MIC values less than 250 µg/ml. Reduced rate of the annual rainfall in some cases increased or decreased the antibacterial activity with clear pattern whereas in other situations rainfall regime did not seem to have any effect. A problem with this project is that inconsistent findings may be attributed to a host of other factors such pathogens and competition rather than rates of annual rainfall. If trees growing in the dry area had better rain than trees, growing in the higher rainfall area before collection the results may also be ambiguous. This could also have led to confusing results if soil water potential was determined during collection. The results demonstrate that stressful conditions may not always enhance antibacterial activity of plants. To test this preliminary conclusion, clone plants of similar age with minimal genetic diversity should be grown under well controlled environmental stress conditions *ex situ* before determining the effect of water stress on antibacterial activity.

Some notes:

The study demonstrated that the different rates of annual rainfall might not have had a sole effect on the antibacterial activity of the tree species, but a host of other factors. The results indicate an inconsistent pattern between rates of annual rainfall and antibacterial activity of the selected tree species. In some cases, rainfall had no effect and in other cases there were statistically significant difference but there was no clear trend. It appears that several other factors including genetic diversity, edaphic factors may have played a role in the antimicrobial activity. The results also indicate in some cases that there is a substantial difference in the antimicrobial activity of the same species growing in different areas, an argument against using plants collected in nature to develop herbal products with a consistent quality. To limit the effects of dynamic natural environment and genetic diversity, the effect of water stress on antibacterial activity of plant species with limited genetic diversity and short growth cycle was determined in the next chapter. .

Chapter 3 Does water stress affect antibacterial activity of *Tulbaghia violacea* and *Hypoxis hemerocallidea*?

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Abstract

Many conservative traditional practitioners in South Africa still believe that medicinal plant species growing in nature will lose their biological activity if they are cultivated. This perception has led to overexploitation of dwindling wild populations of medicinal plants. It is because there is insufficient knowledge regarding the effects of environmental stress on the biological activity of plants. The exploratory study evaluated the effects of different watering intervals on the dry mass of *Tulbaghia violacea* Harv. and *Hypoxis hemerocallidea* Fish., C.A.Mey. & Avé-Lall. and the antibacterial activity of their leaf extracts. The study subjected plants to watering intervals of 3, 14 and 21 days based on irrigation with 1000 ml of distilled water every two days. Air-dried leaves were finely ground and extracted with acetone. Minimum inhibitory concentration (MIC) was determined by using a microplate serial dilution technique with four bacterial strains. Thin layer chromatography (TLC) and bioautography determined chemical constituents and bacterial growth inhibition, respectively. Dry mass was reduced significantly ($P \leq 0.05$) with watering interval of 21 days indicating that plants were stressed. However, the same stress conditions had very little effect on the quantity extracted (mg/g), MIC (mg/ml) and total activity (ml/g) values as well as chemical constituents of plants. The results suggest that cultivating medicinal plants *ex situ* under optimal watering intervals may not necessarily have adverse effect on the antibacterial activity of extracts. It is therefore likely that optimal water regimes may maintain or enhance the productivity and antibacterial activity of good chemotypes. Undertaking further studies to confirm these results is fundamental.

Keywords: Bioautography, *Hypoxis hemerocallidea*, minimum inhibitory concentration, *Tulbaghia violacea*, total activity value, water stress conditions

3.1 Introduction

The traditional healers' belief that medicinal plants growing in nature will lose their biological activity if they are cultivated *ex situ* (Keirungi & Fabricius, 2005) motivated this study. This perception has some conservation implications. Many resource users continue with unsustainable extraction of medicinal products from dwindling wild populations. This dependence puts many South African popular medicinal plant species at risk of overexploitation and even local extinction.

Different rates of annual rainfall did not have a consistent effect on the antibacterial activity present in acetone extracts of some tree species (Chapter 2). Many other factors could have had an influence on the results. The challenge is lack of sufficient knowledge on how environmental stress conditions affect biological activity of plant species. Therefore, the current study was aimed at evaluating the effect of water stress conditions on the antibacterial activity of the selected medicinal plant species with a view to promote their *ex situ* cultivation. The selection of plant species was based on the following criteria: must have known antimicrobial activity, be widely used, have a narrow genetic diversity, be fast growing and accessible, and easy to propagate. *Tulbaghia violacea* Harv. and *Hypoxis hemerocallidea* Fish., C.A.Mey. & Avé-Lall. were finally selected because they met most of the set criteria.

T. violacea, commonly known as the wild-garlic, is a member of the Alliaceae family. Local people use it as folk medicines for a variety of infections (Thamburam *et al*., 2006). It treats type-1 diabetes, fever and colds, paralysis, hypertension, asthma, rheumatism, sinus headaches, tuberculosis, oesophageal cancer, inflammation and gastrointestinal ailments including expulsion of intestinal worms (Lyantagaye & Rees,

2003). Many studies indicate that *T. violacea* has both antifungal and antibacterial properties as in the case of the culinary garlic plant (Harris, 2004; Nteso & Pretorius, 2006). The inhibitory activity of the root extracts of *T. violacea* against various pathogens such as *Mycobacterium tuberculosus*, *Mycobacterium smegmatis*, *Candida albicans and Escherichia coli* is evident in Burbidge (1978). Crude extracts of *T. violacea* significantly inhibits the growth of bacteria, *Clavibacter michiganensis*, *Ralstonia solanacearum* and *Xanthomonas campestris* (Nteso & Pretorius, 2006). The same extracts also inhibit growth of fungi such as *Botrytis cinera*, *Botryosphaeria dothidea*, *Sclerotium rolfsii*, *Pythium ultimum* and *Rhizoctonia solani*, *Mycosphaerella pinodes* (Lindsey & van Staden, 2004; Nteso & Pretorius, 2006). There is also an indication that aqueous and ethanol extracts of the *T. violacea* has anthelminthic activity (McGaw et al., 2000).

H. hemerocallidea, commonly known as African potato, is a member of a member of Hypoxidaceae. The plant is a perennial with long, strap-shaped leaves and yellow, star-shaped flowers. Its previous botanical name was *H. rooperi* (van Wyk et al., 1997). It has a wide distribution in the grassland areas of South Africa. The plant inhabits coastal areas of the Eastern Cape and KwaZulu-Natal as well as major parts of Gauteng, Mpumalanga and Limpopo provinces (van Wyk et al., 1997). It is an important plant species in traditional medicine in southern Africa and has some antibacterial activity (Katerere & Eloff, 2008). It is a part of herbal medicines used in the Botswana (Watt and Breyer-Wijk, 1962). People use extracts of corms (tubers-like) as dietary supplement and for a diversity of ailments (Nair & Kanfer, 2008). It can also treat prostate cancer (Gillmer & Symmonds, 1999). It is one of the 50 most important medicinal plants in the African Herbal Pharmacopoeia (AAMPS, 2010).

3.2 Materials and methods

3.2.1 Planting material preparation

Similar aged plantlet tissue culture clones of *Tulbaghia violaceae* and *Hypoxis hemerocallidea* were obtained from the Vegetable and Ornamental Plant Institute (VOPI) of the Agricultural Research Council (ARC) in Pretoria. The decision to use clones was to minimise possible genetic variability.

3.2.2 Growth of plantlets under water stress treatments

Plantlets of *H. hemerocallidea* and *T. violaceae* were raised in the growth trays filled with pine-bark medium for about three weeks. After plantlets reached a height of 10 to 15 cm with at least two leaves, they were transplanted into pots (27 cm diameter x 25 cm height, volume *c.* 14 L) filled with potting-mix. The potting mix comprised four parts loam soil, two parts sand, one part manure and two parts compost (Netshiluvhi, 1999). Water stress treatments comprised of irrigating with 1000 ml of distilled water per plant in intervals of 3, 14 and 21 days. Each treatment had ten plantlet replicates. There was no fertilizer application during the plant growth tests. In the greenhouse, room temperature (25°C) was maintained during the day and night. The experiment was carried out for a period of 240 days under natural light and darkness in the greenhouse.

3.2.3 Dry matter and voucher specimens

Recently mature fresh leaves (g) of 30 individual plants (10 per treatment) per plant species during vegetative and flowering stage were harvested at the end of the experiment. Leaves were air-dried in a ventilated storeroom at room temperature to constant mass. Voucher specimens of *T. violacea* (117131) and *H. hemerocallidea* (117132) plants were collected. Mrs Elsa van Wyk, the curator, verified and stored voucher specimens in the H.G.W.J. Schweickerdt Herbarium situated at the University of Pretoria in South Africa.

3.2.4 Extraction procedure

Dried leaves were separated from stems and then ground into a fine powder in a Jankel and Kunkel Model A10 mill. Acetone was used to extract the leaf samples because it is the best extractant for a wide series of compounds in leaves (Eloff, 1998a). It is also the least toxic to organisms in bioassays (Eloff et al., 2007). One gram of the finely ground leaves of each tree was extracted with 10 ml acetone in 50 ml centrifuge tubes. The tubes were shaken vigorously in a Labotec model 20.2 machine for 3-5 minutes at high speed to ensure uniform samples (Eloff, 1998a). The extracts were centrifuged at 3000 x g for 10 minutes and the supernatant was filtered through Whatman No. 1 filter paper into a pre-weighed glass vials. The same process was repeated twice in order to exhaustively extract the plant material and the extracts were combined. Five ml of the filtrate was removed and placed into a pre-weighed vial under a stream of air at room temperature in a fume cupboard. That was done to remove the acetone and to determine the

concentration of the combined extract. The required quantity of acetone in the combined extract was removed to yield a concentration of 10 ml/mg (Eloff, 2004). This process limits problems experienced in redissolving dried extracts.

3.2.5 Test bacterial strains

Gram-positive [*Staphylococcus aureus* (ATCC 29213) and *Enterococcus faecalis* (ATCC 29212)] and Gram-negative [*Pseudomonas aeruginosa* (ATCC 25922) and *Escherichia coli* (ATCC 27853)] bacterial strains were used to evaluate the antibacterial activity of both plant and tree species. The bacteria species have been identified as the most important nosocomial pathogens (Sacho & Schoub, 1993). The strains were obtained from the Central Microbiology Laboratory, Faculty of Veterinary Science at the University of Pretoria. The strains were grown at 37°C in Mueller-Hinton broth (Merck chemicals) (Eloff, 1999).

3.2.6 Phytochemical analysis

Chemical constituents of the extracts were analysed by thin layer chromatography (TLC) using aluminium-backed TLC plates (Merck, silica gel 60 F_{24}). The TLC plates were developed in the three mobile systems of differing polarity established in the Phytomedicine Laboratory of the University of Pretoria (Kotze & Eloff, 2002). The mobile systems used were; chloroform/ethyl acetate/formic acid (CEF: intermediate) (5:4:1), benzene:ethyl acetate:ammonia (BEA: non-polar) (9:1:0.1) and ethyl acetate:methanol:water (EMW: polar) (40:5.4:5). The TLC plates were visualized under UV light (250 and 360 nm, Camac Universal lamp TL-600) to detect UV active absorbing sports or plant constituents. The plates were then sprayed with vanillin spraying reagent (0.1% vanillin dissolved in 28 ml methanol and 1 ml sulphuric acid) and heated at 100°C to optimal colour development. The position of the visible compounds on the TLC plate was established by

calculating the retardation factor (R_f), which is the distance compound travelled divided by the distance the solvent had travelled from the origin.

3.2.7 Bioautography assay

The TLC plates (not sprayed with vanillin spray reagent) were left overnight in a stream of air to remove traces of the eluents and then sprayed with a concentrated suspension of actively growing cells of bacteria. This method relies on the direct growth inhibition or killing of pathogens on contact with the active band (Begue & Kline, 1972). The sprayed plates were incubated overnight at 38°C in a chamber at 100% relative humidity to allow the pathogens to grow on the plates. After overnight incubation, bioautograms were sprayed with an aqueous solution of 2 $mg.ml^{-1}$ ρ-iodonitrotetrazolium violet (INT) (Sigma) and incubated for 30 minutes for observation of clear zones on the plates indicating growth inhibition of pathogens by bioactive compounds in the extracts. A set of chromatograms sprayed with vanillin was used as reference for bioautograms displaying areas of inhibition. The R_f values of active zones were correlated with those bands on the reference chromatograms.

3.2.8 Minimum inhibitory concentration

The Minimum inhibitory concentration (MIC) values (mg/ml) were determined by two-fold serial dilution (e.g. 10, 5, 2.5, 1.25, 0.63, 0.32, 0.16, 0.08, etc) of extracts beyond where no inhibition of growth of test bacteria was observed (Eloff, 2001). This method was used to evaluate the antibacterial activity of extracts (Eloff, 1998a). Sufficient acetone was added to dilute the extract to a concentration of 10 mg/ml. Plant extracts (100 µl) in triplicate for each experiment were serially diluted two-fold in a 96-well microlitre plates. A similar

volume 100 µl of the actively growing test organism cultures was added to each well and the cultures were incubated overnight at 37°C under 100% relative humidity. As an indicator of bacterial growth, 40 µl of 0.2 mg/ml of ρ-iodonitrotetrazolium violet (INT) dissolved in water was added to each microplate well before being incubated for an hour or two (Eloff, 1998b). The MIC value was recorded as the lowest concentration that inhibited growth of bacteria. The colourless tetrazolium salt acts as an electron acceptor. It is reduced to a red-coloured formazan product by biologically active pathogens. Clear zones on the chromatogram indicated inhibition of the growth of bacteria after incubation with INT. The experiment was repeated thrice to confirm the results, and three replicates were included in each experiment.

3.2.9 Total activity

The total activity of a plant is calculated by taking into account the antibacterial activity as well as the quantity extracted from the plant material. This is calculated by dividing the quantity extracted in mg from 1 g of extracts of plants with the MIC in mg/ml. The result in ml/g indicates the degree to which the active compounds in one gram of plant material can be diluted and still inhibit growth of test pathogens (Eloff, 2000, 2004). This is a useful measure in comparing different plants as well as in isolating bioactive compounds. The higher the total activity, the more effective is the plant.

3.2.10 Statistical analysis

Data were statistically analysed using GenStat ® for Windows ® (2003). Results for antimicrobial activity of all plants were reported as means ± standard error (SE). Results with 5% level of confidence were regarded as statistically significant. Significant differences for comparisons were determined by a one-way analysis of variance (ANOVA) procedure.

3.3 Results and discussion

3.3.1 Dry mass

The dry mass of *H. hemerocallidea* and *T. violacea* was significantly ($p \leq 0.05$) reduced under an irrigation interval of 21 days by close to 50% (Table 3.1) suggesting that both sets of plants were under water stress. These results are consistent with those of other studies such as Dunford & Vazquez (2005), Gomez-del-Campo (2007) and Aziz et al. (2008). Reduction in dry mass is sometimes due to a decrease in the quantity of minerals (calcium and potassium) and ascorbic acid necessary for plant growth and development (Osuagwu *et al*., 2010).

Table 3.1. Effects of water treatments on aboveground dry matter of *Tulbaghia violaceae* and *Hypoxis hemerocallidea*. Values (means ± standard error; n=10) showing the same superscripts in the same column are not significantly different at the 5% confidence level.

Watering intervals (days) per fed of 1000 ml	**Dry matter (g)**	
	Tulbaghia violacea	*Hypoxis hemerocallidea*
3	28.7± 3.3^{a}	41.3 ± 6.7^{a}
14	19.5 ± 4.2^{ab}	20.6 ± 5.3^{b}
21	13.7± 2.9^{b}	20.6 ± 7.1^{b}

The stress strongly affects photosynthesis by increasing leaf senescence (Manske, 1998). The reduced photosynthetic activity leads to reduced plant herbage. That takes place when carbon allocation is diverted to non-photosynthetic organs of plants (Chaves et al., 2002). Prolonged water stress can be lethal to plants particularly when cell turgidity and biochemical activity cannot be maintained (Brown, 1995), which probably was not yet the case in this study. Olive trees protect their internal metabolism from water stress by closing the stomata (Fernandez & Moreno, 1999). Closing stomata inevitably decreases photosynthetic rate and therefore growth. The results give and indication that water stress on plants may significantly reduce their production capacity (Dunford & Vazquez, 2005).

3.3.2 Antibacterial activity

The dry mass of *H. hemerocallidea* and *T. violacea* was significantly reduced by different water regimes (Table 3.1). However, there were no statistically significant differences in the MIC values of the extracts of the same plants against any of the test bacteria (Table 3.2). There were also few differences in the chemical composition and antimicrobial activity of compounds separated by TLC (Appendix A). Leaf extracts of *T. violacea* and *H. hemerocallidea* had good antibacterial activity as attested by low MIC values (< 1 mg/ml) across watering intervals. The activity of *T. violacea* (average MIC = 0.26 mg/ml) and *H. hemerocallidea* (MIC = 0.32 mg/ml) in this study is comparable with that recorded in Ncube et al. (2011) and Katerere & Eloff (2008), respectively. Leaf extracts of *H. hemerocallidea* also had good antibacterial activity against *E. coli* and *S. aureus* in Steenkamp et al. (2006).

Table 3.2. Minimum inhibitory concentration of medicinal plant species subjected to water treatments. Values (means ± standard error; n=8) showing the same superscripts in the same column are not significantly different at the 5% confidence level.

Plant species	**Watering intervals (days)**	**Minimum inhibitory concentration (mg/ml)**			
		Staphyloccocus aureus	*Escherichia coli*	*Pseudomonas aeruginosa*	*Enteroccocus faecalis*
Hypoxis hemerocallidea	3	0.55 ± 0.14[a]	0.18 ± 0.06[a]	0.29 ± 0.06[a]	0.41 ± 0.24[a]
	14	0.51± 0.20[a]	0.25 ± 0.08[a]	0.16 ± 0.00[b]	0.31 ± 0.00[a]
	21	0.39 ± 0.10[a]	0.16 ± 0.00[a]	0.31± 0.00[a]	0.31 ± 0.00[a]
Tulbaghia violacea	3	0.37 ± 0.16[a]	0.22 ± 0.08[a]	0.18 ± 0.24[a]	0.35 ± 0.18[a]
	14	0.31 ± 0.00[a]	0.16 ± 0.00[a]	0.16 ± 0.07[a]	0.31± 0.00[a]
	21	0.43 ± 0.16[a]	0.22 ± 0.08[a]	0.15± 0.03[a]	0.23 ± 0.03[a]

There was also no clear trend between the majority of activity of extracts and water treatments. The same was the case when the total activity was determined (Table 3.3). Total activity values also incorporated extractable plant material that did not show any significant differences between water treatments. Leaf extracts of *T. violacea* and *H. hemerocallidea* also had uniform antimicrobial activity across four seasons in Ncube et al. (2011). Differences in thymol and carvacrol compounds of young and old Mexican oregon plants (*Lippia berlandieri* Schau.) are also not statistically significant between water stress conditions (Dunford & Vazquez, 2005). Therefore, the results clearly suggest that watering intervals had very little or no effect on the antibacterial activity of plant extracts.

Different rates of annual rainfall too did not seem to have a consistent effect on the antibacterial activity of field tree species in an unpublished work. Measuring changes in specific active compounds of different

plants subjected to water stress could have given much detail in this study, as was the case in Kirakosyan et al. (2004).

Table 3.3. Total activity of plant extracts against test bacteria under different water treatments and quantity extracted from 1.00 g of dries material. Values (means ± standard error; n=8) with the same superscripts in the same column are not significantly different at the 5% confidence level.

Plant species	**Watering intervals (days)**	**Quantity extracted (mg)**	**Total activity against bacteria (ml/mg)**			
			Staphyloccocus aureus	*Escherichia coli*	*Pseudomonas aeruginosa*	*Enteroccocus faecalis*
Hypoxis hemerocallidea	3	15.5 ± 1.0^{a}	18.1 ± 9.0^{a}	51.6 ± 5.7^{a}	25.8 ± 2.9^{a}	30.9 ± 19.9^{a}
	14	14.3 ± 1.3^{a}	14.4 ± 6.9^{a}	28.8 ± 13.8^{b}	40.8 ± 7.8^{b}	20.4 ± 3.9^{a}
	21	16.5 ± 2.1^{a}	22.9 ± 7.4^{a}	52.0 ± 13.5^{a}	32.6± 6.2^{ab}	25.8 ± 2.9^{a}
Tulbaghia violacea	3	9.8 ± 2.7^{a}	37.9 ± 13.7^{a}	64.5 ± 25.2^{a}	73.4 ± .0.0)^{a}	42.6 ± 20.3^{a}
	14	10.8 ± 1.3^{a}	41.0 ± 4.0^{a}	82.0 ± 8.0^{a}	99.4 ± 0.0^{a}	41.0 ± 4.0^{a}
	21	9.8 ± 2.2^{a}	29.9 ± 10.3^{a}	61.3 ± 13.7^{a}	81.8 ± 0.0^{a}	56.4 ± 23.7^{a}

There were few differences in chemical constituents present in the bioautograms supporting the overall antibacterial activity results (data not shown). There were at least five relatively non-polar antibacterial compounds present in the leaf extracts of *T. violaceae* based on the bioautograms separated by the non-polar BEA solvent system. In the case of the *H. hemerocallidea,* acetone leaf extracts had at least four intermediate polarity antibacterial compounds present.

The results in this study are also in disagreement with several other studies where authors investigated the effect on certain compounds. Water stress increases carvacrol content of *Satureja hortensis* (Baher et al., 2002) and *Oreganum vulgare* (Vazquez & Dunford, 2005). It has also enhances the levels of harpagoside of Devil's claw (Schneider et al., 2006) and polyphenolics of *Crataegus laevigata* and *C. monogyna* (Kirakosyan et al., 2004). The production of some activity of *Pisum sativum* plants was also induced by water stress in Plumbe & Willmer (1985).

Contrasting results between this and other studies may be due to many other factors. Water availability, exposure to soil pathogens and variations of soil pH and nutrients affect the accumulation of secondary metabolites (Economakis et al., 2002). The absence of fungal biomass cannot trigger the production of secondary metabolites for defence against microbes (Rajakaruna et al., 2002). The environmental factors such as temperature, rainfall and day length affect the efficacy of the medicinal properties (Dubey et al., 2004). Type and amount of fertilisers applied to plants also have an effect on biological activity as demonstrated in van den Heever et al. (2007).

In conclusion, water stress significantly reduced dry mass of all study plant species but not their antibacterial activity. The results demonstrate that different water stress does not influence the antibacterial activity of these species. Contrasting results between this and other studies cited in the text call for further in depth evaluation of the antibacterial and antifungal activity of more plant extracts.

Some notes:

The results in chapter 3 demonstrated that different watering intervals applied had very little effect on the antibacterial activity of the study plant species. The water stress intervals of 3, 14 and 21 days may have been too far apart. Therefore, Chapter 4 will address these challenges by using confirmed clone plants of the same age, additional plant species, additional microorganisms, more methods to determine stress levels and wider irrigation water.

Chapter 4 Effect of induced water stress on leaf dry matter, stomatal conductance and antimicrobial activity of three medicinal plants

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Abstract

Some traditional practitioners and scientists still believe that domestic cultivation would reduce the quality of medicinal material. The cause for this is probably due to poor understanding of how environmental factors affect antimicrobial activity of plant extracts. This has serious conservation implications. Therefore, the study evaluated the effect of four different water supply levels (50, 100, 200 and 500 ml) on dry mass, stomatal conductance and antimicrobial activity of *Leonotis dysophylla* Benth., *Bulbine frutescens* (L.) Willd. and *Tulbaghia violacea* Harv. The stomatal conductance and dry mass was determined. Leaves were harvested, air-dried, measured and finely ground. The antimicrobial activity of acetone extracts was determined through minimum inhibitory concentration (MIC), thin layer chromatography (TLC) and bioautography. The study used four bacterial and three fungal strains. Plants receiving 50 ml of distilled water every two days had significant reduction in dry mass production and stomatal conductance compared with plants given up to 500 ml of water. In general, all plant extracts had good antimicrobial activity with all MIC values less than 2.5 mg/ml. However, there were hardly any significant differences in MIC and total activity values of plants between water treatments. In those few cases where there were differences, there was no correlation between antimicrobial activity and water treatments. At least with these three species it appears that water stress does not necessarily lead to a considerable change in antimicrobial activity. Plants grown under optimal conditions are as active as plants grown under water stress.

Keywords: Antibacterial activity; antifungal activity; *Bulbine frutescens*; *Leonotis dysophylla*; minimum inhibitory concentration; total activity; *Tulbaghia violacea*; water stress

4.1 Introduction

The increasing demand for herbal medicines encourages collectors and traders to decimate natural populations of important medicinal plants. Global interest particularly from the Western pharmaceutical industry exacerbates exploitation (Van de Kope et al., 2006). Some South African medicinal plants are already on the brink of extinction in the wild (Dold & Cocks, 2002). Domestic cultivation could offer a long-term solution to this challenge. However, the Nqabara community in the Eastern Cape Wild Coast believes that plants growing under cultivation lose “power” (Keirungi & Fabricius, 2005). Some researchers also believe that domestic cultivation would reduce the quality of medicinal material (Guo et al., 2009). This claim has major implications for conservation. Although much work has been done on the influence of environmental factors on the production of certain metabolites, it seems that there is limited understanding of how environmental factors affect biological activity of plant extracts. Therefore, the study evaluated the effects of induced water stress conditions on dry mass production, stomatal conductance and antimicrobial activity of *Leonotis dysophylla* Benth., *Bulbine frutescens* (L.) Willd. and *Tulbaghia violacea* Harv.

Leonotis dysophylla, commonly known as woody lion's-ear or klip-dagga, is a member of the Lamiaceae or Mint family. There is very little coverage on the use of this plant species as a herbal medicine. Its leaf infusions treat headaches (Hutchings & van Staden, 1994) and common colds (Watt and Breyer-Brandwijk, 1962). It is also used as a tonic (Watt and Breyer-Brandwijk, 1962). Leaf extracts of *L. dysophylla* have good antibacterial activity (MIC < 0.10 mg/ml) against *Staphylococcus aureus, Pseudomonas aeruginosa*, *Escherichia coli* and *Enterococcus faecalis* (Eloff, 2010).

T. violacea, commonly known as wild-garlic, is a member of the Alliaceae family. It is used tp treat type-1 diabetes, fever and colds, paralysis, hypertensive, asthma, rheumatism, sinus headaches, tuberculosis, oesophageal cancer, inflammation and gastrointestinal ailments (Lyantagaye & Rees, 2003). The plant has both antifungal and antibacterial activity, as is the case of culinary garlic plant (Harris, 2004). Tuber extracts inhibit bacterial growth of *Mycobacterium tuberculosus*, *M. smegmatis* and *Escherichia coli (*Burbidge, 1978). Plant extracts also inhibit fungal growth of *Candida albicans* (Motsei et al., 2003; Thamburan et al., 2006), *Botrytis cinera*, *Pythium ultimum* and *Rhizoctonia solani* (Lindsey & Van Staden, 2004). Furthermore, extracts inhibit fungal growth of *Sclerotium rolfsii*, *Mycosphaerella pinodes* and *Botryosphaeria dothidea* (Nteso & Pretorius, 2006). Aqueous and ethanol extracts of tubers also has anthelminthic activity (McGaw et al., 2000).

B. frutescens, commonly known as the snake flower or grass-aloe, is a member of the Asphodelaceae (previously Liliaceae) family. Its leaf gel treats insect bites, wounds, rashes, acne, blisters, burns, mouth ulcers, cracked lips, cold sores, acne and ringworm (Dyson, 1998). Roots treat diarrhoea, colic, urinary tract and venereal diseases (Van Wyk et al., 1995). Anthraquinones, knipholone and isoknipholone isolated from roots are some of the chemical constituents of *B. frutescens* (Van Staden & Drewes, 1994). The isolated compounds have antiplasmodial and antitropanosomal activity. Furthermore, phenylanthraquinones and isofuranonaphthoquinones extracted from the same species have antiparasitic and antioxidant activity (Abegaz et al., 2002).

4.2 Materials and methods

4.2.1 Plant material

Plant clones of the same age were prepared for the greenhouse experiment under room temperature (25°C), light, irrigation and growth medium before trial commencement. To generate vegetative clones, plantlets were prepared through division of mother clumps of *B. frutescens* and *T. violacea*. Plantlets were established in the growth trays filled with vermiculite comprising pine-bark medium. The two mother plant species were obtained from the Vegetable and Ornamental Plant Institute (VOPI) of the Agricultural Research Council (ARC). Seeds of *L. dysophylla* were collected from a wild population in the Akasia municipality west of Pretoria-North and germinated in the growth trays filled with vermiculite at a depth of 1.25 cm. Almost 100% seed germination was achieved after two weeks. Seeds from one of the mature *L. dysophylla* plants in the greenhouse were germinated again under the same conditions to minimise genetic variability. Approximately 100% germination was again achieved within two weeks. Seedlings were grown for a period of up to six weeks in the greenhouse at the Experimental Farm of the University of Pretoria. Seedlings were irrigated with 500 ml of distilled water every two days.

4.2.2 Growth of vegetative and seedling clones

After reaching a height of 10 to 15 cm with at least two leaves, seedlings were transplanted into large pots (27 cm diameter x 25 cm height, c. 14 L capacity) filled with potting-mix. Potting-mix comprised four parts loam soil, two parts sand, one part manure and two parts compost (Netshiluvhi, 1999). Seedlings were then subjected to different water stress conditions comprising irrigation with 500, 200, 100 and 50 ml of distilled

water every two days under room temperature in the greenhouse. Irrigation was in the morning for the entire period of the experiment. Each water treatment consisted of four pots containing one plant per pot. The entire experiment ran for a period of 26 weeks.

4.2.3 Stomatal conductance

The study used stomatal conductance (mmol/m^2/s^1) parameter to determine the extent of transpiration of plants. The stomatal conductance determined the stress levels of plants under different irrigation treatments. The instrument, SC-I Leaf Porometer instrument (ICT Plant Science Instrumentation), measured the conductance at the abaxial (basal) position of randomly selected leaves of each replicate plant. Abaxial position is an area with a relatively large density of stomata. After 26 weeks of growth experiment, readings of stomatal conductance of the mature plants were recorded. That was done to avoid effects of soil heterogeneity that may affect water availability.

4.2.4 Preparation of leaf samples

Harvesting of recently mature fresh leaves of twelve plants (4 per species) during vegetative and flowering stage per each water treatment took place at the end of the experiment. Leaves were air-dried in a ventilated storeroom at room temperature before removing stems. Thereafter, dried leaves were finely ground in a Jankel and Kunkel Model A10 mill. Voucher specimens of *T. violacea* (117131), *L. dysophylla* (117130) and *B. frutescens* (117129) plants were collected. Mrs Elsa van Wyk, the curator, verified and stored voucher specimens in the H.G.W.J. Schweickerdt Herbarium situated at the University of Pretoria in South Africa.

4.2.6 Extraction procedure

One gram of the finely ground leaves of each tree was extracted with 10 ml acetone in 50 ml centrifuge tubes. The tubes were shaken vigorously in a Labotec model 20.2 machine for 3-5 minutes at high speed to ensure uniform samples (Eloff, 1998a). The extracts were centrifuged at 3000 xg for 10 minutes and the supernatant was filtered through Whatman No. 1 filter paper into a pre-weighed glass vials. The same process was repeated twice in order to exhaustively extract the plant material and the extracts were combined. Five ml of the filtrate was removed and placed into a pre-weighed vial under a stream of air at room temperature in a fume cupboard. That was done to remove the acetone and to determine the concentration of the combined extract. The required quantity of acetone in the combined extract was removed to yield a concentration of 10 ml/mg (Eloff, 2004).

4.2.7 Test bacterial strains

Gram-positive [*Staphylococcus aureus* (ATCC 29213) and *Enterococcus faecalis* (ATCC 29212)] and Gram-negative [*Pseudomonas aeruginosa* (ATCC 25922) and *Escherichia coli* (ATCC 27853)] bacterial strains were used to evaluate the antibacterial activity of both plant and tree species. These species have been identified as the most important nosocomial pathogens (Sacho & Schoub, 1993). The strains were obtained from the Central Microbiology Laboratory, Faculty of Veterinary Science at the University of Pretoria. The strains were grown at 37°C in Mueller-Hinton broth (Merck chemicals) (Eloff, 1999).

4.2.8 Test fungal strains

The fungal strains used in the study were:

(a) Yeasts (*Candida albicans* and *Cryptococcus neoformans*), important pathogens affecting the health of immunocompromised patients.

(b) Mould (*Aspergillus fumigatus*), one of the most common and important disease-causing fungus of animals.

The strains were obtained from the Central Microbiology laboratory (Faculty of Veterinary Science, University of Pretoria). They were maintained in Sabouraud Dextrose (SD) agar at 4°C and inoculated in SD broth at 37°C. Strains were incubated prior to conducting bioautography and microdilution assays.

4.2.9 Phytochemical analysis

Chemical constituents of the extracts were analysed by thin layer chromatography (TLC) using aluminium-backed TLC plates (Merck, silica gel 60 F_{24}). The TLC plates were developed in the three mobile systems of differing polarity established in the Phytomedicine Laboratory of the University of Pretoria (Kotze & Eloff, 2002). The mobile systems used were; chloroform/ethyl acetate/formic acid (CEF: intermediate) (5:4:1), benzene:ethyl acetate:ammonia (BEA: non-polar) (9:1:0.1) and ethyl acetate:methanol:water (EMW: polar) (40:5.4:5). The chromatograms were examined under UV light (250 and 360 nm, Camac Universal lamp TL-600) to detect UV active absorbing spots. The plates were then sprayed with vanillin spraying reagent (0.1% vanillin dissolved in 28 ml methanol and 1 ml sulphuric acid) and heated at 100°C to optimal colour development. The position of the visible compounds on the TLC plate was established by calculating the

retardation factor (R_f), which is the distance compound travelled divided by the distance the solvent had travelled from the origin.

4.2.10 Bioautography assay

The chromatograms (not sprayed with vanillin spray reagent) were left overnight to dry in a draft of cold air to remove the eluents and then sprayed with a concentrated suspension of actively growing cells of bacteria or fungi (Masoko & Eloff, 2006). This method relies on the direct growth inhibition or killing of pathogens on contact with the active band (Begue & Kline, 1972). The sprayed plates were incubated overnight at 38°C in a chamber at 100% relative humidity to allow the pathogens to grow on the plates. After overnight incubation, bioautograms were sprayed with an aqueous solution of 2 mg/ml ρ-iodonitrotetrazolium violet (INT) (Sigma). Thereafter, bioautograms were incubated for 30 minutes to observe clear zones indicating growth inhibition of pathogens by bioactive compounds in the extracts. A set of chromatograms sprayed with vanillin-sulphuric acid was used as reference for bioautograms displaying areas of inhibition. The R_f values of active zones were correlated with those bands on the reference chromatograms.

4.2.11 Minimum inhibitory concentration

The minimum inhibitory concentration (MIC) values (mg/ml) were determined after two-fold serial dilution (e.g. 10, 5, 2.5,1.25, 0.63, 0.32, 0.16, 0.08) of extracts with a concentration of 10 mg/ml beyond where no inhibition of growth of test bacteria was observed. This method was used to evaluate the antibacterial activity of extracts (Eloff, 1998a). Plant extracts (100 µl) in triplicate for each experiment were serially

diluted two-fold with water in 96-well microlitre plates. A similar volume 100 µl of the actively growing test organism cultures was added to each well and the cultures were incubated overnight at 37°C under 100% relative humidity. As an indicator of bacterial growth, 40 µl of 0.2 mg/ml of ρ-iodonitrotetrazolium violet (INT) dissolved in water was added to each microplate well before being incubated for an hour or two (Eloff, 1998b). The MIC value was recorded as the lowest concentration that inhibited growth of bacteria. The colourless tetrazolium salt acts as an electron acceptor. It is reduced to a red-coloured formazan product by biologically active pathogens (Eloff, 1998b). Clear zones indicated inhibition of the growth of bacteria after incubation with INT. The experiment was repeated twice to confirm the results, and three replicates were included in each experiment.

4.2.12 Total activity

Total activity was also used as a parameter to measure the effects of temperature stress on plant activity. Total activity value (ml/g) measures the total antibacterial activity present in different plants by dividing the quantity extracted (mg) from 1 gram of plant material with the MIC value in mg/ml (Eloff, 2000). It indicates the degree to which the active compounds in one gram of plant material can be diluted and still inhibit growth of pathogens. Total activity value is calculated by dividing the quantity in mg extracted from 1 gram of plant material (mg/g) with the MIC in (mg/ml). The higher the total activity in ml/g of a plant extract, the more effective the plant is.

4.2.13 Statistical analysis

Data were statistically analysed using GenStat ® for Windows ® (2003). Results for antimicrobial activity of all plants were reported as means ± standard error (SE). Results with 5% level of confidence were regarded as statistically significant. Significant differences between treatments were determined by a one-way analysis of variance (ANOVA) procedure.

4.3 Results

4.3.1 Dry mass production

The dry mass production of all plants decreased significantly (P≤0.05) with a reduced water supply level (Table 4.1). Especially in the case of *B. frutescens*, there was a linear dose response (R^2= 0.98) between dry mass yield and water supply (Fig 4.1).

Table 4.1. Influence of water treatments on the aboveground dry matter of plants grown for 26 weeks. Values (means ± standard error; n=4) showing the same superscripts in the same row are not significantly different at the 5% confidence level.

Plant species	**Leaf dry mass (g) under different water supply levels**			
	50 ml	100 ml	200 ml	500 ml
L. dysophylla	77 ± 18^{b}	95 ± 38^{b}	97 ± 27^{b}	142 ± 43^{a}
T. violacea	86 ± 41^{b}	84 ± 16^{b}	164 ± 40^{a}	185 ± 38^{a}
B. frutescens	333 ± 121^{d}	586 ± 74^{c}	930 ± 51^{b}	1618 ± 272^{a}

The results indicate that the selected water supply levels did indeed lead to water stress in all cases (Fig. 4.1). It is somewhat surprising that *B. frutescence*, a relatively succulent plant was the most sensitive to decreasing watering from 500 ml to 200 ml every two days. With the other two species, water was not a limiting factor at levels higher than 200 ml every second day (Table 4.1; Fig. 4.1). These species may be

more amenable to cultivation under arid conditions, whereas the productivity of *B. frutescence* could be high if sufficient water supply was available.

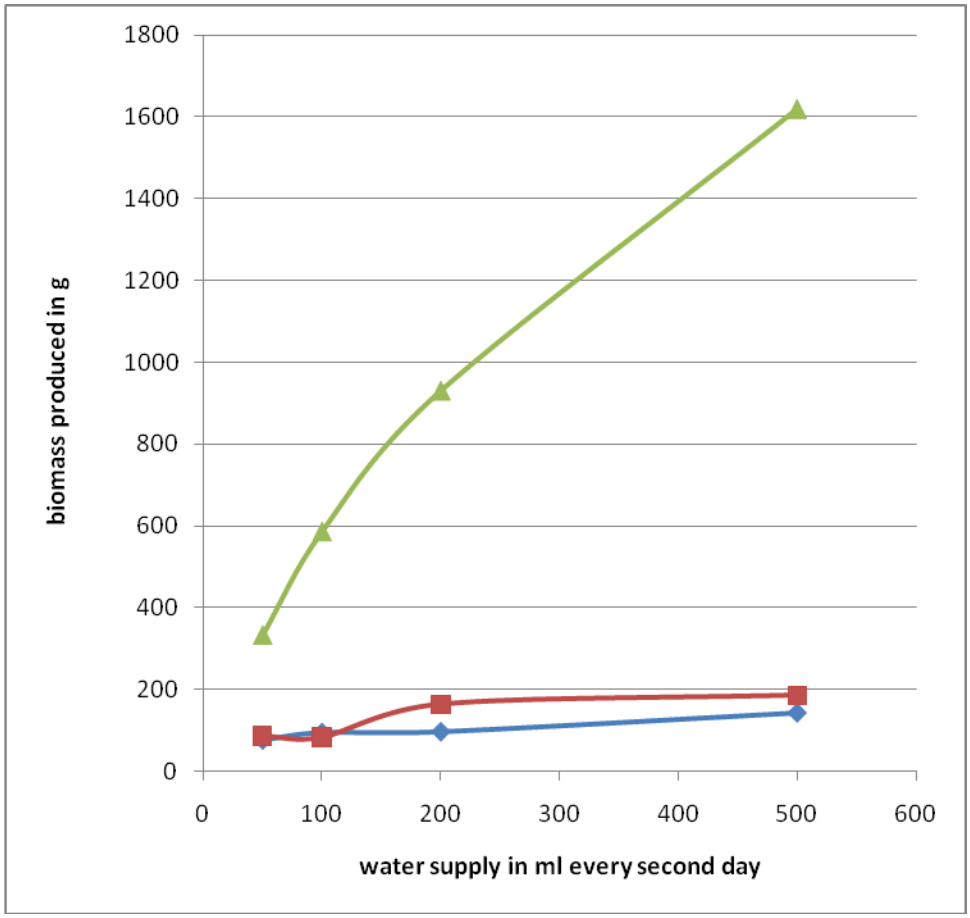

Figure 4.1. Influence of different water supply levels on the aboveground dry matter of plants grown for 26 weeks. Green, red and blue lines represent *B. frutescens*, *T. violacea and L. dysophylla*, *respectively*.

4.3.2 Stomatal conductance

Mean stomatal conductance (mmol/m^2/s^1) of all plant species is presented in Table 4.2. As with dry mass, stomatal conductance of all plants decreased significantly with the reduction of water supply. *L. dysophylla* had a higher stomatal conductance than two other plant species possibly because of having a relatively

larger leaf surface area. The reduction in dry mass and stomatal conductance proves that plants were under stress because even the stomata of *B. frutescens* were relatively closed under the lowest water supply level of 50 ml.

Table 4.2. Influence of water treatments on stomatal conductance of medicinal plants measured during flowering stage. Values (means ± standard error; n=4) showing the same superscripts in the same row are not significantly different at the 5% confidence level.

Plant species	**Mean stomatal conductance (mmol/m²/s) under different water supply levels**			
	50 ml	100 ml	200 ml	500 ml
L. dysophylla	19 ± 8^{a}	28 ± 4^{a}	53 ± 13^{b}	192 ± 64^{c}
T. violacea	4 ± 1^{a}	9 ± 1^{b}	13 ± 2^{b}	21 ± 9^{c}
B. frutescens	0	2 ± 1^{a}	5 ± 1^{b}	5 ± 1^{b}

4.3.3 Mass extracted from samples for antimicrobial activity

There were no statistically differences in the mass extracted from 1 g samples of plants subjected to different water supply levels (Table 4.3). The results suggest that stress conditions had no noteworthy effect on the acetone solubility of plant metabolites.

Table 4.3. Quantity extracted from 1.00 g of dried and ground leaf samples of plants. Values (means ± standard error; n=4) showing the same superscripts in the same column are not significantly different at the 5% confidence level.

Water supply levels (ml)	Acetone leaf extracts (mg) under different water supply levels		
	T. violaceae	*L. dysophylla*	*B. frutescens*
50	113 ± 35[a]	137 ± 12[a]	120 ± 27[a]
100	133 ± 15[a]	110 ± 27[a]	133 ± 15[a]
200	117 ± 6[a]	127 ± 142[a]	120 ± 10[a]
500	97 ± 15[a]	120 ± 20[a]	97 ± 15[a]

4.3.4 Minimum inhibitory concentration and total activity

4.3.4.1 Antifungal activity

All plant extracts yielded MIC values that were between 0.3 and 2.1 mg/ml) (Table 4.4) indicating the presence of some antifungal activity. However, there was no clear correlation between the MIC values of species and water supply levels. The different water stress had no statistically significant effect (P≤0.05) on the MIC values of *L. dysophylla* and *B. frutescens* extracts. There were also no significant differences of the water stress on the MIC values of the *T. violacea* extract against *Candida albicans*. Different water stress did have an effect on the MIC values of *T. violacea* against *Cryptococcus neoformans* and *Aspergillus fumigatus* were statistically significant (P≤0.05). There was however, a slight trend that antifungal activity

decreased under increased water stress. The anomalous value at 100 ml for the *T. violacea* experiment cannot be explained easily.

Table 4.4. Minimum inhibitory concentration (mg/ml) of plants against test fungi under water treatments. Values (means ± standard error; n=4) showing the same superscripts in the same column are not significantly different at the 5% confidence level.

		Minimum inhibitory concentration (mg/ml) under different water supply levels		
Plant species	**Water supply levels (ml)**	*Candida albicans*	*Cryptococcus neoformans*	*Aspergillus fumigatus*
L. dysophylla	50	0.5 ± 0.1[a]	0.8 ± 0.2[a]	0.8 ± 0.2[a]
	100	0.4 ± 0.1[a]	0.8 ± 0.2[a]	0.6 ± 0.2[a]
	200	0.5 ± 0.1[a]	0.8 ± 0.2[a]	0.7 ± 0.2[a]
	500	0.5 ± 0.1[a]	0.8 ± 0.2[a]	1.0 ± 0.2[a]
B. frutescens	50	1.5 ± 0.3[a]	0.4 ± 0.1[a]	0.6 ± 0.2[a]
	100	0.7 ± 0.3[a]	0.3 ± 0.1[a]	0.8 ± 0.2[a]
	200	0.8 ± 0.3[a]	0.4 ± 0.1[a]	0.7 ± 0.2[a]
	500	0.8 ± 0.3[a]	0.4 ± 0.1[a]	0.5 ± 0.2[a]
T. violacea	50	0.9 ± 0.6[a]	2.3 ± 0.7[a]	2.1 ± 0.7[a]
	100	1.0 ± 0.6[a]	1.3 ± 0.7[b]	0.8 ± 0.7[b]
	200	1.4 ± 0.5[a]	1.0 ± 0.6[b]	1.7 ± 0.6[ac]
	500	0.8 ± 0.5[a]	1.3 ± 0.6[b]	1.4 ± 0.6[ac]

Because the different water regimes did not affect the quantity extracted, water stress also did not have a significant effect on the total antifungal activity. In the few cases where there were significant differences, there was no clear pattern between total activity values and water supply levels.

Table 4.5. Total activity values of plants against test fungi under water treatments. Values (means ± standard error; n=4) showing the same superscripts in the same column are not significantly different at the 5% confidence level.

Plant species	Water stress levels (ml)	Antifungal total activity (ml/g) under different water supply levels		
		Candida albicans	*Cryptococcus neoformans*	*Aspergillus fumigatus*
L. dysophylla	50	450 ±149[a]	183 ± 34[a]	183 ± 48[ab]
	100	379 ± 149[a]	148 ± 34[a]	175 ± 48[a]
	200	436 ± 149[a]	257 ± 34[a]	339 ± 48[b]
	500	245 (149)[a]	154 ± 34[a]	127 ± 48[a]
B. frutescens	50	121 ± 53[a]	327 ± 68[a]	191 ± 63[a]
	100	238 ± 53[a]	430 ± 68[a]	172 ± 63[a]
	200	159 ± 53[a]	321 ± 68[a]	230 ± 63[a]
	500	125 ± 53[a]	268 ± 68[a]	214 ± 63[a]
T. violacea	50	112 ± 19[a]	148 ± 25[a]	74 ± 23[a]
	100	138 ± 19[a]	107 ± 20[a]	212 ± 84[b]
	200	183 ± 23[a]	139 ± 25[a]	70 ± 23[a]
	500	125 ± 19[a]	77 ± 20[a]	153 ± 34[ab]

4.3.4.2 Antibacterial activity

All plant extracts had a reasonable antibacterial activity (0.31 - 1.88 mg/ml) against all test bacteria across water supply levels (Table 4.6). The different watering regimes did not lead to statically significant differences ($P \leq 0.05$) in MIC values of any plant extract against the test bacteria. There were few differences in chemical composition and antimicrobial activity of compounds separated by TLC (Appendix B).

Differences in the majority of the total activity values of *T. violacea* against all test bacteria under different water stress conditions were also not statistically significant (Table 4.7). The only significant differences observed in the total activity values were for *B. frutescens* against *E. coli* and *E. faecalis* as well as *L. dysophylla* against *P. aeruginosa*. However, there was no clear trend between all total activity values and water supply levels.

Table 4.6. Minimum inhibitory concentration (mg/ml) of plants against test bacteria under water treatments. Values (means ± standard error; n=4) showing the same superscripts in the same column are not significantly different at the 5% confidence level.

Plant species	**Water supply levels (ml)**	**Antibacterial minimum inhibitory concentration (mg/ml) under different water supply levels**			
		Staphyloccocus aureus	*Escherichia coli*	*Pseudomonas aeruginosa*	*Enteroccocus faecalis*
L. dysophylla	50	0.84 ± 0.4[a]	0.52 ± 0.4[a]	1.04 ± 0.2[a]	0.63 ± 0.3[a]
	100	1.25 ± 0.4[a]	0.63 ± 0.4[a]	1.04 ± 0.2[a]	0.63 ± 0.3[a]
	200	1.46 ± 0.4[a]	1.04 ± 0.4[a]	0.84 ± 0.2[a]	1.04 ± 0.3[a]
	500	1.88 ± 0.4[a]	0.73 ± 0.4[a]	1.04 ± 0.2[a]	1.46 ± 0.3[a]
B. frutescens	50	0.73± 0.2[a]	0.52 ± 0.1[a]	1.15 ± 0.7[a]	0.73 ± 0.2[a]
	100	0.42 ± 0.2[a]	0.31 ± 0.1[a]	1.25 ± 0.7[a]	0.31 ± 0.2[a]
	200	0.52 ± 0.2[a]	0.63 ± 0.1[a]	1.77 ± 0.7[a]	0.31 ± 0.2[a]
	500	0.52 ± 0.2[a]	0.63 ± 0.1[a]	1.77 ± 0.7[a]	0.42 ± 0.2[a]
T. violacea	50	1.88 ± 0.4[a]	2.50 ± 0.0[a]	1.88 ± 0.5[a]	0.94 ± 0.2[a]
	100	1.04 ± 0.3[a]	2.50 ± 0.0[a]	1.67 ± 0.4[a]	0.84 ± 0.2[a]
	200	1.25 ± 0.4[a]	2.50 ± 0.0[a]	1.25 ± 0.5[a]	0.63 ± 0.2[a]
	500	1.67 ± 0.3[a]	2.50 ± 0.0[a]	1.67 ± 0.4[a]	0.84 ± 0.2[a]

Table 4.7. Total activity values of plants against test bacteria under water treatments. Values (means ± standard error; n=4) showing the same superscripts in the same column are not significantly different at the 5% confidence level.

		Antibacterial total activity (ml/g) under different water supply levels			
Plant species	**Water supply levels (ml)**	*Staphyloccocus aureus*	*Escherichia coli*	*Pseudomonas aeruginosa*	*Enteroccocus faecalis*
L. dysophylla	50	177 ± 37^{a}	288 ± 76^{a}	143 ± 32^{a}	217± 35^{a}
	100	88 ± 37^{a}	175 ± 76^{a}	112 ± 32^{a}	175 ± 35^{a}
	200	166 ± 37^{a}	364 ± 76^{a}	257 ± 32^{b}	218 ± 35^{a}
	500	103 ± 37^{a}	219 ± 76^{a}	133 ± 32^{a}	106 ± 35^{a}
B. frutescens	50	244 ± 54^{a}	273 ± 35^{a}	211 ± 62^{a}	246 ± 46^{a}
	100	348 ± 54^{a}	430 ± 35^{b}	164 ± 62^{a}	430 ± 46^{b}
	200	261 ± 54^{a}	190 ± 35^{ac}	170 ± 62^{a}	387 ± 46^{cb}
	500	214 ± 54^{a}	154 ± 35^{c}	142 ±62^{a}	268 ± 46^{ac}
T. violacea	50	82 ± 37^{a}	52 ± 5^{a}	82 ± 20^{a}	148 ± 33^{a}
	100	146 ± 30^{a}	53 ± 4^{a}	87 ± 16^{a}	177 ± 27^{a}
	200	92 ± 37^{a}	46 ± 5^{a}	92 ± 20^{a}	183 ± 33^{a}
	500	63 ± 30^{a}	39 ± 4^{a}	63 ± 16^{a}	125 ± 27^{a}

4.4. Discussion

4.4.1. Dry mass

The lowest water supply level of 50 ml significantly reduced dry mass production of all study plants. With specific reference to *B. frutescens*, there was a linear dose response (R^2= 0.98) between dry mass yield and water supply levels. Although plants and water treatments used were different, other studies (Ramadoss et al., 2008) are in agreement with these results. The reduced dry mass is normally due to inhibition of division and enlargement of plant cells (Kusaka et al., 2005). Water stress reduces CO_2 assimilation and the production of growth promoting cytokinins and gibberelic acid in plants (Kujawski, 2002). It also minimises photosynthetic rate (Sinaki et al., 2007), which leads to reduced shoot growth and leaf area (Luvaha et al., 2008). The inhibited cell expansion and cell division of water stressed cassava plants accounts for 40% leaf area reduction in Alves & Setter (2000).

Bulbine frutescens is highly resistant to drought and other types of stress (Joffe, 1993). It is, however, not clear, why *B. frutescence*, a relatively succulent plant, was the most sensitive to decreasing watering from 500 ml to 200 ml. It may be related to the effect on water on the opening of stomata. At the lowest water treatment the stomata were completely closed. This may also be due to lack of sufficient water reserves in the storage tissue to protect the leaves from sudden wilting and severe shrinkage (Larcher, 2001). However, other plants such as olive trees have the ability to stop shoot growth but not their photosynthetic activity (Xiloyannis et al., 2009). This is to maximise biomass production (Williams et al., 1998) while conserving water (Kalapos et al., 1996).

With *L. dysophylla* and *T. violacea*, water was not a limiting factor at water supply levels higher than 200 ml every second day. Water stored in other parts of these plants might have provided buffer for maintaining the water balance as was the case in Larcher (2001). These species may be more amenable to cultivation under arid conditions, whereas the productivity of *B. frutescence* would be high if sufficient water was available.

4.4.2 Stomatal conductance

The stomatal conductance of all plants in general decreased significantly ($P \leq 0.05$) with water supply level of 50 ml. This proves that plants were under water stress. The results are also in agreement with those based in mango tree species (Luvaha et al., 2008) and cassava plants (Alves & Setter, 2000). The stomata of *B. frutescens* seemed closed under the lowest water supply level of 50 ml. Stomatal closure minimises excessive evapotranspirational (Raven & Edwards, 2001) but it also leads to limited growth because carbon dioxide uptake depends on open stomata. *Bulbine frutescens* also had by far the lowest value of stomatal conductance under high water supply level. A possible explanation could be that *B. frutescens* is a Crassulacean Acid Metabolism (CAM) plant as a member of Asphodelaceae which is one of the seven major CAM families. The CAM plants close their stomata during the day to save water and only open them at night to allow carbon dioxide to enter (Drennan & Nobel, 2000). These characteristics enable CAM plants to grow in arid regions. Abscisic acid (a stress hormone) formed in the roots in response to water stress is normally transported to leaves of plants to trigger stomatal closure (Sharp, 2002). *Leonotis dysophylla*, in this study, had higher stomatal conductance than of the other two plant species probably because it had relatively larger leaf area surface with more stomata. It is possible that *B. frutescens* and *T. violacea*

respond to water stress through sunken stomata that enable them to withstand increased water stress by withholding excessive water loss.

4.4.3 Antifungal and antibacterial activity

Most plant extracts had reasonable MIC values (<1 mg/ml) against all test fungi and bacteria across water supply levels. The results were indicative of presence of good antimicrobial activity. In general, there were no statistically significant differences in almost all MIC (or total activity) values of plants against fungi and bacteria between water treatments. Also evident was the lack of clear correlation between MIC (total activity) values against pathogens and water treatments. All this suggests that water supply levels used in this study do not have any noteworthy effect on the antimicrobial activity of plant extracts. Although different methods were used, the data in chapter 3 based on *Tulbaghia violacea* and *Hypoxis hemerocallidea* are in agreement with these results. Similarly, water stress did not have any significant effect on the thymol and carvacrol content of the oil extracted from Mexican oregano (Dunford & Vazquez, 2005). This could probably be due to absence of effects of pathogens, herbivores and allelopathy present in the field environment. Abiotic and biotic stresses in the field environment regulate the levels of secondary metabolites in plants (Dixon & Paiva, 1995). Secondary metabolites accumulated in plants have clear ecological roles such as protection against pathogens including fungi (Rios et al., 1988).

Other studies investigating the effect of water on individual chemicals compounds do not support our results. The relative percentage of thymol compound of *Thymus vulgaris* plant was highest under the longest irrigation interval of 10 days (Aziz et al., 2008). That led to a decrease in content of p-cymene compound due to its transformation into thymol. Water stress has also increased the levels of terpenoids of

pine plants (Turtola et al., 2003) and some phenolic compounds of *Hypericum brasiliense* Choisy (de Abreu & Mazzafera, 2005). Earlier water stress increases the production of secondary compounds and then reduces it when severe stress is prolonged (Kujawski, 2002). For example, the amount of carvacrol increases under moderate stress, while γ-terpinene content decreases under moderate and severe water stress treatments (Baher et al., 2002). In our experience, however, antimicrobial activity of plant extracts depends on synergistic interactions. However, the increased levels of compounds of extracts may not necessarily translate into increased activity because in the Phytomedicine Programme it has frequently been found that antimicrobial activity in plant extracts depends on a combination of compounds rather than a single compound. Wild plant materials however had a higher antibacterial activity than the cultivated plants of the same species (Luseba et al., 2011). In this study the MIC values found using the same techniques were unfortunately not provided and the statistical analysis was very complicated to understand.

Although low water supply significantly reduced dry mass of plants, the same condition had very little effect on the antimicrobial activity of extracts. At least with these species cultivation under an optimal irrigation regime may not reduce the antimicrobial activity of plants. Cultivation with controlled irrigation equivalent to the 200 - 500 ml provided here is therefore likely to increase dry mass and maintain or enhance the antimicrobial activity of extracts. It also appears that if chemotypes with good activity were cultivated, it would yield effective products with the potential of good quality control.

Some notes:

The results obtained in this chapter supported the results in Chapter 3 that water stress has little influence on antibacterial and antifungal activity. In the next chapter, the influence of temperature on antimicrobial activity of different plant extracts will be examined. The purpose is to see if temperature treatments would yield similar or different results as was found with water treatments.

Chapter 5 Effect of temperature stress on antimicrobial activity of three medicinal plants

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Abstract

Ever increasing dependence on natural populations of medicinal plants has led to local extinction of some species in South Africa. Cultivation is one of the interventions that could address this problem. However, some traditional healers believed that cultivation would reduce the quality of herbal medicines. This is partly due to insufficient knowledge of how environmental factors affect the biological activity of the plants. The study evaluated the effects of temperature stress conditions on dry mass production, leaf area, respiration and antimicrobial activity of *Leonotis dysophylla* Benth., *Bulbine frutescens* (L.) Willd. and *Tulbaghia violacea* Harv. Leaf area, respiration and dry mass were determined by leaf area meter, porometer and a balance, respectively. Thin layer chromatography (TLC), bioautography and minimum inhibitory concentration (MIC) determined antimicrobial activity. High temperature (30°C) led to a considerably reduced dry mass, leaf area and stomatal conductance suggesting that plants, particularly *L. dysophylla* and *T. violacea*, were under stress. In contrast, the dry mass of *B. frutescens* increased considerably with high temperature confirming its resilience to temperature stress. Stomatal conductance readings of *B. frutescens* were not registered. Fresh leaves were harvested, air-dried and finely ground. Acetone leaf extracts were screened for antimicrobial activity against four bacterial and three fungal strains. Differences observed in the majority of the MIC values of plants against test pathogens were not statistically significant between temperature treatments. However, total activity values indicated that the activity of *B. frutescens* and *T. violacea* had higher activity under higher temperatures whereas for *L. dysophylla* the opposite was the case.

Keywords: Leaf area; minimum inhibitory concentration; stomatal conductance; temperature stress; total activity

5.1 Introduction

Overutilization of wild populations of medicinal plants has led to local extinction. Cultivation is one of the interventions that could address this persisting challenge. However, conservative communities (Keirungi & Fabricius, 2005) and some researchers believe that domestic cultivation would reduce the quality of medicinal material (Guo et al., 2009). Even in the use of herbal medicines in some developed countries, there is preference for plants growing in nature (Canter et al., 2005). These authors point out other arguments for the cultivation of medicinal plants i.e. better quality control, no misidentification of species, optimal production and less phenotypic variation.

Dependence only on natural populations for herbal medicines may be due to insufficient understanding of how environmental factors affect the biological activity of the plants. This study evaluated the effects of induced temperature treatments on growth and respiration parameters and antimicrobial activity of *Leonotis dysophylla* Benth., *Bulbine frutescens* (L.) Willd. and *Tulbaghia violacea* Harv. Temperature is one of the abiotic stresses known to affect biological activity of plants (Nagesh & Devaraj, 2008). The results may also provide some information on the effects of global warming on the bioactivity of medicinal plants as emphasised in Cavalier (2009).

T. violacea, popularly known as wild-garlic, is a member of the Alliaceae family. The plant is common in the Eastern Cape and the local communities use it to cure a number of ailments (Motsei et al., 2003). Its leaf extracts treat are used to treat type-1 diabetes, fever and colds, paralysis, hypertensive, asthma, rheumatism, sinus headaches, tuberculosis, oesophageal cancer, inflammation and gastrointestinal

ailments (Lyantagaye and Rees, 2003). Several studies mention that the plant has antimicrobial properties (Nteso & Pretorius, 2006; Lindsey & Van Staden, 2004) and anthelminthic activity (McGaw et al., 2000).

B. frutescens, commonly known as the snake flower or grass-aloe, is a member of the Asphodelaceae (previously Liliaceae) family. Its leaf gel is used to treat insect (mosquito) bites, cuts, grazes, burns (Abegaz, 2002), wounds, rashes, acne, blisters, mouth ulcers, cracked lips, cold sores, acne and ringworm (Van Staden & Drewes, 1994). Root extracts treat diarrhoea, colic, urinary tract, venereal diseases (Van Wyk et al., 1995) and fever (Abegaz, 2002). Similar extracts contain gaboroquinones, phenylanthraquinones and isofuranonaphthoquinones, as some of its chemical constituents (Abegaz, 2002; Abegaz et al., 2002). Such compounds possess antiplasmodial, antiparasitic and antioxidant properties.

Several *Leonotis* species, members of the Lamiaceae or mint family, treat various ailments such as high blood pressure, asthma (Van Wyk et al., 1997), leprosy and tuberculosis (Hutchings et al., 1996)., *L. dysophylla*, popularly known as the Klip-dagga in Afrikaans, is used traditionally as a tonic and to treat colds (Watt & Breyer-Brandwijk, 1962). *L. dysophylla* has some good antibacterial activity (110, 95, 113 and 63 µg/ml against *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli* and *Enterococcus faecalis*, respectively*)* (Eloff, 2010).

5.2 Materials and methods

5.2.1 Planting material

Leonotis dysophylla was obtained from the roadside in the Akasia Municipality in the west of Pretoria-North while *B. frutescens* and *T. violacea* were collected from the Vegetable and Ornamental Plant Institute (VOPI) of the Agricultural Research Council (ARC). Voucher specimens of *T. violacea* (117131), *L. dysophylla* (117130) and *B. frutescens* (117129) plants were collected. Mrs Elsa van Wyk, the curator, verified the identity and stored voucher specimens in the H.G.W.J. Schweickerdt Herbarium of the University of Pretoria in South Africa.

To generate vegetative clones, plantlets were obtained through division of mother clumps of *Bulbine frutescens* and *Tulbaghia violacea*. Plantlets were established in the growth trays filled with vermiculite comprising pine-bark medium under the same room temperature (25°C), light, irrigation and growth medium before trial commencement. Seeds of *L. dysophylla* were collected from the wild population in the Akasia municipality in the west of Pretoria-North and germinated in the growth trays filled with vermiculite at a depth of 1.25 cm. Germination took place under controlled greenhouse conditions. Almost 100% seed germination was achieved after two weeks. Seeds from one of the mature *L. dysophylla* plants in the greenhouse were germinated once again under the same conditions to minimise genetic variability. Approximately 100% germination was again achieved within two weeks. Seedlings were raised for a period of up to six weeks in the greenhouse of the Experimental Farm of the University of Pretoria. They were irrigated with 500 ml of distilled water every two days.

After reaching a height of 10 to 15 cm with at least two leaves, seedlings were transplanted into large pots (27 cm diameter x 25 cm height, volume c. 14 L) filled with potting-mix. Potting-mix comprised four parts loam soil, two parts sand, one part manure and two parts compost (Netshiluvhi, 1999). Seedlings were subjected to temperatures of 15°C and 30°C in the growth chambers. Each treatment had four replicate clones (one clone per pot) of each plant species. Chambers fitted with 24 fluorescent (215 watt) and incandescent (60 watt) bulbs were prepared to provide 12 hours of light and 12 hours of darkness. Seedlings were watered with 500 ml distilled water every second day before midday. The experiments were run for a period of 26 weeks.

5.2.3 Stomatal conductance

After 26 weeks of growth, readings of stomatal conductance (mmol/m^2s) were taken. The conductance was measured using a SC-I Leaf Porometer (ICT Plant Science Instrumentation) at the abaxial (basal) position of randomly selected leaves of each replicate plant. Basal position of the leaf is an area with relatively high density of stomata. Stomatal conductance was used to determine the rate of transpiration that gave indication of the extent to which plants were temperature-stressed.

5.2.4 Leaf area

Another parameter used in this study to measure the effect of temperature stress conditions on plants was a leaf area produced in cm^2. The portable Leaf Area Meter (LI-3100C model) was used (LI-COR

Biosciences, 2004). The instrument is designed for biological applications requiring rapid, precise area (length or width) measurements. To determine leaf area, leaves of each replicate plant were fed into the instrument one after another and readings were taken.

5.2.5 Extraction procedure

Recently mature fresh leaves were harvested from all study plants during vegetative and flowering stage. Leaves were then air-dried in a ventilated enclosure under room temperature of 25°C before being separated from stems. The dry mass was determined before grinding it into a fine powder in a Jankel and Kunkel Model A10 mill. One gram of the finely ground plant material was weighed into 50 ml centrifuge tubes. Ten ml acetone was added as extractant into the centrifuge tubes. Acetone is a good extractant (Eloff, 1998a) and least toxic to organisms in bioassays (Eloff et al., 2007). It also gives good results with several other plant species. The centrifuge tubes were vigorously shaken in a Labotec model 20.2 machine for *c.* 5 min at high speed to ensure rapid extraction. The extracts were centrifuged at 3000 x g for 10 min. The supernatant was filtered through Whatman No. 1 filter paper into pre-weighed labelled glass vials. The extraction was repeated twice in order to exhaustively extract plant material. Exactly 5 ml of the filtrate was removed and placed into a pre-weighed vial under a stream of air at room temperature in a fume cupboard to remove acetone. The solvent was allowed to dry until residue remained. The yield of extract was calculated.

5.2.6 Test bacterial strains

Gram-positive [*Staphylococcus aureus* (ATCC 29213) and *Enterococcus faecalis* (ATCC 29212)] and Gram-negative [*Pseudomonas aeruginosa* (ATCC 25922) and *Escherichia coli* (ATCC 27853)] bacterial strains were used to evaluate the antibacterial activity of plant species. The strains were obtained from the Central Microbiology Laboratory, Faculty of Veterinary Science at the University of Pretoria. The strains were grown at 37°C in Mueller Hinton (MH) broth (Merck chemicals).

5.2.7 Test fungal strains

The fungal strains used in the study were *Candida albicans* and *Cryptococcus neoformans* (Yeasts) and *Aspergillus fumigatus* (Mould), common and important animal fungal pathogens. The strains were obtained from the Central Microbiology laboratory (Faculty of Veterinary Science, University of Pretoria). They were maintained in Sabouraud Dextrose (SD) agar at 4°C and inoculated in SD broth at 37°C. Strains were then incubated prior to conducting bioautography and microdilution assays.

5.2.8 Thin layer chromatography analysis of plant extracts

The residue was made up to 10 mg/ml with acetone. The plant extracts were analysed by thin layer chromatography (TLC) on Merck TLC F254 plates with chloroform/ethyl acetate/formic (CEF) acid (5:4:1) as a solvent system. The TLC plates were prepared and then loaded with 10 µl extract (100 µg) (Stahl,

1969). Plates were then developed in saturated TLC tank (closed tank with saturated filter paper). The solvent front on the plates was marked with a pencil. The TLC plates with developed plant extracts were visualised under UV light (254 and 365 nm). Thereafter, plates were sprayed with 0.5 g vanillin dissolved in 100 ml sulphuric acid/ethanol (40:10) and heated at 100°C until optimal colour development. The TLC plates were scanned to obtain record of results.

5.2.9 Minimum inhibitory concentration

In order to determine minimum inhibitory concentration (MIC), 96-well microtitre plate was labelled before placing 100 µl distilled water in each well using multichannel micropipette (Eloff, 1998b). About 100 µl of extract (at 10mg/ml dissolved in acetone) was added to the first well of the column. Then a multichannel micropipette was used to remove 100 µl from the first well and place it in the next well down the column. The plunger was pushed gently up and down three or four times to ensure that the content of the well was properly mixed. The process continued down to the bottom of the plate. The final 100 µl was discarded to ensure that all wells contained 100 µl of extract. The columns contained a series of two-fold dilutions of extracts (e.g. 5, 2.5, 1.3, 0.63, 0.32, 0.16, 0.08 mg/ml, and so on) in a 96-well microtitre plates. Overnight bacterial cultures were prepared before being diluted with fresh Mueller-Hinton (MH) broth (1:100). Then 100 µl of bacterial cultures was placed into each well before mixing by squirting bacteria into wells. A similar volume of 100 µl of actively growing bacterial cultures was added to each well before being incubated overnight at 37°C under 100% relative humidity. About 40 µl of 0.2 mg/ml of ρ-iodonitrotetrazolium violet (INT) solution was added to each microplate well before being incubated for an hour or two. After further incubation, bacterial growth was denoted by the red colour of the INT formazan produced. The MIC value was recorded as the lowest concentration that inhibited bacterial growth.

5.2.10 Bioautography

Cultures of bacteria were prepared in MH broth. The TLC plates were developed and then the eluent was allowed to evaporate in a stream of air over a period of two days. The bacterial culture was centrifuged at 3000 x g for 10 min. The TLC plates were sprayed with bacterial suspensions using a glass spray gun. Plates were incubated under 100% relative humidity at 37°C overnight (Hamburger & Cordell, 1987). Plates were then dried before being sprayed with 2 mg/ml INT (ρ-iodonitrotetrazolium violet, Sigma) solution. Thereafter, plates were incubated again at 100% relative humidity at 37°C. The inhibition of bacterial growth was indicated by clear zones on the chromatogram (Begue & Kline, 1972).

5.2.11 Total activity

Total activity was also used as a parameter that would be applied to measure the effects of temperature stress on plant activity. It indicates the degree to which the active compounds in one gram of plant material can be diluted and still inhibit growth of pathogens. Total activity value (ml) measures the total antibacterial activity present in the plant by dividing the quantity (mg) extracted from 1 gram of plant material with the MIC value in mg/ml (Eloff, 2000). The result in ml indicates the volume to which the compounds present in 1 g of plant material can be diluted and still inhibit the growth of the microorganism.

5.2.12 Statistical analysis

Data were statistically analysed using GenStat ® for Windows ® (2003). Results for antimicrobial activity of all plants were reported as means ± standard error (SE). Results with 5% level of confidence ($P \leq 0.05$) were regarded as statistically significant. Significant differences for comparisons were determined by a one-way analysis of variance (ANOVA).

5.3 Results and discussion

5.3.1 Dry mass

The dry mass of *Leonotis dysophylla* and *Tulbaghia violacea* was reduced significantly (P≤0.05) at 30ºC. Surprisingly the mass of *Bulbine frutescens* increased considerably under same high temperature of 30°C (Table 5.1). *Bulbine frutescens* is a member of Asphodelaceae family well adapted to arid environments characterised by high temperatures. The other two plant species seemed to be sensitive to high temperatures. In the case of *L. dysophylla* and *T. violaceae* it was clear that the plants were under temperature stress, but this did not appear to be the case with *B. frutescence*.

Table 5.1. Influence of temperature treatments on the leaf dry matter production of plants grown for 26 weeks. Values (mean ± standard error; n=4) showing the same superscripts in the same row are not significantly different at the 5% confidence level.

Plant species	**Leaf dry mass (g) under temperature treatments**	
	15°C	**30°C**
L. dysophylla	74 ± 33[a]	52 ± 27)[b]
T. violacea	164 ± 40[a]	83 ± 18[b]
B. frutescens	549 ± 37[a]	711 ± 74[b]

Another parameter of stress is the leaf area of the plants under different temperatures. This is discussed next.

5.3.2 Leaf area

The mean leaf area of *Leonotis dysophylla* significantly decreased from 37 to 21 cm^2 under high temperature of 30°C (Table 5.2). The results are in agreement with those in many studies such as Wahid et al. (2007). It is another indication that *L. dysophylla* was indeed stressed. Limited growth resources because of heat stress lead to reduced leaf sizes (Fischer, 1984). This is a strategy used by plants to reduce transpiration surface (Luvaha et al., 2008). The reduction in leaf area also prevents a possible damage of photosynthetic apparatus (Navari-Izzo and Rascio, 1999). Leaves of study plants especially *L. dysophylla* growing under high temperature turned pale in colour because of desiccation. That may have been due to decreasing chlorophyll content through photooxidation (Kirnak et al., 2001).

Table 5.2. Influence of temperature treatments on leaf area of plants after 26 weeks treatment. Values (means ± standard error; n=4) showing the same superscripts in the same row are not significantly different at the 5% confidence level.

Plant species	**Leaf area (cm^2) under temperature treatments**	
	15°C	**30°C**
L. dysophylla	37 ± 13[a]	21 ± 3[b]
T. violacea	28 ± 11[a]	20 ± 7[a]
B. frutescens	20 ± 6[a]	27 ± 7[a]

Leonotis dysophylla seems to be more sensitive to high temperatures than *T. violacea* and *B. frutescens*. The leaf areas of *T. violacea* were reduced and of *B. frutescens* were increased but not statistically significantly under high temperature stress. *Leonotis dysophylla* is adapted to cool temperatures, as it is

evidently abundant during winter season. The leaf area of *B. frutescens* increased slightly at higher temperature indicating agains that it was not stressed. This may be because it could be a crassulacean acid metabolism (CAM) plant. CAM plants are adapted to arid conditions that are characterised by very hot temperatures (Drennan & Nobel, 2000).

Plants respond to water stress and to temperature stress by the closing of stomata. This is discussed next.

5.3.3 Stomatal conductance

Stomatal conductance of *L. dysophylla* (from 66 to 27 mmol/m^{2}s) and *T. violacea* (from 29 to 14 mmol/m^{2}s) was significantly reduced ($P \leq 0.05$) under high temperature stress of 30°C. The same was the case in studies on other plant species (Downes, 1969 and Black et al., 1969). Closing stomata is an adaptive response to temperature stress (Raven & Edwards, 2001). The adaptive response includes the production and accumulation of free amino acids and sugars by plant tissue (Mostajeran & Rahimi-Eichi, 2009).

As for *B. frutescens*, the stomatal conductance reading recorded by the porometer was zero. Again, the reason could be that *B. frutescens* is a CAM plant because it is a member of Asphodelaceae which is one of the seven major CAM families. The CAM plants close their stomata during the day to save water and only open them at night to allow carbon dioxide to enter (Drennan & Nobel, 2000). The reduction in transpiration may also increase internal temperature of certain plant and subsequently cause oxidative injury (Williams et al., 1998).

5.3.4 Antimicrobial activity

All plant extracts across temperature treatments had some antimicrobial activity against all bacteria and fungi with MIC values not exceeding 2.5 mg/ml [Tables 5.3(a) & (b)]. The increase in temperature did not lead to any statistically significant differences ($P \leq 0.05$) in the MIC values against any of the bacterial and fungal pathogens. There were also few differences in the chemical composition and antimicrobial activity of compounds separated by TLC (Appendix B). These results were similar to those based on water stress conditions and similar species in our unpublished data. It is a pity that the availability of growth chambers did not make it possible to use three temperatures to determine if there are clear trends. Perhaps the set of temperatures used in this study formed an optimal range (15 – 30°C), which phytoconstituents of plants could withstand. Probably the sensitivity of different enzymes responsible for inducing the production of biological activity in this study was stable and uniform across temperature treatments used. Relatively high temperature (40°C) significantly increased certain enzymes (glutathione peroxidase) of *Phalaenopsis* while significantly reducing others (superoxide dismutase) (Ali et al., 2005).

Another study has shown that bioactive compounds of some plants are unstable to high temperatures (Wong et al., 2009). Temperatures of 30°C and 35°C in Wang & Zheng (2001) and Zobayed et al.(2005) significantly increased antioxidant capacities of strawberry plants and peroxidase activity of St. John's wort, respectively. High temperatures accelerate the transformation of terpinene and p-cymene to phenolic compounds of extracts (Said-Al et al., 2009). Temperatures of up to 70°C have reduced the biological activity of extracts of plants in Jabeen et al. (2008). This happens when heat stress proteins (HSPs) responsible for the production of biological activity are denatured by heat (Efeoglu, 2009), which was apparently not the case in this study.

Table 5.3(a). Minimum inhibitory concentration of plant extracts against test bacteria under temperature treatments. Values (means ± standard error; n=4) showing the same superscripts in the same column are not significantly different at the 5% confidence level.

Plant species	**Temperature treatments (°C)**	**Minimum inhibitory of concentration (mg/ml)**			
		S. aureus	*E. coli*	*P. aeruginosa*	*E .faecalis*
L. dysophylla	15	0.52 ± 0.1[a]	0.73 ± 0.2[a]	0.73 ± 0.3[a]	0.73 ± 0.3[a]
	30	0.63 ± 0.1[a]	0.84 ± 0.2[a]	0.94 ± 0.3[a]	0.94 ± 0.3[a]
B. frutescens	15	0.52 ± 0.2[a]	0.52 ± 0.1[a]	1.15 ± 0.7[a]	0.42 ± 0.1[a]
	30	0.62 ± 0.2[a]	0.52 ± 0.1[a]	1.15 ± 0.7[a]	0.52 ± 0.1[a]
T. violacea	15	1.25 ± 0.3[a]	2.50 ± 0.0[a]	1.67 ± 0.4[a]	1.15 ± 0.7[a]
	30	1.67 ± 0.3[a]	2.50 ± 0.0[a]	1.67 ± 0.4[a]	1.35 ± 0.7[a]

Table 5.3(b). Minimum inhibitory concentration of plant extracts against test fungi under temperature treatments. Values (means ± standard error; n=4) showing the same superscripts in the same column are not significantly different at the 5% confidence level.

Plant species	Temperature treatments (°C)	Minimum inhibitory concentration (mg/ml)		
		Candida albicans	*Cryptococcus neoformans*	*Aspergillus fumigatus*
L. dysophylla				
	15	1.04 ± 0.1[a]	0.47 ± 0.2[a]	0.84 ± 0.2[a]
	30	1.25 ± 0.1[a]	1.04 ± 0.2[b]	1.04 ± 0.2[a]
B. frutescens	15	1.04 ± 0.2[a]	0.42 ± 0.1[a]	0.63 ± 0.1[a]
	30	0.73 ± 0.2[a]	0.42 ± 0.1[a]	1.04 ± 0.1[a]
T. violacea	15	1.04 ± 0.4[a]	1.25 ± 0.1[a]	2.50 ± 0.3[a]
	30	1.46 ± 0.4[a]	0.84 ± 0.1[a]	1.67 ± 0.3[a]

Although the MIC values per se were not different between temperatures [Table 53(a) & (b)], the total activity values, which also incorporated the quantity extracted, showed some notable differences. Metabolites present in *B. frutescens* and *T. violacea* were more soluble were probably more soluble under high temperatures than metabolites of *L. dysophylla*, which grows relatively well under autumn and winter seasons. A consequence of this is that the total activity of *B. frutescens* and *T. violacea* extracts was considerably higher at the higher temperature of 30°C whereas there was no effect or even a reduction in one case for *L. dysophylla* [Table 54(a) & (b)].

Table 5.4(a). Total activity of plant extracts against test bacteria under different temperature treatments and quantity extracted from 1.00 g of dried material.Values (means ± standard error; n=4) with the same superscripts in the same column are not significantly different at the 5% confidence level.

Plant species	Temperature (°C)	Quantity extracted (mg)	Antibacterial total activity (ml/g)			
			S. aureus	*E. coli*	*P. aeruginosa*	*E. faecalis*
L. dysophylla	15	110.0 ± 10.0[a]	240 ± 53[a]	211 ± 68[a]	198 ± 80[a]	200 ± 48[a]
	30	100.0 ± 10.0[a]	164 ± 53[a]	141 ± 68[a]	172 ± 80[a]	88 ± 48[b]
B. frutescens	15	101.2 ± 32.1[a]	208 ± 80[a]	213 ± 66[a]	158 ± 85[a]	284 ± 86[a]
	30	204.3 ± 63.5[b]	440 ± 80[b]	421 ± 66[b]	412 ± 85[b]	421 ± 86[b]
T. violacea	15	103.3 ± 32.1[a]	71 ± 26[a]	41 ± 12[a]	71 ± 17[a]	185 ± 39[a]
	30	206.7 ± 63.5[b]	165 ± 26[b]	83 ± 12[b]	128 ±17[b]	353 ± 39[b]

Table 5.4(b). Total activity of plant extracts against fungi under temperature treatments and quantity extracted from 1.00 g of dried material. Values (means ± standard error; n=4) showing the same superscripts in the same column are not significantly different at the 5% confidence level.

Plant species	**Temperature treatments (°C)**	**Quantity extracted (mg)**	**Mean antifungal total activity (ml/g)**		
			Candida albicans	*Cryptococcus neoformans*	*Aspergillus fumigatus*
L. dysophylla	15	110.0 ± 10.0^{a}	114 ± 16^{a}	330 ± 106^{a}	143 ± 26^{a}
	30	100.0 ± 10.0^{a}	80 ± 16^{a}	106 ± 26^{b}	106 ± 26^{a}
B. frutescens	15	101.2 ± 32.1^{a}	104 ± 76^{a}	256 ± 28^{a}	164 ± 35^{a}
	30	204.3 ± 63.5^{b}	347 ± 76^{b}	513 ± 28^{b}	210 ± 35^{a}
T. violacea	15	103.3 ± 32.1^{a}	119 ± 57^{a}	83 ± 64^{a}	41 ± 32^{a}
	30	206.7 ± 63.5^{b}	187 ± 57^{a}	283 ± 64^{b}	143 ± 32^{b}

In conclusion, high temperature of 30°C significantly reduced dry mass, leaf area and stomatal conductance of *L. dysophylla* and *T. violaceae* suggesting that plants were under temperature stress. In contrast, the dry mass and leaf area of *B. frutescens* increased under high temperature showing that it behaved like a true CAM plant. In the majority of cases, the antimicrobial activity of all study plants against test pathogens between temperature treatments did not differ statistically significantly. The results in general suggest that temperature treatments used in the study have very little effect on the antimicrobial activity of plants. However, because different quantities were extracted under different temperatures the total antibacterial activity of *B. frutescens* and *T. violacea* was better under high temperature there was no effect or even a reduction at higher temperatures in the case of *L. dysophylla*. This clearly relates to the quantity dissolved from 1 g of plant material that was used in the calculation of total activity. This means that the increased temperature led to a larger percentage of the plant material being soluble in acetone but little change in the composition of the antibacterial compounds. There was close to a doubling of the soluble material at the higher temperature in the case of *B. frutescens* and *T. violacea*. This aspect may be worth investigating in depth. In the case of the antifungal activity (Table 5.4b) there was a much higher effect against *C. neoformans* and *A. fumigatus.* From the difference in response between *C. albicans*, *C. neoformans* and *A. fumigatus*, it appears that the compounds responsible for the antifungal activity are not general metabolic toxins.

Some notes:

The results presented in chapters 4 – 5 indicated that with these plant species generally water and temperature treatments had no effect on the antimicrobial activity. There were however differences between different plant species. In these chapters, only one biological activity was investigated but it would be unwise to generalise on only one biological activity with a limited number of species. The results presented in Chapter 2 on long-lived plant species growing in nature were not that clear. The differences might have been caused by edaphic or genetic differences. In many plant species, the biological activity may be related to antioxidant activity. It therefore seemed logical to investigate the influence of induced water and temperature stress on antioxidant activity of extracts to be discussed in the next chapter.

Chapter 6 Antioxidant activity of acetone leaf extracts of plants growing under induced temperature and water stress conditions

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Abstract

Several papers indicate that stress causes metabolic changes leading to differences in some metabolite pools. It does appear in some cases that antimicrobial activity depending on many compounds in the extract is not so dependent on stress conditions. This study investigated the effect of different water and temperature treatments on antioxidant activity of *Leonotis dysophylla* Benth., *Bulbine frutescens* (L.) Willd. and *Tulbaghia violacea* Harv. Air-dried and finely ground leaves were extracted with acetone. In order to screen for antioxidants, the 2,2, diphenyl-2-picryl-hydrazyl (DPPH) was sprayed onto the thin layer chromatograms in methanol. Antioxidant activity of extracts was assessed against 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) and L-ascorbic acid standard oxidants. In doing that, two free radicals, 2,2'-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid (ABTS) and DPPH, respectively. Results indicate that IC_{50} values (0.03 – 0.76 mg/ml) of all plant extracts across temperature and water treatments represented lower antioxidant activity than of Trolox (0.002 mg/ml) and ascorbic acid (0.004 mg/ml). The antioxidant activity of all plant extracts decreased significantly under high temperature treatment of 30°C. Differences between the IC_{50} values of *L. dysophylla* and *B. frutescens* under different water treatments were not statistically significant, which indicated uniform antioxidant activity. However, the antioxidant activity of *T. violacea* extracts was significantly reduced towards water treatment of 50 ml. Results in general suggest that antioxidant activity of plant extracts is more likely to be reduced by high temperatures than low water supplies.

Keywords: ABTS free radical, ascorbic acid, antioxidant activity, *Bulbine frutescens*, DPPH free radical, *Leonotis dysophylla*, temperature stress, Trolox, *Tulbaghia violacea*, water stress.

6.1 Introduction

Several studies have demonstrated the importance of indigenous herbal medicines in the healthcare systems of developing countries (Canter et al., 2005). In several developed countries herbal medicines plays an increasingly important role in maintaining health. Many mechanisms explain the therapeutic use. Antioxidant activity of plant extracts can be implicated in many potential uses of medicinal plants by protecting against deleterious oxidative processes. In many cases antioxidant activity may be related to immune stimulation that aids the patient in self-healing processes. Herbal medicines and food are natural sources of antioxidant activities. The antioxidant activity of plant extracts comes mainly from phenolic constituents such as flavonoids, phenolic acids and polyphenolic compounds (Maltas & Yildiz, 2011). Polyphenols are the major antioxidant constituents isolated from many medicinal and edible plants (Wan et al., 2011). The antioxidant compounds neutralize or scavenge free radicals including hydrogen peroxide (H_2O_2), superoxide (O^{-1}), hydroxyl (OH), peroxyl (ROO) by different mechanisms including metal chelation and electron donation as reducing agent (Iqbal & Bano, 2009). The antioxidant activity of a plant extract in particular has the ability to prevent oxidative damage caused by free radical such as the reactive oxygen species (ROS). The oxidative damage caused by free radical has been implicated in several chronic human diseases such as diabetes mellitus, cancer, arthritis and aging process (Patel et al., 2010).

The need to respond to prevalent chronic diseases has led to the excessive demand for and intake of herbal medicines in the developing countries (Sathisha et al., 2011). The challenge, though, is that the medicinal plant products continue to be extracted from dwindling wild populations (Rojas et al., 2006). This practice often leads to local extinctions (Guo et al., 2009) as can be witnessed in several parts of South Africa. Some scientists after investigating the effect of stress conditions on the presence of certain

metabolites also believe that the biologically active plant compounds are formed under stress conditions. Furthermore, traditional healers and local communities also believe that medicinal properties derived from wild populations are superior (Keirungi & Fabricius, 2005). To a certain extent, this has been the case in some studies (McChesney, 1999; Souri et al.,2008; Luseba et al., 2011). For example, oxidative stress induces the formation of enzymatic compounds such as ascorbate, glutathione and α-tocopherols (Yordanov et al., 2003). The elevated levels of glutathione reductase activity (enzymatic) of spinach (*Spinacia oleracea* L.) were also produced as an adaptive response to water stress in Gamble & Burke (1984).

According to our unpublished data, water and temperature treatments in general have very little effect on antimicrobial activity of acetone leaf extracts of *Leonotis dysophylla* Benth., *Bulbine frutescens* (L.) Willd. and *Tulbaghia violacea* Harv. Antioxidant activity depends on a mixture of several compounds in a plant extract. This study investigated whether water and temperature stress conditions would have any noteworthy effect on the antioxidant activity of extracts of selected medicinal plants.

6.2 Materials and methods

6.2.1 Preparation of plant material

Leonotis dysophylla was obtained from the roadside in Akasia Municipality in the west of Pretoria-North while *B. frutescens* and *T. violacea* were collected from the Vegetable and Ornamental Plant Institute (VOPI) of the Agriclutural Research Council (ARC). Voucher specimens of *T. violacea* (117131), *L. dysophylla* (117130) and *B. frutescens* (117129) plants were prepared. Mrs Elsa van Wyk, the curator, verified and kept specimens in the H.G.W.J. Schweickerdt Herbarium situated at the University of Pretoria.

Clone plants were prepared for the study to minimise genetic variability, as was the case in Section 4.2. Plantlets of 10 to 15 cm height with at least two leaves were transplanted from growth trays into pots (27 cm diameter x 25 cm height, volume *c.* 14 L) filled with potting-mix described in Netshiluvhi (1999). Plants were all grown for a period of 26 weeks under controlled water and temperature stress conditions. After 26 weeks of growth, recently mature leaves from vegetative and flowering plants were harvested, air-dried and finely ground before extraction. A known mass of each of the powdered material was then extracted with ten volumes of acetone at room temperature for 24 hours and filtered. Acetone was used as extractant, as it has been found to extract large quantities of bioactive plant material (Eloff, 1998a). The extracts obtained were concentrated under vacuum at 40°C using a rotary evaporator (Buchi®, Switzerland) to give the crude extracts of each plant material. The dry extracts were stored in sealed vials in the refrigerator prior to evaluation of antioxidant activity.

6.2.2 Chemicals

Chemicals used were L-ascorbic acid (Merck), potassium persulphate (Sigma), 2,2'-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) (Sigma), 6-hydroxy-2, 5,7,8-tetramethylchromane-2-carboxylic acid (Trolox®) (Fluka), 2,2-diphenyl-1-picryl hydrazyl (DPPH) (Sigma) and absolute ethanol (Merck).

6.2.3 Qualitative and quantitative determination of antioxidant activity

In determining the antioxidant activity of extracts, the study applied the methodology cited by several authors in Bizimenyera et al. (2007). Qualitative screening for antioxidant activity was determined by using 2, 2-diphenyl-1-picryl hydrazyl (DPPH) according to several studies such as Takao et al. (1994). The DPPH (0.2%) was sprayed on the thin layer chromatograms (TLC) of extracts developed in EMW (ethyl acetate/methanol/water (10/1.35 /1) solvent system in methanol. Antioxidant activity was detected on the chromatograms when the initially purple DPPH background turns yellow in bands where an antioxidant compound is present.

Quantification of antioxidant activity (AOXA) was determined spectrophotometrically using two radicals, ABTS and DPPH and a Versa-max® microplate reader (Labotec). In one method, use was made of the Trolox equivalent antioxidant capacity (TEAC) assay based on the scavenging of the ABTS radical into a colourless product (Re et al., 1999). The absorbance was read at 734 nm. Trolox (6-hydroxy-2, 5,7,8-tetramethylchromane-2-carboxylic acid) is a Vitamin-E analogue. If an extract had antioxidant activity equivalent to Trolox, its TEAC value would be 1 and if the extract were more active its TEAC would be greater than 1.

The second method employed the DPPH free radical assay (Mensor et al., 2001). Different concentrations of the extracts were prepared between 20.0 and 1.0 µg/ml. About 10 µL of 0.4 mM DPPH in ethanol was added to 25 µL of each concentration of extract tested and allowed to react at room temperature in the dark for 30 minutes. Blank solutions were prepared with each test sample solution (25 µL) and 10 µL ethanol only while the negative control was DPPH solution, 10 µL plus 25 µL ethanol. L-ascorbic acid was the positive control. The decrease in absorbance was measured at 518 nm. Values obtained were converted to percentage antioxidant activity (AOXA%) using the formula: -

$$AOXA\% = 100 - \{[(Abs_{sample} - Abs_{blank}) \times 100] / Abs_{control}\}$$

Abs_{sample} is the absorbance of the sample, Abs_{blank} is the absorbance of the blank and $Abs_{control}$ is the absorbance of the control.

L-ascorbic acid (vitamin C) was used as a positive control (antioxidant agent). The antioxidant activity is expressed as inhibitory concentration (IC_{50}) values. The lower the IC_{50} value the more effective antioxidant activity. The IC_{50} value, defined as the concentration of the sample leading to 50% reduction of the initial DPPH concentration, was calculated from the linear regression of plots of concentration of the test extracts (µg/mL) against the mean percentage of the antioxidant activity obtained from three replicate assays. The IC_{50} is half maximal (50%) inhibitory concentration (IC) of a substance. For statistical analysis, the results were expressed as mean ± SEM (standard error of mean) and the IC_{50} values obtained from the linear regression of plots of concentration of the test compounds (µM) against the mean percentage of the

antioxidant obtained from the three replicate assays. Such plots show a good coefficient of determination, with most values being $r^2 \geq 0.910$ (SigmaPlots^R 2001, SPSS Science).

6.2.4 Statistical analysis

Data were statistically analysed using GenStat ® for Windows ® (2003) and SA® PROC GLM. The results for antioxidant activity of all tree species were reported as means ± standard error (SE). Significant differences for comparisons were determined by a one-way analysis of variance (ANOVA) procedure. The results with 5% level of confidence ($P \leq 0.05$) were regarded as statistically significant.

6.3 Results and discussion

The results with the qualitative antioxidant determination were disappointing (results not shown). Even the most polar solvent system used (EMW) did not separate the different antioxidant compounds present in extracts of all three species well. In the case of *T. violaceae* the extract from the plant treated with 500 ml water did not have an antioxidant compound with an R_f of 0.05 separated with EMW that all other treatments had. We could not therefore determine if the water or temperature stress led to a change in the composition of the antioxidant compounds in any of the extracts. It appears that the antioxidants present in these species, must be polyphenolic or tannin-like compounds that adhere very strongly to silica gel on the TLC plates.

The quantitative antioxidant results are presented in Table 6.1 and Figures 6.1 - 6.3. The DPPH radical-scavenging activity and ABTS free radical decolourisation assay of acetone leaf extracts of *T. violacea*, *B. frutescens* and *L. dysophylla* growing under different temperature and water treatments were evaluated. The IC_{50} values (0.03 – 0.76 mg/ml) of all plant extracts across temperature and water treatments were considerably higher and therefore had a much lower anti-oxidant capacity than of Trolox (0.002 mg/ml) and ascorbic acid (0.004 mg/ml). The results therefore indicate a relatively very low antioxidant activity. The relatively low antioxidant activity may explain the disappointing results obtained in the Similarly, compounds (separated by EMW and sprayed by DPPH) had small polar bands (yellow) on DPPH chromatograms across all treatments, suggesting presence of weak scavenging activity of plant extracts (data not shown here).

High temperature (30°C) significantly (P≤0.05) decreased antioxidant activity of all plant extracts (Table 6.1) with values between 75 and 433% of the activity at 15°C. The results from which the IC_{50} values were calculated are shown in Figures 6.1 – 6.3. The exposure of plants to high temperature stress in lowers the enzymatic activity, which also reduces the antioxidant activity of plant extracts (Gamble & Burke 1984). Decrease of the antioxidant activity could be due to compounds that become unstable under high temperatures due to antioxidant enzymatic decomposition (Wong et al., 2009). High temperature stress has the potential to enhance inactivation of catalase by preventing synthesis of new antioxidant enzymes (Hertwig et al., 1992). The presence of trace elements such as non-phenolic compounds may also reduce the antioxidant activity of a plant (Vinson et al., 1998).

Water treatments did not have a significant (P≤0.05) effect on the antioxidant activities of extracts of *L. dysophylla* and *B. frutescens*. The same was also the case in other studies where water stress did not lead to changes in the antioxidant activity of the extracts of certain plants (Ferreira et al., 2002; Wong et al. 2007). Low water supply did however decrease the antioxidant activity of *T. violacea* extracts significantly. There appears to be different responses to water stress on antioxidant activity. It could also be due to the reaction of some plants to stress which may differ depending on the sensitivity of antioxidant enzymes of different plant extracts (Panchuk et al., 2002). Sometimes the reactive oxygen species (ROS) are unstable and able to change rapidly to non-radical products (Michalak, 2006). Water stress has potential to cause oxidative damage at a cellular level because of increased accumulation of superoxide and hydrogen peroxide (Robinson & Bunce, 2000; Allen, 1995). As a result, plants may produce excessive ROS rather than fix CO_2 (Mittler & Zilinskas, 1994). During various abiotic stresses, the extent of reactive oxygen species (ROS) production exceeds the antioxidant defence capability of the cell, resulting in cellular damage (Almeselmani et al., 2006). The quenching activity of antioxidant of plant extracts is upset, or

inhibited by the effect of environmental stress such as water or drought (Ali & Alqurainy, 2006). The results in general suggest that the radical scavenging activity of plant species is more sensitive to temperature stress than to water stress.

Our results disagree with those of either author where water stress increased the antioxidant activity of plant extracts (Babu & Devaraj, 2008, Zhang et al., 2006 and Liu and Huang, 2002). This kind of response may depend on tolerant genotype to stress, duration and intensity of stress (Iqbal & Bano, 2009). The elevated levels of glutathione reductase activity (enzymatic) of Spinach (*Spinacia oleracea* L.) form as an adaptive response to environmental stress (Gamble & Burke (1984).

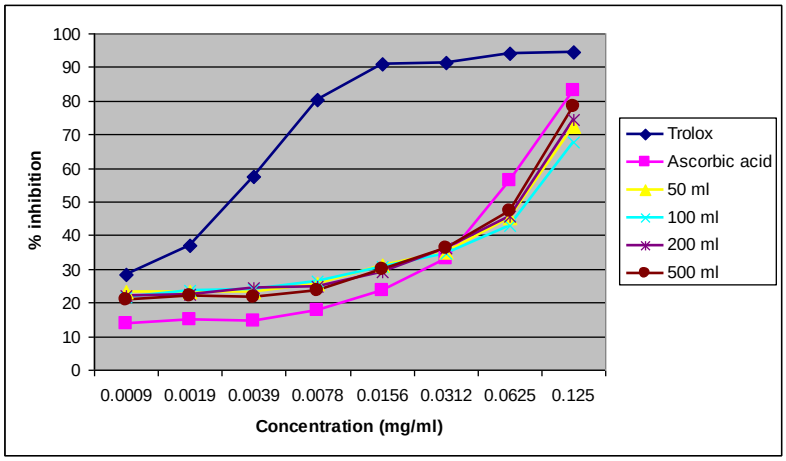
Temperature stress induces cellular responses such as production of stress proteins that increase antioxidants (Rani et al, In Press). Another study argues that early exposure to environmental stress induces the production of increased antioxidant activity of a plant extract (Khalil et al., 2009). That may partly explain why long term exposure (26 weeks) to high water and temperature stress yielded no effect on and reduced antioxidant activity of plant extracts in this study, respectively.

Table 6.1. Effects of higher temperature treatment of 30°C and lowest water treatment of 50 ml on dry leaf mass (adapted from Chapters 4 and 5) and DPPH radical scavenging activity of acetone extracts of plant species. Values (means ± standard error; n=4) showing the same superscripts in the same row are not significantly different at the 5% confidence level ($P \leq 0.05$).

Plant species	Treatments	Dry leaf mass (g)	Antioxidant activity (IC_{50} in mg/ml)
	Water 500 ml	142 ± 43[a]	0.10 ± 0.02[a]
	Water 200 ml	97 ± 27[b] b	0.12 ± 0.05[a]
L. dysophylla	Water 100 ml	95 ± 38[b]	0.14 ± 0.06[a]
	Water 50 ml	77 ± 18[b]	0.12 ± 0.06[a]
	Temp 15°C	74 ± 33[a]	0.03 ± 0.01[a]
	Temp 30°C	52 ± 27[b]	0.13 ± 0.04[b]
	Water 500 ml	185 ± 38[a]	0.24 ± 0.08[a]
	Water 200 ml	164 ± 40[a]	0.38 ± 0.29[a]
T. violacea	Water 100 ml	84 ± 16[b]	0.76 ± 0.31[b]
	Water 50 ml	86 ± 41[b]	0.64 ± 0.31[b]
	Temp 15°C	164 ± 40[a]	0.24 ± 0.01[a]
	Temp 30°C	83 ± 18[b]	0.42 ± 0.14[b]
	Water 500 ml	1618 ± 272[a]	0.19 ± 0.01[a]
	Water 200 ml	930 ± 51[b]	0.18 ± 0.05[a]
B. frutescens	Water 100 ml	586 ± 74[c]	0.20 ± 0.05[a]
	Water 50 ml	333 ± 121[d]	0.25 ± 0.06[a]
	Temp 15°C	549 ± 37[a]	0.19 ± 0.12[a]
	Temp 30°C	711 ± 74[b]	0.34 ± 0.22[b]

Standards: Trolox = 0.002 mg/ml; Ascorbic acid = 0.004 mg/ml.

A



B

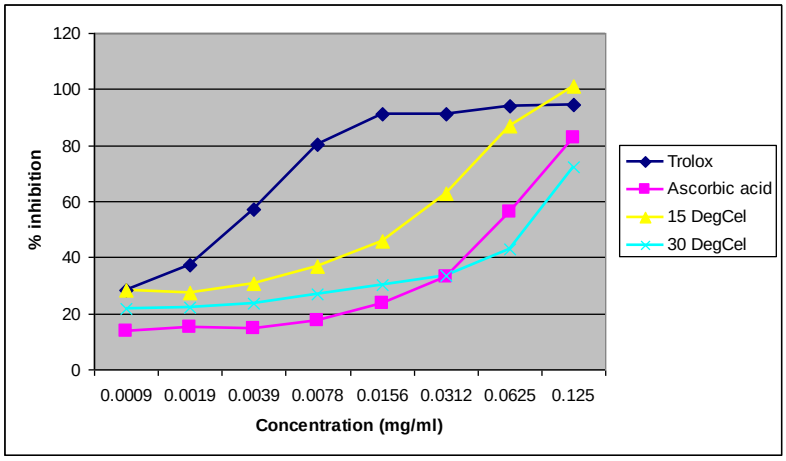
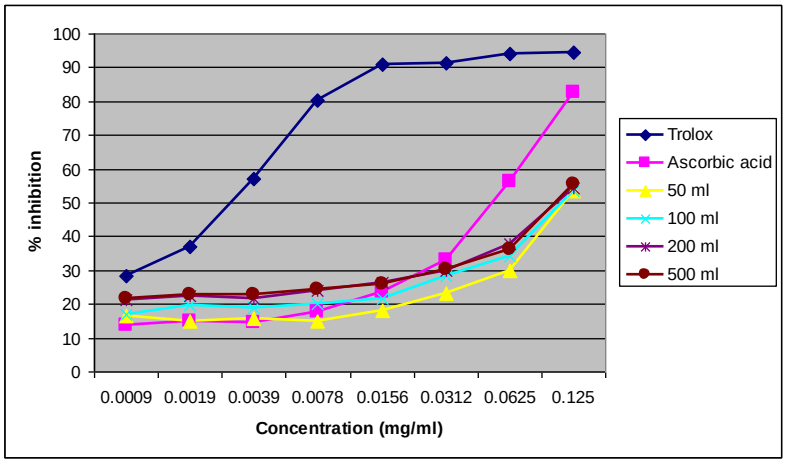


Figure 6.1. DPPH free scavenging activity of standards (Trolox and Ascorbic Acid) and acetone extracts of *Leonotis dysophylla* growing under different water **(A)** and temperature **(B)** treatments.

A



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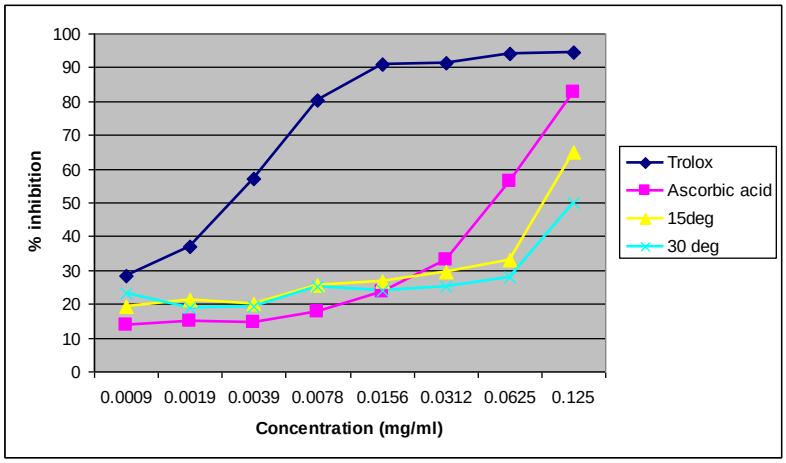
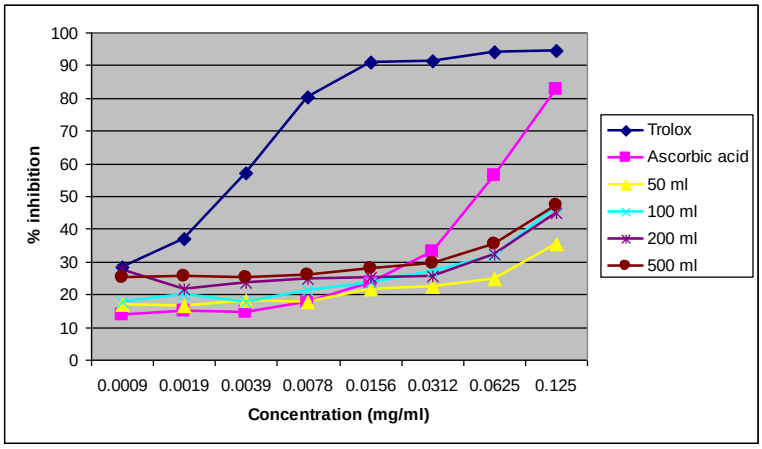


Figure 6.2. DPPH free scavenging activity of standards (Trolox and Ascorbic Acid) and acetone extracts of *Bulbine frutescens* growing under different water **(A)** and temperature **(B)** treatments.

A



B

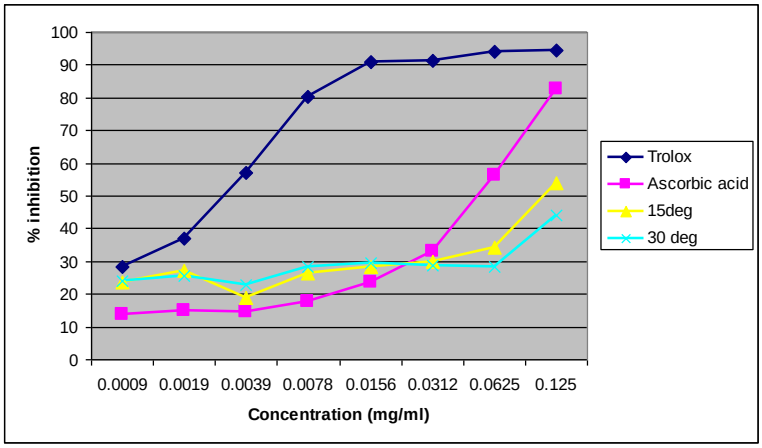


Figure 6.3. DPPH free scavenging activity of standards (Trolox and Ascorbic Acid) and acetone extracts of *Tulbaghia violacea* growing under different water **(A)** and temperature **(B)** treatments.

The plants examined had a relatively low antioxidant activity compared to Trolox and L-ascorbic acid. This may explain the disappointing results obtained in the qualitative analysis aof antioxidant compounds. It may also explain differences between our results and those of other authors.

In the context of this thesis, it appears that growing at least these species under temperature stress conditions decreased the antioxidant activity and consequently the potential health benefits of grown under natural stress conditions rather than under good agricultural production conditions. Similarly, water stress did not have any effect on the antioxidant activities of the species examined. Under the highest water stress conditions, the antioxidant activity of *T. violacea* was reduced significantly.

Some notes:

As in the previous chapters, it appears with the different species investigated that water and temperature stress does not necessarily lead to extracts with higher antibacterial, antifungal or antioxidant activities. The implications of these results will be discussed in Chapter 7.

Chapter 7 General discussion

The overall aim of this study was to evaluate to what degree different environmental conditions influenced antimicrobial and antioxidant activities of plants cultivated outside their natural environment. This section discusses the implications of the overall study results on traditional practitioners and scientists' beliefs that cultivation weakens medicinal properties and that the concentration of secondary metabolites only increases under environmental stress.

Acetone extracts of long-lived woody (*C. collinum*, *T. sericea* and *S. birrea*) and clone short-lived herbaceous (*H. hemerocallidea*, *T. violacea*, *L. dysophylla* and *B. frutescens*) plants subjected to different rates of annual rainfall and controlled environment conditions (water and temperature) possessed good biological activity against test pathogens and oxidants, respectively. This is probably one of the reasons, why these plants are widely used within local communities for medicinal purposes by traditional practitioners to treat various illnesses and conditions (Chapters 2 - 6).

However, leaf extracts of long-lived plants yielded inconsistent antibacterial results between different rates of annual rainfall (Chapter 2). For example, *C. collinum* and *S. birrea* against *A. aureus*, *E. coli* and *P. aeruginosa* had significantly increased antibacterial activity towards the lowest rate of rainfall. Extracts of *T. sericea* against *P. aeruginosa* and *E. faecalis* had a significant increase and decrease in antibacterial activity towards lowest rate of rainfall, respectively. With extracts of *C. collinum* and *S. birrea* against *E. faecalis* as well as *T. sericea* extracts against *S. aureus* and *E. coli* there was no correlation between antimicrobial activity and rates of annual rainfall. These results indicate that the antimicrobial activity of plants in nature varies widely depending on several other factors not just different rates of annual rainfall. Those factors may include pathogens, herbivores (Banchio et al., 2007), genetic variability (Guo et al.,

2009; Yuan et al., 2010), allelopathy (Jahangir et al., 2009; Blanco, 2009), age (Dunford & Vazquez, 2005), substrate and habitat (Rajakaruna et al., 2002). If the decreased rainfall was associated with water stress, the results indicate a wide variability in antibacterial activity of different plants against different pathogens. These results could not clearly support or refute the claim made by certain traditional healers that plants growing in nature are more active than cultivated plants. These results also show the wide variability in activity of leaf extracts of the same tree species, which is actually an argument against using plants growing in nature to deliver a product with consistent biological activity. The next logical step was to minimise the genetic variability and to grow plants under well-controlled environmental parameters before determining the antimicrobial activity.

In contrast to the above results, in nearly all cases there were no significant differences in antimicrobial and antioxidant activities of short-lived plants under different water and temperature stress treatments (Chapters 3 - 6). These findings suggest that different treatments had very little effect on antimicrobial and antioxidant activities of extracts. Different water supplies on *Lippia berlandieri* did not have any significant effect on thymol and carvacrol content of extracts (Dunford & Vazquez, 2005). Therefore, the findings do not agree fully with scientists and traditional practitioners' general beliefs. However, the antioxidant activity of extracts was significantly reduced under high temperature indicating the sensitivity of extracts to high temperatures. Strangely in this case the temperature stress did not lead to an increase in antioxidant activity, but rather to a statistically significant decrease. Similarly, the antioxidant activity of only *T. violacea* was also lower significantly under the highest water stress conditions. It is evident that the latter findings also disagree partially with the scientists' belief. It could probably be true under certain circumstances, but may not always be the case. For example, amounts of phenolic compounds of *T. violacea* and *H. hemerocallidea* ethanol extracts were higher in spring than any other seasons (Ncube et al., 2011). In another study, extracts of highly water stressed *Thymus vulgaris* yielded the highest and lowest

percentages of thymol and p-cymene compounds, respectively (Aziz et al., 2008). It is because the rate of transformation of p-cymene to thymol is relatively high under stress conditions. A study review has also demonstrated that drought can trigger a change in plant metabolism resulting in an increase in concentration of phenols and terpenes of different plant species (Khan et al., 2011). The effectiveness of extracts may sometimes depend on the different sensitivity of Gram-positive or Gram-negative bacteria (Rajakaruna et al., 2002). Interesting differences between the different species examined were found under different temperature and water regimes. This could be explained by the crassulacean acid metabolism of one of the species used. The results indicate the danger of making wide generalizations from results using a limited number of species.

After this study was nearly completed, other authors (Luseba et al., 2011) also examined the same problem. What they did was to compare the biological activity of some plant species growing in the medicinal plant collection of the Onderstepoort Veterinary Institute with the activity of the same plant species growing in nature. Unfortunately, they did not provide their raw data but used a complicated statistical analysis to show that five species growing in nature had a higher activity than the cultivated plants and that dried plant material had a higher activity than fresh plant material. The major criticism against this work is that the potential genetic differences between the cultivated and natural plants were not taken into consideration. It may be worthwhile to compare the biological activity of extracts of plants growing in good botanical gardens where the origin of the tree is known with trees from the original location.

The overall findings suggest that the biological activity of plants is more likely to vary widely in nature than under controlled conditions outside the natural environment. The biological activity of plants growing in

nature is susceptible to external factors such pathogens, herbivores and a host of other factors. This is an indication that natural environment may not always guarantee high and stable biological activity. As a result, belief by some traditional practitioners cannot be widely substantiated.

Therefore, the findings encourage cultivation (under controlled environmental conditions) because it has great potential to regulate and enhance the biomass production and desired biological activity of extracts. It may optimise yield of biomass production, and ensure uniform and quality biological activity and limit misidentification (Guo et al., 2009). If a chemotype with a higher concentration is stable, plant breeders' rights may be obtained. Furthermore, delivery of a plant product with a high or stable biological activity will have a strong competitive advantage if handled properly. For the findings to have a lasting impact in the healthcare sector of developing countries, the following recommendations can be made:

- Disseminate and share findings with all stakeholders including scientists, poor communities, traditional practitioners, relevant government departments and the private sector in order to remove misconceptions regarding the influence of environmental conditions on biological activities of extracts;
- Discuss advantages and disadvantages of cultivation with stakeholders in order to provide options;
- Integrate cultivation with cultural beliefs, rituals and indigenous knowledge related to use of herbal medicines to ensure buy-in and full support for cultivation by local communities and traditional practitioners; and
- Repeat the study with the same and or other plants to confirm these results and expand the study areas to analysing the different antimicrobial compounds present in extracts of various plant species and related enzymes that trigger the production thereof.