Cape Peninsula University of Technology

THE ANTIMICROBIAL AND ASSOCIATED ANTIOXIDANT ACTIVITY OF ROOIBOS (*ASPALATHUS LINEARIS)* AND HONEYBUSH (*CYCLOPIA INTERMEDIA)* HERBAL TEAS

By

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PREFACE

This thesis is submitted in fulfillment of the requirements for the degree Master of Technology in the discipline of Biomedical Technology. In chapter 1 a description of the research problem, brief background of the research project and the projected aims and objectives is provided. Chapter 2 provides a literature review that discusses basic concepts related to the project and supplies a rationale for the performance of the project. Two articles which will be submitted for publication are chapters 3 and 4. The two chapters highlight the main aims of this project. They both have separate abstracts, introductions, methods and materials, results, discussions and conclusions. It should be noted that these two chapters contain similar methods and materials. Chapter 5 is the general discussion summarising the intergrated results of the entire thesis. Each chapter has separate numbering systems and references, according to the relevant journal requirements as the thesis is written in an article-based format.

DECLARATION

I, Phumuzile Dube, declare that the contents of this dissertation/thesis represent my own unaided work, and that the dissertation/thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

Signed **Date**

ABSTRACT

The increase in antibiotic resistant bacterial and fungal infections and the prevalence of oxidative stress-related conditions including cancers, cardiovascular diseases and diabetes has led to a consensus among pharmaceutical companies, clinicians and researchers that novel antimicrobial and antioxidant approaches are needed. These should be ideally efficacious, non toxic, easily accessible and affordable. There has been an increased interest in the identification of medicinal plants that possess both these bioactivities in an intrinsically related manner, allowing the simultaneous prevention of these ailments. Two South African herbal teas, rooibos and honeybush have been associated with a long history of medicinal use, hence their consideration for the current study. Numerous studies have been performed to evaluate the antioxidant activities of these South African herbal teas, however limited information about their antimicrobial activity currently exists.

The antimicrobial activity against pathogenic bacteria *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus pyogenes* and fungus *Candida albicans,* total antioxidant capacity, phenolic and flavonoid constituents of water, methanol, chloroform and ethyl acetate extracts of green/unfermented and fermented rooibos and honeybush were investigated. Among the 16 extracts, 12 herbal extracts exhibited antimicrobial activity. The methanol and water extracts were active against all the Gram-positive bacteria (*S. aureus* and *S. pyogenes*) and the fungus *C. albicans*. The chloroform extracts on the other hand displayed selective activity, inhibiting the growth of only *S. pyogenes* whilst ethyl acetate extracts were inactive against all the nosocomial pathogens under study. The Gram-negative *P. aeruginosa* displayed resistance to all the extracts. Regardless of the antimicrobial activities observed, the MIC values (>1 mg/ml) observed for each crude extract against the various microorganisms resulted in them not being labelled as noteworthy antimicrobial agents. Thin layer chromatography-bioautography further allowed the identification of the antimicrobial active fractions present in the most potent of the extracts against specific microbial agents. The further identification of these active fractions is a recommendation for future studies. Generally the rooibos methanol extracts were most potent against *S. aureus* and *C. albicans,* whilst the chloroform extracts were most active against *S. pyogenes*. The total antioxidant capacity of the herbal tea extracts were analyzed using three assays namely the oxygen radical absorbance capacity (ORAC), ferric reducing ability of plasma (FRAP) and trolox equivalence antioxidant capacity (TEAC). For both rooibos and honeybush extracts the assays revealed that the green extracts displayed the highest antioxidant capacity coupled with a high flavonoid and total polyphenol content. The antimicrobial activity observed

coupled with the generally strong antioxidant activity of the extracts, resulted in the confirmation of a direct relation of these two bioactivities in both rooibos and honeybush.

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DEDICATION

To the Lord my God, to you be all the Glory!!!

And

To my family, God bless you all!!!!!

LIST OF OPERATIONAL TERMS AND CONCEPTS

Antimicrobial	a molecule or substance that has the ability to either kill or inhibit the growth of microorganisms
Antioxidant	a substance thought to protect body cells from the damaging effects of oxidation
Aspalathus linearis	South African shrub (commonly known as rooibos) used to make a herbal tea
Candida albicans	a dimorphic fungal organism normally present in the mucous membranes of the mouth, intestinal tract, and vagina of healthy people; capable of causing infection in immunocompromised individuals
Candidiasis	a yeast infection of the mucous membranes caused by *Candida albicans*
Catechins	flavonoid phytochemical compounds found principally in green tea and are considered potent antioxidants
Commensal	organisms that cause no harm to the host
Cyclopia intermedia	botanical name for the South African shrub honeybush that has flowers that smell of honey and a distinctive sweet taste, therefore used as a herbal tea
Immunocompromised	individual who has an impaired or weakened immune system
Infectious disease	an illness caused by a specific infectious agent or its toxic product that result from transmission from an infected source
Nosocomial	infection or disease acquired whilst in a hospital or healthcare setting
Oxidative stress	disturbance in the balance between the production of reactive oxygen species (free radicals) and the antioxidant defences
Polyphenols	a poly-hydroxy phenol possessing antioxidant ability, which tends to prevent or neutralize the damaging effects of free radicals
Pseudomonas aeruginosa	a Gram-negative motile bacteria that can cause a wide range of infections in immunocompromised individuals
Staphylococcus aureus	a Gram-positive bacterial species normally found on nasal mucous membranes that produces exotoxins capable of causing a range of infections

Streptococcus pyogenes	a Gram-positive bacterium that is the cause of group A streptococcal infections

LIST OF ABBREVIATIONS

A	
AIDS	Acquired Immunodeficiency Syndrome
AMPK	AMP- activated protein kinase phosphorylation
ANOVA	Analysis of Varience
APPH	2,2‘-Azobis(2–amidinopropane)dihydrochloride
ATCC	American Type Culture Collection
B	
BA	Blood agar
C	
°C	Degrees Celsius
CA-MRSA	Community Acquired- Methicillin Resistant *Staphylococcus aureus*
CBA	Cooked blood agar
CF	Cystic fibrosis
CFU	Colony-Forming Unit
CNS	Central Nervous System
D	
DNA	Deoxyribose Nucleic Acid
DMSO	Dimethylsufoxides
F	
FL	Fluorescence
FRAP	Ferric Reducing Ability of Plasma Assay
$FeCl_3$	Iron trichloride
G	
GAE	Gallic Acid Equivalents
GAS	Group A Streptococci
H	
HIV	Human Immunodeficiency Virus
HPLC	High Performance Liquid Chromatography
HA-MRSA	Hospital acquired- Methicillin Resistant *Staphylococcus aureus*
I	
ICU	Intensive Care Unit
M	
MIC	Minimum Inhibitory Concentration

MRSA	Methicillin Resistant *Staphylococcus aureus*
MBN	Methylbenzylnitrosamine
N	
NMR	Nuclear Magnetic Resonance
NBT	Nitrotetrazolium Blue Chloride
NAD(P)H	Nicotine adenine dinucleotide (phosphate)
O	
ORAC	Oxygen Radical Absorbance Capacity
$OH^{\cdot}$	Hydroxyl radical
P	
PBP	Penicillin- binding proteins
R	
R_f	Retardation Factor
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
RR	Response Regulators
S	
SDA	Saubauraud Dextrose Agar
SD	Standard Deviation
SEM	Standard Error of the Mean
SOD	Superoxide Dismutase
STSS	Streptococcal Toxic Shock Syndrome
STZ	Streptozotocin
T	
TEAC	Trolox Equivalent Antioxidant Capacity Assay
TLC	Thin Layer Chromatography
TP	Total Polyphenol
TPC	Total Polyphenol Content
U	
UV	Ultraviolet
V	
VAP	Ventillator- associated Pneumonia

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CHAPTER ONE

INTRODUCTION

1.1 Statement of the research problem

i. The rise of antimicrobial resistance in infectious human pathogens poses a growing challenge to medicine and public health. Due to the high prevalence of Human Immunodeficiency Virus (HIV) and the Acquired Immunodeficiency Syndrome (AIDS) in Africa, and South Africa in particular, the immunocompromised population (who are more susceptible to bacterial and fungal infections) is increasing daily, contributing to the high morbidity and mortality caused by infectious diseases (Pizzo, 1999; Coetzee *et al*., 2013; Mudau *et al.*, 2013).

ii. The adverse effects of oxidative stress on human health are a serious issue. Under oxidative stress, our bodies produce more reactive oxygen/nitrogen species leading to a redox imbalance that may lead to damage of important cellular components, i.e. genetic material, lipids and proteins; and is consequently the start of many health issues (Bancirova, 2010). A disturbed redox status in the body facilitates the development of degenerative diseases such as certain cancers, cardiovascular diseases, neurodegenerative diseases and various inflammatory diseases; and also contributes to the weakening of patients' immune system, increasing their risk of developing infectious diseases (De la Fuente and Victor, 2000; Sena and Chandel, 2012).

1.2 Background of research problem

In recent times, the prevalence of infectious diseases caused by antimicrobial resistant bacteria (e.g. *Pseudomonas* aeruginosa, *Staphylococcus* aureus, *Streptococcus* pyogenes) and fungi (e.g. *Candida albicans*) has increased (Ncube *et al*., 2008). These microorganisms have developed numerous defense mechanisms against antimicrobial agents. The resistance stems from beneficial structural components, multidrug resistance pumps and genetic mutations. Exasperating this problem is the increase in the immunocompromised population, particularly in Africa, which is greatly susceptible to infectious diseases. The immune system may be compromised by different disease states and conditions e.g. HIV/AIDS and surgical procedures including bone marrow transplants and splenectomy. The correlation between infectious diseases and an increased immunocompromised populace results in elevated morbidity and mortality worldwide.

Oxidative stress can be described as a shift in the balance between generation of reactive oxygen species (ROS) and the activity of the antioxidant defences (Aruoma, 1998). Most oxidants are derived directly or indirectly from oxygen (O_2), existing as free radicals or non-radicals and can also include reactive nitrogen species (RNS) (Bancirova, 2010). The generation of oxidative compounds in the body is physiologically relevant as an important step in inflammation and tissue processes (Locatelli *et al.*, 2003). However, if there is an excessive generation of these compounds and a deficient antioxidant defence system, damage to important cellular components (e.g. genetic material, proteins and lipids) occurs, which may result in the start of many health complications (Sies, 1997). Oxidative stress plays a major role in the development of chronic and degenerative illnesses such as atherosclerosis, coronary heart diseases, neurodegenerative diseases, aging and cancer (Finkel, 2011; Madhavi *et al.*, 1996). This suggests that an overwhelmed antioxidant defence system initiates and propagates processes involved in the pathogenesis of many diseases (Block *et al*., 2002). The adverse effects of oxidative stress on human health are a great concern. Under oxidative stress, the body produces more ROS than the enzymatic and non-enzymatic antioxidants can counteract (Pham-Huy *et al.*, 2008). Severe oxidative stress can cause cell damage and eventual cell death (Aruoma, 1998). The immunity cells can also be affected, resulting in an immunocompromised state due to the oxidative damage (De la Fuente and Victor, 2000). Sources of oxidative stress include exogenous factors, e.g. exposure to environmental pollutants, cigarette smoke, excessive ultraviolet light, bacterial, fungal and viral pathogens, as well as endogenous factors such as oxidative bursts from activated macrophages, energy production or detoxification reactions in cells (Block *et al.,* 2002).

As traditional antibiotics become ineffective against microbials including *Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus pyogenes* and *Candida albicans*, the use of plant-derived antimicrobials is increasingly being accepted by orthodox medicine. Recent research also emphasizes on supplementing the diet with antioxidant compounds contained in natural plant sources as an approach to solving oxidative stress-linked problems. Plant extracts, widely associated with numerous medicinal benefits on humans, could help limit these problems and be of great significance in therapeutic treatments. In fact, these natural plants have an important function in preventive medicine approaches (Jaberian *et al.,* 2013). *Aspalathus linearis* (Rooibos) and *Cyclopia intermedia* (Honeybush) are two fynbos plants endemic to the South African plant kingdom. Studies have reported them to possess a wide variety of bioactive potential including anti-diabetic, antimicrobial, antioxidant, anti-cancer and anti-obesity activity

(Son *et al.,* 2013, Coetzee *et al.,* 2008). However these studies did not investigate the antimicrobial and antioxidant content of different solvent extracts prepared from *Aspalathus linearis* (Rooibos) and *Cyclopia intermedia* (Honeybush) herbal teas and they did not evaluate the relation of these two bioactivities. The analysis of these parameters may provide further evidence to support the antimicrobial and antioxidant activity of both Rooibos and Honeybush herbal teas.

1.3 Research aim and objectives

The overall aims of this study were to:

1. Evaluate the antibacterial effects of different solvent extracts prepared from *Aspalathus linearis* (Rooibos) and *Cyclopia intermedia* (Honeybush) on *Pseudomonas aeruginosa, Staphylococcus aureus* and *Streptococcus pyogenes.*
2. Evaluate the antifungal effects of different solvent extracts prepared from *Aspalathus linearis* (Rooibos) and *Cyclopia intermedia* (Honeybush) on *Candida albicans.*
3. Evaluate the antioxidant contents and capacity of different solvent extracts prepared from *Aspalathus linearis* (Rooibos) and *Cyclopia intermedia* (Honeybush).
4. Evaluate the possible relation of the antimicrobial and associated antioxidant activity of the *Aspalathus linearis* (Rooibos) and *Cyclopia intermedia* (Honeybush) extracts.

The main objectives of this study were to:

1. Evaluate the antibacterial activity of different green and fermented *Aspalathus linearis* (Rooibos) and *Cyclopia intermedia* (Honeybush) solvent extracts against medically important Gram-negative and Gram-positive bacteria.
2. Evaluate the antifungal activity of different green and fermented *Aspalathus linearis* (Rooibos) and *Cyclopia intermedia* (Honeybush) solvent extracts against the opportunistic *Candida albicans* fungus.
3. Evaluate the antioxidant activity of different green and fermented *Aspalathus linearis* (Rooibos) and *Cyclopia intermedia* (Honeybush) solvent extracts.
4. Determine the total polyphenol content of different solvent extracts of the green and fermented forms of *Aspalathus linearis* (Rooibos) and *Cyclopia intermedia* (Honeybush).
5. Determine the main polyphenolic components in different solvent extracts of the green and fermented forms of *Aspalathus linearis* (Rooibos) and *Cyclopia intermedia* (Honeybush).

6. Determine the relation existing between the antimicrobial and antioxidant activity of the green and fermented rooibos and honeybush extracts.

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CHAPTER TWO

LITERATURE REVIEW

2.1 Infectious diseases

The epidemiology of invasive infectious diseases has increased in recent times. This escalation has been associated with the urbanization of the world leading to elevated migration rates and exponential increase in the world's human population whose lifestyle has greatly evolved (Zarrin and Jundishapur, 2009; Alirol *et al*., 2011). Infectious diseases are illnesses caused by specific micro-organisms e.g. bacteria, fungi, virus or parasites, developing as a result of a weakened immune system.They have the ability not only to exert noteworthy impact on the human species but also to profoundly affect food safety and security (Jones *et al*., 2008; Fauci and Morens, 2012; Schmidhuber and Tubiello, 2007). Approximately 15 million (25.5%) of an estimated 58.8 million annual deaths worldwide, are assumed to be caused by infectious diseases (Fauci and Morens, 2012; WHO, 2011).

Infectious diseases have played a crucial role in history, from influencing the course of wars to the determination of civilization progress. The most important infectious diseases reported emerged within the past 11 000 years (Wolfe *et al*., 2007). With the current advanced biological knowledge pool and long history of infectious diseases, their complete eradication is an expected assumption. However this is not the case; instead new infections continue to emerge whilst those of the past still exist. This is mainly explained by certain microorganisms' ability to develop mechanisms that allow them to evade the antimicrobial effects of specific conventional treatments. This antimicrobial resistance phenomenon is identified as one of the immense threats to the health of mankind worldwide (IDSA, 2011; Laxminarayan *et al*., 2013). Exacerbating this problem is the increase in the number of immunocompromised individuals at high risk of bacterial and fungal infections (Mudau *et al.*, 2013). Individuals may be immunocompromised because of cancer or its treatment, infection with the Human Immunodeficiency Virus (HIV) or Acquired Immunodeficiency Syndrome (AIDS), surgical procedures such as splenectomy, bone marrow or solid organ transplant (Pizzo, 1999). A breach of skin or mucosal barriers of the body may also result in immunosuppression. In these individuals, an increased risk of infection exists (Pizzo, 1999). The major concern in South Africa is that about 5.6 million individuals are infected with HIV (UNAIDS, 2011; Masevhe *et al*., 2015).

The resurgence of highly infectious microbes resistant to conventional treatment is an important cause of morbidity, mortality and economical strains in health care systems globally, particularly in the low income countries (Coetzee *et al*., 2013; Mudau *et al.*, 2013; Moodley *et al*., 2010). The rise of antibiotic resistant nosocomial infections in both public and private hospitals across South Africa is one of the greatest threats to patient safety (Mendelson, 2014). Bereket *et al* (2012), defined nosocomial infections as any infection a patient acquires during their admittance in any clinical or hospital setup. These infections are absent in patients during their initial stay at the clinical facility, and become discernible during their hospitalization period or after being discharged. Control of nosocomial pathogens, which are the causative agents of these infections, are currently a topic of great concern, with numerous research studies focused on their management (Masevhe *et al.*, 2015; Chastre *et al*., 2014). Many nosocomial pathogens including bacteria (e.g. *Pseudomonas aeruginosa*, *Staphylococcus aureus,* and *Streptococcus pyogenes*) and fungi (e.g. *Candida albicans*) are currently posing a great challenge to the medical sector, due to their widespread antibiotic resistance (Bereket *et al*., 2012). These infectious diseases represent a significant cause of morbidity and mortality in developing countries, South Africa included, necessitating the urgent need for alternative antimicrobial agents that are easily accessible, safe and efficacious requiring limited financial resources (Geffers and Gastmeier, 2011; Masevhe *et al*., 2015).

2.1.1 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa (Schroeter, 1872) is an aerobic Gram-negative bacillus (rod shaped) of the *Pseudomonadaceae* family (Figure 2.1). A pure culture of *Pseudomonas aeruginosa* (*P. aeruginosa*) was first isolated from skin wounds in 1882 by Gessard (Hugh and Leifson, 1964; Vasil, 1986). It is mainly associated with soil and water, posing a great challenge as it is one of the leading nosocomial pathogens worldwide (Van Delden and Iglewski, 1998; Wilson and Dowling, 1998; Obritsch *et al*., 2005; Strateva *et al.*, 2007; Falkinham *et al*., 2015). It has a single flagellum for motility and efficient attachment to surfaces (O'Toole and Kolter, 1998). Unlike other Gram-negative bacilli, *P. aeruginosa* can survive in harsh environmental conditions including high temperatures (up to 43 ^{0}C) and has the ability to utilize over 50 biological chemicals for growth (permitting it to grow on a variety of agar plates as illustrated by Table 2.1), allowing it to grow in disinfectant and distilled water. Its main survival mechanism is the biofilm, characterised by clustering of microcolonies of the *P. aeruginosa* surrounded by a biopolymer matrix attached to a surface, which protects it from unfavourable environmental conditions, host antibodies and phagocytes. *P. aeruginosa* produces many virulence factors that include enzymes (elastase, haemolysin-phospholipase C, proteases), exotoxins, cytotoxins,

exoenzymes (ExoS, ExoT, ExoU, ExoY) and a blue phenozine pigment (pyocyanin) which is a distinguishing factor from other pseudomonads giving it a blue colour pus characteristic (Driscoll *et al*., 2007; Ma *et al*., 2009; Fujitani *et al*., 2011).

Out of 18 studies conducted to investigate the sources of *P. aeruginosa* outbreaks in hospitals, 14 concluded that sinks, tap water and faucets could be the likely source. Eight of the studies indicated the source as transmission between healthcare workers and/or patients, whilst 2 studies identified the source as the internal flora within the individual patients (Fujitani *et al*., 2011).

Table 2.1: Growth of *P. aeruginosa* on various media

Media	Growth results	Pigmentation
Luria-Bertani (LB)	+	No pigmentation
Nutrient agar	+	Bluish green
Muller Hinton agar	+	Bluish green
Pseudomonas medium F	+	Greenish
Pseudomonas medium P	+	Fluorescent bluish green p
Modified F medium	+	Fluorescent bluish green
Pseudomonas isolation agar	+	Fluorescent bluish green
Acetamide agar	+	Bluish green green
Succinate medium with $FeCl_3$	+	Bluish green
Succinate medium without $FeCl_3$	+	Bluish green
Hi Fluoro Pseudomonas agar	+	Fluorescent greenish

Adapted from Ningthoujam and Shovarani, 2008

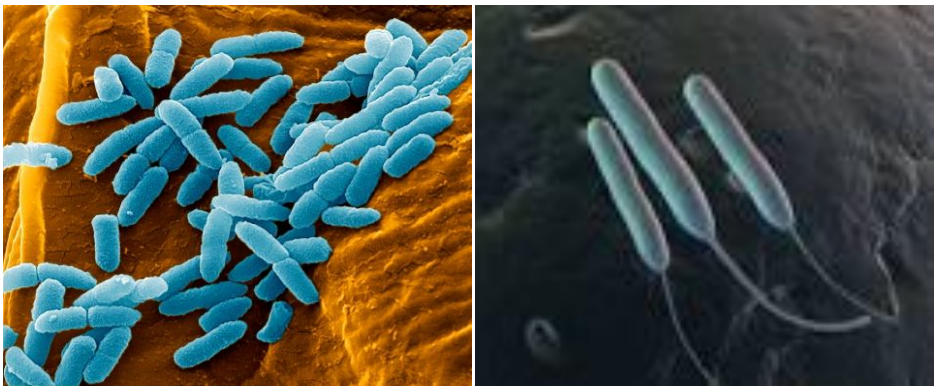

Figure 2.1: Scanning electron micrograph of the bacillus *P. aeruginosa*
(Adapted from https://lookfordiagnosis.com)

2.1.1.1 Bacterial Pathogenesis

P. aeruginosa is a significant cause of both community-acquired (e.g. ulcerative keratitis, otitis external, skin and soft tissue infections) and hospital-acquired (e.g. pneumonia, urinary tract infections, severe blood-stream infections, surgical site infections and skin infections in the setting of burn injuries) infections (Driscoll *et al*., 2007). It is more commonly hospital acquired than community acquired. *P. aeruginosa* infections show high prevalence in the following patient groups:

- those whose immune systems are compromised, e.g. neutropenic, HIV infected and bone marrow transplant patients (Coetzee *et al.*, 2013; Van Delden and Iglewski, 1998)
- those with prolonged hospitalization (Obritsch *et al*., 2005; Driscoll *et al*., 2007)
- those diagnosed with cystic fibrosis (Driscoll *et al*., 2007)
- those exposed to antimicrobial therapies for prolonged durations (Obritsch *et al*., 2005)

These infections may sometimes be life-threatening (Van Delden and Iglewski, 1998*)*. However, in the absence of an impaired immune system, *P. aeruginosa* is incapable of causing any form of disease in humans, thereby confirming its opportunistic nature.

A recent prospective, observational study conducted in 11 countries from four regions namely United States, Europe, Latin America and Asia Pacific, showed that the prevalence of ventilator-associated pneumonia (VAP) caused by *P. aeruginosa* is 15.6% globally (Kollef *et al*., 2014). *P. aeruginosa* VAP is responsible for crude and attributable mortality rates of up to 87% and 42.8%, respectively (Fujitani *et al*., 2011). A retrospective case-control study conducted in a haematology ward of a tertiary academic hospital in South Africa reported 80% fatality rate in neutropenic patients infected with *P. aeruginosa* (Mudau *et al*., 2013).

2.1.1.2 Treatment

P. aeruginosa has been traditionally treated using the broad spectrum drug ciprofloxacin (Hodson *et al*., 1987; Taccetti *et al*., 2012). However prolonged use of this drug has been associated with the development of different conditions in patients; tenditis, drug fever (hypersensitive reaction to the drug) and tendon rupturing (Cunha, 2001). *P. aeruginosa* is capable of developing resistance to antimicrobial agents it was formally noted to be sensitive to because of prolonged exposure e.g. third and fourth generation cephalosporins and aminoglycosides (gentamicin and amikacin). Early detection of *P. aeruginosa* infections can improve the prognoses but cannot necessarily guarantee the success of treatment. Hence the use of less common and less detrimental antimicrobial agents, like medicinal plants, is fast becoming a more preferred approach in the treatment of antimicrobial resistant *P. aeruginosa* infections (Driskoll *et al*., 2007)

2.1.1.3 Resistance mechanisms

It has been reported that nosocomial infections due to *P.aeruginosa* occur with increased frequency in patients with advanced HIV infections due to their immunocompromised state (Ali *et al.*, 1995). These infections are often life threatening and difficult to treat due to the microorganisms remarkable ability of acquiring further mechanisms of resistance to multiple groups of antimicrobial agents coupled with its natural resistance (Strateva and Yordanov, 2009; Obritsch *et al*., 2005).

P. aeruginosa is intrinsically resistant to many structurally unrelated antimicrobial agents (Mesaros *et al.,* 2007) because of the:

- low permeability of its outer membrane (Livermore, 1984),
- constitutive expression of various efflux pumps with wide substrate specificity (Livermore, 2001)
- naturally occurring chromosomal class C Beta-lactamase (AmpC*β*-lactamase), enzymes capable of degrading *β*-lactams antimicrobial agents (Nordmann and Guibert, 1998)
- alteration of target structures and ability to inhibit entry of antimicrobial agents (Schweizer, 2003).

The innate resistance of the species relates to many *β*-lactam antibiotics, e.g.ceftazidime, and penicillin-G, as well as first and second generation cephalosporins; which poses serious therapeutic problems, increasing the demand for alternative treatment approaches.

2.1.2 *Staphylococcus aureus*

In comparison to *P. aeruginosa, Staphylococcus aureus* (*S. aureus*) has a higher pneumonia causing frequency, making it a very dangerous pathogen since pneumonia is the chief cause of death worldwide in children (Rasigade *et al.*, 2014; Reed *et al*., 2012). Isolated in the 1800's, *S. aureus* is still a highly pathogenic bacterium today. It is a Gram-positive opportunistic cocci (round shaped) that is both a commensal organism and a pathogen (Lowy, 1998; Akoda *et al.,* 2012; Gordon and Lowy, 2008, Tong *et al*., 2015) (Figure 2.2). As commensals, up to 30% of the world population carry *S. aureus* on their skin or mucous membranes (Stefani *et al*., 2012). Its pathogenic nature is due to its complex virulence factors; toxic shock syndrome toxin- 1, staphylococcal enterotoxins, DNase, α-haemolysin, β-haemolysin, σ-haemolysin and epidermolytic toxins A and B (Mastunaga *et al*., 1993; Lowy, 1998). These are responsible for the development of diseases in high risk individuals, i.e:

- those at both age extremities (the young and very old) (Laupland *et al*., 2013)
- immunocompromised patients especially HIV infected (Larsen *et al*., 2012; Burkey *et al*., 2008)
- previously infected individuals (Allard *et al*., 2008)

In 2007, the mortality rate of *S. aureus* was 20%, a value that varied with different studies (Klevens *et al*., 2007; DeLeo and Chambers, 2009; Grundmann *et al*., 2010; Stefani *et al*., 2012).

Its basic structure consists of a peptidoglycan cell wall, microcapsule, surface proteins and circular chromosome (Lowy, 1998). Identification of *S. aureus* is a costly process involving a variety of tests, which is an economic strain to the healthcare systems of developing countries. The combination of tube coagulase test, Mannitol salt agar and DNase is a more affordable approach in identifying *S. aureus*, with proven 100% specificity and 75% sensitivity rates (Kateete *et al*., 2010). These three tests together with the gold pigmentation of the *S. aureus* colonies on agar plates differentiate it from the other staphylococcal species (Lowy, 1998).

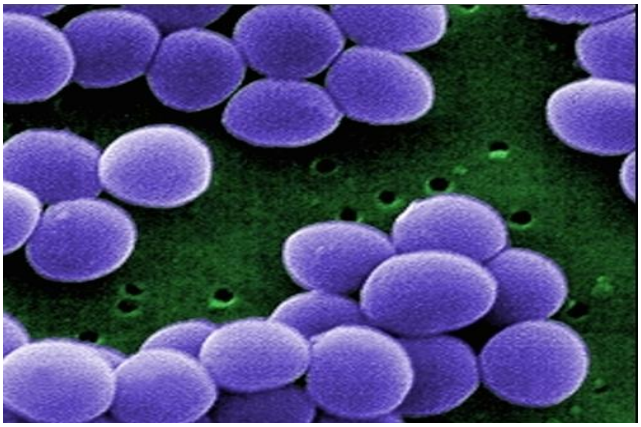

Figure 2.2: Scanning electron micrograph for *S. aureus*
(Adapted from http://www.bacteriainphotos.com)

2.1.2.1 Bacterial Pathogenesis

S. aureus produces a multiplex of toxins and has the ability to display antimicrobial traits (Stefani *et al*., 2012). Isolated from humans, it is an important causative agent of a wide range of infections, from localised skin and soft tissue eruptions to life-threatening conditions such as bacteraemia, endocarditis, osteomyelitis, bone, joint and central nervous system infections and sepsis (Lowy, 1998; Bamberger and Boyd, 2005; David and Daum, 2010; Tong *et al*., 2015). In paediatric patients, it is the main pathogen in bloodstream infections and is considered to be the main pathogen causing nosocomial infections (Tohidpour *et al.,* 2010). In a period of 5 years (2007-2011), 72% of all nosocomial infections and 26% of bacteraemia cases in a children's hospital in Cape Town (South Africa) were caused by *S. aureus*, whilst the fatality rate of *S. aureus* caused bacteraemia recorded was 8.8% (Naidoo *et al*., 2013). Respiratory infections are a major cause of mortality in developing countries (Madikizela *et al*., 2013). *S. aureus* is the most significant bacterium that leads to the development of noteworthy respiratory infections. It also contributes to the severity of the condition cystic fibrosis (CF). In this disorder, the *S. aureus* damages the epithelium of the bronchi and bronchiole of young CF patients, paving way for infections by other microorganisms' especially *P. aeruginosa* (Sadowska *et al*., 2002).

2.1.2.2 Treatment

All *S. aureus* infected patients require prolonged antibiotic therapy, to prevent relapse of infection (Tong *et al*., 2015). Different studies have reported on *S. aureus* strains that are sensitive and resistant to specific antibiotics. A study carried out in the Mafikeng area, South Africa identified *S. aureus* isolates from cows milk that were resistant to ampicillin, penicillin,

methicillin, oxytetracycline, erythromycin, streptomycin and sulphamethoxazole, but sensitive to vancomycin (Ateba *et al*., 2010). Vancomycin has been the ideal antibiotic in the treatment of *S. aureus* infections for more than 40years (Van Hal and Fowler, 2013). However Pantosti and Venditi (2009) noted strains that have decreased vancomycin sensitivity, meaning an expected emergence of vancomycin resistant *S. aureus* strains in the future. When high doses of vancomycin were given to patients with methicillin resistant *S. aureus* (MRSA) infections, it was reported the high doses displayed antimicrobial activity, but the dosages implemented nephrotoxicity in patients (Hidayat *et al*., 2006). New antibiotics are available, which have shown significant antimicrobial potential against *S. aureus*. These include tigecycline, daptomycin and linezolid but due to their high cost, their routine prescription is not established (Bamberger and Boyd, 2005). These factors increase the global demand for more efficacious, affordable and less detrimental antimicrobial agents against the multi-drug resistant *S. aureus*.

2.1.2.3 Resistance mechanisms

The microcapsule surrounding *S. aureus* is made out of microcapsular polysaccharide serotype (type 5), which is an antiphagocytic polysaccharide. This feature protects the bacterium from the host's phagocytic component of the immune system (Lowy, 1998).

The emergence of virulent antibiotic-resistant strains of *S. aureus* with infection outbreaks among hospitalized patients is a weighty predicament worldwide (Mastoraki *et al.,* 2008; Geha *et al.,* 1994). These include methicillin, fluoroquinolone, macrolide, tetracycline and aminoglycoside resistant *S. aureus,* with methicillin resistant *S. aureus* being the most common (Pantosti and Venditi, 2009; Duran *et al*., 2012). These outbreaks are a result of the variety of mechanisms possessed by *S. aureus* enabling it to evade antibiotic pathways a (Gordon and Lowy*,* 2008). These mechanisms include the:

- enzymatic inactivation of the antibiotic e.g. penicillanase targeting penicillin (Pantosti and Venditi, 2009)
- alteration of the target to decrease affinity for the antibiotic e.g. PBP replaced by PBP2a in methicillin- resistant *S. aureus* (MRSA) (Duran *et al*., 2012)
- trapping of the antibiotic and spontaneous gene mutations rendering them insensitive to the antibiotic present (Pantosti *et al.,* 2007).

Methicillin is a modified penicillin, developed in pursuit to counteract *S. aureus* resistance to penicillin. Methicillin's mode of action involves the blocking of penicillin-binding proteins (PBP), which function to maintain the bacterial cell wall of *S. aureus*. Resistance of *S. aureus* to methicillin is through the expression of the *mec A* gene encoding for a modified penicillin-binding protein (PBP2a), a replacement of PBP, resulting in a lowered affinity of the bacterium to β-lactams. Production of modified PBP2a makes *S. aureus* resistant to all β-lactams: penicillins, cephalosporins and carbapenems (Pinho *et al*., 2001; Pantosti and Venditi, 2009; Monecke *et al*., 2011; Stefani *et al*., 2012).

In 2011, the prevalence of MRSA in South Africa was reported at 24% (Falagas *et al*., 2013). Epidemiological studies that allow for the full assessment of the pandemic in Africa are still lagging and poorly documented, but an assumption of intermediate to high prevalence rates have been reached. The highest rates of >50% were reported in Asia, Malta, North and South America (Stefani *et al*., 2012). In earlier years, MRSA had been considered a hospital-acquired pathogen (HA-MRSA), targeting patients that have established risk factors. However recently it has been isolated in the community and also from livestock. The community acquired MRSA (CA-MRSA) is being predicted by mathematical models to displace HA-MRSA as the main nosocomial pathogens in a couple of years to come. CA-MRSA has the potential to colonize and cause infections in healthy individuals, increasing the severity of this occurrence (Mediavilla *et al*., 2012; Otter and French, 2011; D'Agata *et al*., 2009; Skov and Jensen, 2009).

2.1.3 *Streptococcus pyogenes*

Streptococcus pyogenes (*S. pyogenes*), also known as group A streptococci (GAS), is an aerobic Gram-positive round shaped bacterium (Johansson *et al.*, 2010) responsible for a wider range of infections from acute to life-threatening (Lamagni *et al.,* 2009) (Figure 2.2). It is a human pathogen involved in respiratory tract infections (pharyngitis, pneumonia), skin and soft-tissue infections, and toxic shock-like syndromes (Gracia *et al.,* 2009). It contributes significantly to the mortality and morbidity in developing countries, with a fatality rate in young children of 15%. GAS adapts in the host's harsh environment. This is accomplished by the complex virulence factors expressed by the microorganism referred to as response regulators (RRs). These RRs are the link between the surrounding environment and the adaptive responses of the bacterium. So they serve as a means of communication, allowing the bacterial cell to protect itself against the host eradication actions (Kreikemeyer *et al*., 2003). About 1 752 protein coding genes have been identified in GAS, with approximately two thirds responsible for known streptococcal function including virulence traits (Ferretti *et al*., 2001).

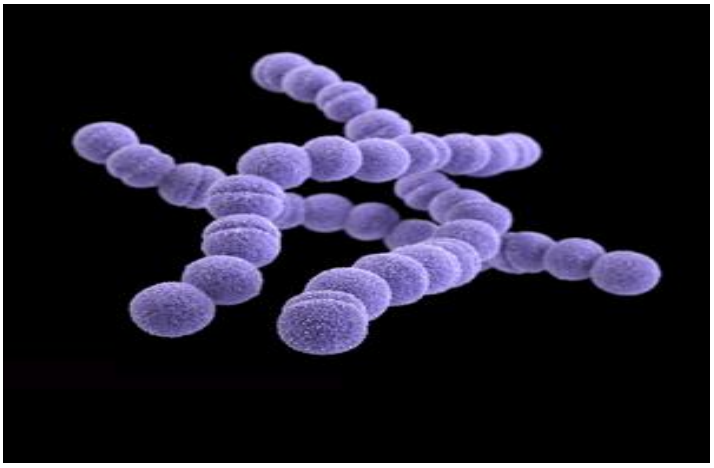

Figure 2.3: **Scanning electron micrograph for *S. pyogenes***
(Adapted from http://www.cdc.gov/media/subtopic/library/diseases.htm)

2.1.3.1 Bacterial pathogenesis

A global renaissance of severe invasive infections due to GAS has recently been reported (Johansson *et al.,* 2010). The two most severe invasive manifestations are streptococcal toxic shock syndrome (associated with multi organ failure) and necrotizing fasciitis (flesh eating disease), both of which are associated with high morbidity and mortality (Johansson *et al.,* 2010). The other common infections due to GAS range in severity from mild upper respiratory tract infections (including streptococcal sore throat), skin and soft tissue infections to severe invasive infections, such as bacteraemia, pneumonia, scarlet fever and post infection rheumatic fever (Efstratiou *et al.,* 2002; Johansson *et al.*, 2010).

2.1.3.2 Treatment

GAS is sensitive to penicillin and other *β*-lactam antimicrobials, such as cephalosporin (Stevens, 2000). However, penicillin has decreased activity against severe invasive *S. pyogenes* infections (Sawai *et al.,* 2007). Treatment of GAS related infections is difficult because different strains exist with varying antibiotic susceptibility and resistance traits. It is of utmost importance that the isolated strain from the patient be tested for susceptibility and/ or resistance to specific antibiotics before their administration (Shulman *et al*., 2012). When 40 strains of GAS were isolated and tested for susceptibility to 17 antibiotics, it was reported that all strains were sensitive to amoxicillin, cefixime, cefotaxime, ceftriaxone, vancomycin, levofloxacin, teicoplanin and cefpodoxime whilst they were resistant to tetracycline. This study also proved the efficiency of the antimicrobial activity of penicillin antibiotic against GAS (Camara *et al*., 2013).

2.1.3.3 Resistance Mechanisms

Approximately a third of the identified 1 752 protein encoding genes have no known function. More than 40 of the genes with known function are responsible for the virulence nature of GAS (Ferretti *et al*., 2001). Expression of some virulence associated genes has resulted in many GAS strains showing resistance to alternative macrolide antibiotics (Richter *et al.,* 2008) and virulent phenotypic switch in response to environmental changes (Kreikemeyer *et al*., 2003). Camara *et al* (2013) reported that 40 GAS strains displayed resistance to tetracycline. The increase in the prevalence of GAS resistant to macrolide antibiotics is posing a growing clinical problem worldwide. Increased administration of the antibiotics is a contributory factor to the elevated resistance (Albrich *et al*., 2004). Target site modification and drug efflux mechanisms within the bacterium also result in antimicrobial resistance.

2.1.4 *Candida albicans*

Fungal infections are also important contributors to morbidity and mortality, due to their significant global increase. The *Candida* genus consists of 17 known species responsible for a variety of clinical manifestations in their human hosts. Of those, *Candida albicans*, *Candida glabrata*, *Candida tropicalis, Candida krusei* and *Candida parapsilosis* are responsible for more than 90% of all reported candidal invasive infections (Pfaller *et al*., 2007). *Candida albicans* (*C. albicans*), a dimorphic fungus that exists as a commensal in humans, is one of the main causes of fungal infections (Zarrin and Jundishapur, 2009; Horn *et al*., 2009; Sardi *et al*., 2013). A unique feature of *C. albicans* is its ability to grow and survive in at least three morphological states; yeast, pseudohyphae and hyphae (Figure 2.4). Changing morphology of *C. albicans* is assumed to be an important virulent characteristic, though experimental data is lacking (Sudbery *et al.,* 2004). Its ability to colonise virtually every body part, from superficial to deep tissue, with varying environmental pressures increases its disease spectrum in comparison to other commensal microorganisms. The increasing mortality rates associated with *C. albicans* infections is a serious economic and health care problem because of the high health care costs and extended hospitalization for infected patients (Lai *et al*., 2012; Sardi *et al*., 2013).

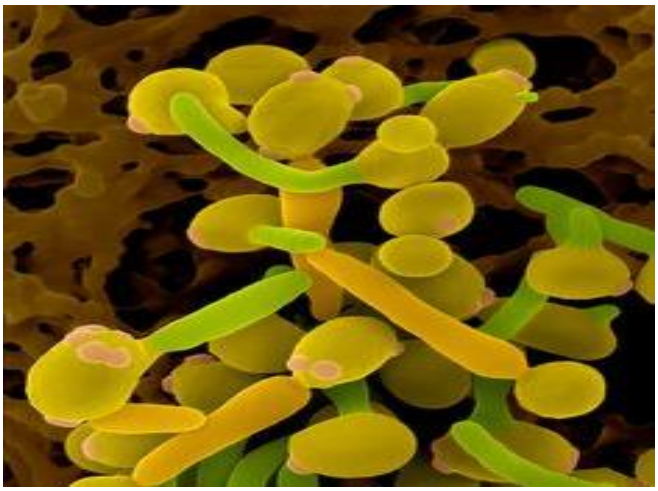

Figure 2.4: **Scanning electron micrograph for *C. albicans***
(Adapted from https://www.pinterest.com/valeriust/virus-bacteria-parasits-bacilli)

2.1.4.1 Candidiasis

Certain virulence factors contribute to the pathogenicity of *C. albicans*:

- ability of the fungi to adhere to the host tissue surfaces (Silva *et al*., 2011)
- evading of the host immune system through morphogenesis (Calderone and Fonzi, 2001)
- biofilm formation on both medical devices and host tissue (Silva *et al*., 2011)
- production of hydrolytic enzymes that damage tissue e.g. proteases, phospholipases, haemolysin (Silva *et al*., 2011; Sardi *et al*., 2013).

Candidiasis is a common yeast infection of the mucous membranes mainly caused by *C. albicans*, often occurring in immune-compromised and diabetic patients. In immunocompromised patients and/or hospitalized patients with severe underlying diseases candididasis is associated with a mortality rate of 10 - 49% (Pfaller and Diekema, 2007; Masevhe *et al*., 2015). The infections range from superficial, e.g. affecting the mucosa of the skin, oral cavity, vagina and gastrointestinal tract, to life-threatening, e.g. deep-seated invasive infections (Pfaller and Diekema, 2010). Increase in the prevalence of conditions that weaken the immune system including HIV/AIDS has consequently led to the epidemiological growth of *C. albicans* infections (Zarrin and Jundishapur, 2009). 90% of HIV/AIDS patients develop oropharyngeal candidiasis during various stages of AIDS (Liu *et al.*, 2011). Preterm, very low weight and extremely low weight infants are highly susceptible to *C. albicans* infections. The organs mainly infected include the lungs, kidneys and those of the central nervous system (CNS). These infants are

placed on antifungal therapy, which may have side effects that are not pleasant to their delicate bodies (Wang *et al*., 2012).

It is also the main pathogen in the oral cavity of HIV infected children, causing oropharyngeal candidiasis (Charone *et al*., 2013). One study showed that a prevalence rate of 79.1% was recorded for HIV infected children with oral candidiasis in Africa (Gaitán-Cepeada *et al*., 2014). In leukemic and solid transplant patients, candidiasis is the main cause of death and is observed more frequently among patients in the intensive care units (ICUs) (Pfaller and Diekema, 2010). Additionally, when burns occur, the skin loses its protective epithelial layer and since *C. albicans* forms part of the skin's natural flora, the wounds may become prone to infection by the fungus (Liu *et al.*, 2011).

2.1.4.2 Treatment

Azole antifungal agents, specifically flucanozole, were considered to be the first choice for treatment in HIV/AIDS patients, however, with the prolonged and frequent exposure to the azoles, drug resistance and therapeutic failure of the *C. albicans* has now become a challenge for clinicians (Liu *et al.*, 2011). However in the case of severe fetal inflammation due to *C. albicans* infection, administration of flucanozole prevents serious fetal injury associated with intra-amniotic infections (Maneenil *et al*., 2015).

2.1.4.3 Resistance mechanisms

Each strain of *C. albicans* can posses more than a single resistant mechanism at any given time. The resistance mechanisms of *C. albicans* continue to complicate patient care management, as they continually evolve (Pfaller, 2012). A study conducted to investigate the scientific explanation for the flucanozole resistance of *C. albicans* showed the presence of energy-requiring efflux pumps (BENr, CDR1 and CRR2) encoded by specific genes isolated in flucanozole-resistant *C. albicans* (Sanglard *et al*., 1995). A closely related study proved that the inactivation of the *ERG3* gene, encoding for sterol $\Delta^{5,6}$-desaturase which is an important enzyme for the synthesis of ergosterol, is a valid mechanism for azole resistance in *C. albicans* (Vale-Silva *et al*., 2012). Another mechanism of resistance is the transformation of antifungal targets through gene mutations, resulting in the alteration of the target, rendering the antifungal non-functional (Pfaller, 2012).

2.2 Oxidative stress

Aerobic respiration is the primary means required for the preservation of aerobic existence. During this process oxygen (O_2) intake mainly results in the mitochondrial production of either energy in the form of adenosine triphosphate (ATP) or oxidants derived directly or indirectly from the oxygen. These oxidants, namely the reactive oxygen species/ reactive nitrogen species (ROS/RNS) (e.g. superoxide, hydrogen peroxide, hydroxyl, nitric oxide, peroxyl, alkoxyl) are highly reactive intermediates derived during the reduction of the inhaled oxygen to water (Figure 2.5) (Sena and Chandel, 2012).

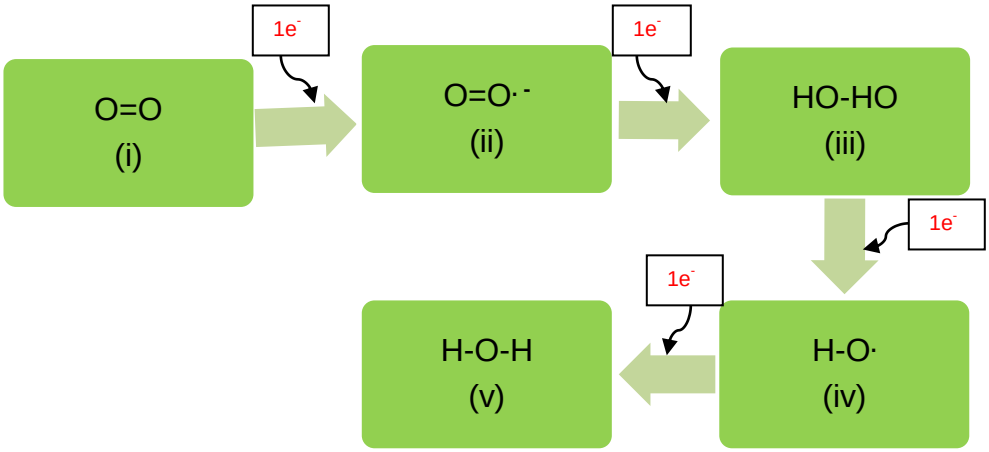

Figure 2.5: Reduction of oxygen (i) to water (v) generating reactive oxygen intermediates superoxide (ii), hydrogen peroxide (iii) and hydroxyl radical (iv)

(Adapted from Mailloux and Harper, 2011).

Oxidative stress can be described as a shift in the balance between generation of ROS/RNS and the activity of the antioxidant defences (Reuter *et al*., 2010). Under oxidative stress, the body produces more ROS/RNS than enzymatic antioxidants (e.g. superoxide dismutase (SOD) and catalase), which are normally naturally produced *in situ* and non-enzymatic antioxidants (e.g. flavonoids, vitamin C, vitamin E), which are normally externally supplied through food and/or supplements (Pham-Huy *et al.*, 2008) (Figure 2.2).

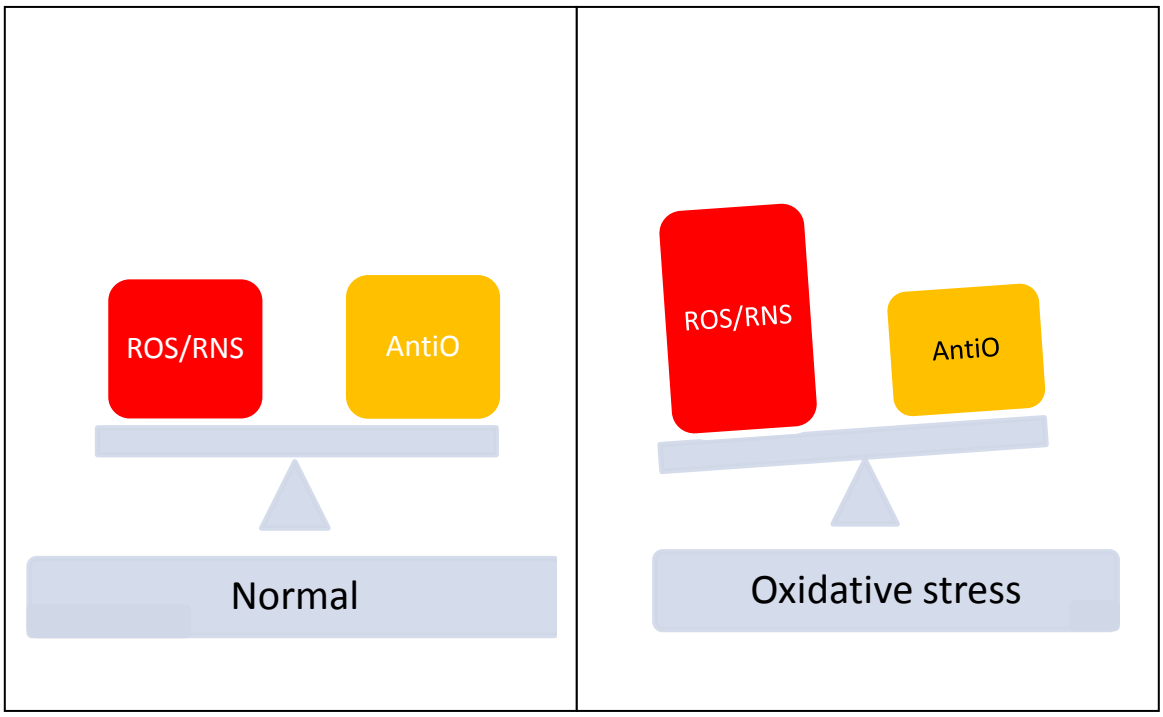

Figure 2.6: Comparison between normal environments and a shift in the balance between ROS/RNS and AntiO. *ROS/RNS (Reactive oxygen/nitrogen species); AntiO (Antioxidants)

The adverse effects of oxidative stress on human health are a great concern. The generation of ROS/RNS in the body is physiologically relevant as an important step in inflammation and tissue processes (Locatelli *et al.*, 2003; Awoniyi *et al.*, 2012). However, an excessive generation of these compounds coupled by a deficient antioxidant system results in damage of important cellular components (e.g. genetic material, proteins and lipids), which may initiate many health complications. Severe oxidative stress can ultimately lead to the death of cells (Aruoma, 1998). Sources of oxidative stress include exogenous factors, e.g. exposure to environmental pollutants; cigarette smoke, excessive ultraviolet light, bacterial, fungal and viral pathogens and high body fat percentage, as well as endogenous factors such as oxidative bursts from activated macrophages, energy production or detoxification reactions in cells (Block *et al.*, 2002).

Oxidative stress plays a major role in the development of chronic and degenerative illnesses such as atherosclerosis, coronary heart diseases, neurodegenerative diseases, ageing and cancer (Finkel and Holbrook, 2000; Madhavi *et al.*, 1996). This suggests that an overwhelmed antioxidant defence system initiates and propagates processes involved in the pathogenesis of many diseases (Block *et al.*, 2002).

2.2.1 Reactive oxygen species

Historical evidence has demonstrated ROS as detrimental and destructive compounds to important cellular components. However, it has recently been noted by numerous studies that whether ROS act as signalling, damaging, or protective factors is strongly dependent on the balance between ROS production and the functional oxidant-scavenging mechanisms (Gill and Tuteja, 2010, Finkel, 2011, Anglada *et al*., 2015). Superoxide anion, hydrogen peroxide and hydroxyl radical are considered important and highly relevant ROS, which at abnormally elevated concentrations, coupled either by an absent/insufficient or non-functional antioxidant defence system, can cause significant cellular damage.

2.2.1.1 Superoxide anion

Superoxide anion is ineluctably produced, toxic in nature and hence considered the ultimate danger for aerobic life. It is referred to as the "primary" ROS formed in the cell which initiates a series of reactions producing the "secondary" ROS, either through enzymatic or metal catalysed processes (Sharma *et al*., 2012). It is generated *in vivo* either through enzymatic action (e.g. NADPH oxidase and xanthine oxidase) or by leakage of electrons from the electron transport system. Superoxide anion is a result of the reduction of O_2 by a single electron as indicated in Equation (1).

$$O_2 + e^- \longrightarrow O_2^{\cdot -} \qquad (1)$$

Superoxide anion is negatively charged making it impermeable to biological membranes. This means it is not able to move from its site of formation to any other sites unless converted to other membrane permeable molecules like hydrogen peroxide (H_2O_2).

Superoxide anion removal systems include superoxide anion conversion to hydrogen peroxide catalysed by the enzyme superoxide dismutase (SOD) (see Equation 2), superoxide anion spontaneous reaction with nitric oxide to produce peroxynitrite (Equation (3)) and superoxide anion oxidation by transition metal ions back to the oxygen molecule as displayed in Equation (4). Hydrogen peroxide and peroxynitrite are non-free radicals that are toxic and possess detrimental potential (Brown and Borutaite; 2012; Radi, 2013; Venditti *et al*., 2013).

$$O_2^{\cdot-} + O_2^{\cdot-} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2 \quad (2)$$

$$O_2^{\cdot-} + NO^{\cdot} \longrightarrow ONOO^- \quad (3)$$

$$O_2^{\cdot-} + Fe^{2+} \longrightarrow O_2 + Fe^{3+} \quad (4)$$

2.2.1.2 Hydrogen Peroxide

Superoxide anion dismutation is unavoidable and involves the addition of an electron and two protons to the superoxide anion ($O_2^{\cdot-}$) resulting in the generation of hydrogen peroxide (H_2O_2). This reaction can occur spontaneously i.e. non-enzymatically as illustrated in Equation (5) or enzymatically in the presence of superoxide dismutase, shown in Equation (2).

$$O_2^{\cdot-} + O_2^{\cdot-} + 2H^+ \longrightarrow H_2O_2 + O_2 \quad (5)$$

Hydrogen peroxide in comparison to other ROS is stable and lacks unpaired electrons (meaning it lacks a charge) enabling it to easily cross biological membranes, causing ROS-induced oxidative damage to sites other than that of its formation (Sharma *et al*., 2012; Brown and Borutaite; 2012) . Hydrogen peroxide hydrolyses to water and oxygen in the presence of catalase and glutathione peroxidases as illustrated by Equations (6) and (7), respectively.

$$2H_2O_2 \xrightarrow{Catalase} 2H_2O + O_2 \quad (6)$$

$$2GSH + H_2O_2 \xrightarrow{GPx} GSSG + 2H_2O \quad (7)$$

2.2.1.3 Hydroxl radical

Hydrogen peroxide and superoxide anion are moderately reactive and cause oxidative cellular damage which is exacerbated by their conversion to more reactive species which include the hydroxyl radical ($OH^{\cdot}$) (Lipinski, 2011). In the presence of a suitable transition metal especially iron, hydroxyl radical is produced from hydrogen peroxide and superoxide anion during the Fenton reaction as shown in Equation (8).

$$H_2O_2 + Fe^{2+} \longrightarrow OH^- + Fe^{3+} + OH^{\cdot} \quad (8)$$

The hydroxyl radical ($OH^{\cdot}$) is the most reactive ROS with a potential of reacting with all biological molecules including DNA, proteins and biological membranes altering their basic function, resulting in extensive cellular damage and destruction. An excessive production of this radical eventually leads to cell death because enzymatic mechanisms to scavenge the hydroxyl radical are absent (Sharma *et al*., 2012; Gill and Tuteja, 2010; O' Brien *et al*., 2012).

2.2.2 Sources of ROS

2.2.2.1 Endogenous sources

ROS are produced as by-products of normal oxygen metabolism in aerobic biological organisms. They can be divided into two groups according to their electron distribution and arrangement. These are free radicals, which are highly reactive molecules that have one or more unpaired electrons, such as superoxide anion and hydroxyl radicals and non-radicals, which are less reactive molecules produced when 2 free radicals share their unpaired electrons, such as hydrogen peroxide. Free radicals cause lipid peroxidation due to their ability to remove a hydrogen molecule from a side chain methylene carbon (Birben *et al.,* 2012).

The addition of a single electron to an oxygen molecule (O_2) forming the superoxide anion is mediated either by nicotine adenine dinucleotide phosphate NAD(P)H oxidase, xanthine oxidase or mitochondrial electron system. The mitochondria is the major site for superoxide anion production, with 1-3% of all electrons being transported by the mitochondrial electron transport system for reduction of oxygen to water leaking from the system to produce superoxide anion. NAD(P)H oxidase is found in phagocytic cells like monocytes, and during phagocytosis these cells produce increased amounts of superoxide anion and other toxic oxygen metabolites leading to bactericidal action (Birben *et al*., 2012, Brand, 2010; O'Brien *et al*., 2012, Venditti *et al*., 2013).

The *in vivo* spontaneous or enzymatic dismutation of the superoxide anion results in the production of hydrogen peroxide. NAD(P)H oxidase and xanthine oxidase also produce hydrogen peroxide. Hydrogen peroxide can then move from its site of formation to a different site, inducing oxidative damage. The hydrogen peroxide can breakdown to form the hydroxyl radical in the presence of transition metals like iron during sequential reactions (Fenton reactions) (Birben *et al*., 2012).

Hydrogen peroxide and superoxide anion have the ability to be converted to other reactive species in the presence of suitable mediating compounds and conditions e.g. conversion of hydrogen peroxide tohypochlorous acid (HOCl) in presence of chlorine, superoxide anion to peroxynitritein the presence of nitric oxide (NO) and hydro-peroxyl radical ($HOO^{-\cdot}$) (Venditti *et al*., 2013).

2.2.2.2 Exogenous sources

Externally produced ROS significantly contributes to the imbalance between oxidants and antioxidants in favour of the former, resulting in the development of oxidative stress. ROS can be present in either food, pollutants in the environment or absorbed through the skin as by-products of chemical processes induced by the sun's UV radiation (Birben *et al*., 2012).

Birben *et al* (2012) concurred with Cantin (2010) that cigarette smoke is a rich source of oxidants, free radicals and organic compounds like superoxide anion and nitric oxide. Exacerbating cigarette smoke's destructive effect is that upon inhalation into the lungs, an increased accumulation of macrophages and neutrophils occurs which produce more superoxide anion.

Hyperoxia, the increased inhalation of oxygen pressure into lungs, results in the upsurge production of the reactive oxygen/ nitrogen species. Oxygen, in the presence of ionizing radiation, is reduced to superoxide anion, which further undergoes reduction to form hydrogen peroxide. The hydrogen peroxide has the potential to react with active metals like iron via Fenton reactions producing hydroxyl radical (Birben *et al*., 2012; Kalyanaraman, 2013).

2.2.3 ROS function and clinical implications.

Depending on the concentration of ROS and the functional state of the antioxidant mechanisms, ROS can either be beneficial or harmful to cells and tissues.

2.2.3.1 ROS in normal tissue function

At low to moderate concentrations, ROS influence important physiological cellular processes. In the presence of certain stimuli e.g. microbial pathogens, phagocytic cells (neutrophils, eosinophils and mononuclear macrophages) tend to take in oxygen at excessively increased rates, a process termed the respiratory burst. During this respiratory burst the immune cells produce excessive amounts of superoxide anion and hydrogen peroxide in order to eliminate

foreign pathogens (Freiters *et al*., 2010; Babior, 1984). At sites of inflammation, ROS function to destroy invading pathogens whilst assisting in the modulation of a controlled inflammatory response (Buonocore *et al*., 2010).

Superoxide anion, hydrogen peroxide and oxygen singlet regulate several gene expressions which encode transcription factors, cell differentiation and development. Furthermore they are involved in cell-to-cell communication through stimulating cell-cell adhesion and cell signalling (Powers and Jackson, 2008; Gomes *et al*., 2012, Suzuki *et al*., 2011).

In order for spermatozoa to be able to penetrate and fertilize an egg during fertilization, it undergoes physiological changes referred to as sperm capacitation, a process which occurs more efficiently in the presence of ROS (Buonocore *et al*., 2010).

2.2.3.2 Clinical implications of ROS

Oxidative stress is a common denominator of numerous pathological conditions. At high concentrations, ROS tend to overwhelm existing antioxidant systems, modifying cellular components such as lipids, proteins and DNA resulting in their loss of normal function. The first step in mutagenesis, carcinogenesis and ageing involves the attack of DNA by ROS, damaging the basic units of DNA including purine and pyrimidine bases (Buonocore *et al*., 2010). Lipid peroxidation, also referred to as the reaction of lipids (especially polyunsaturated fatty acids) with molecular oxygen, is a free radical chain reaction accelerated by ROS. It has been implicated in numerous oxidative stress-linked disorders and diseases, suggesting its possible use as an oxidative stress marker. ROS also oxidizes amino acid side chains and the protein backbone, modifying proteins resulting in their loss of normal biological function (Yin *et al*., 2011; Pandey and Rizvi, 2010; Buonocore *et al*., 2010). The attack of vital cellular components by ROS marks the development of innumerable chronic and degenerative illnesses, a characteristic feature of oxidative stress. This suggests that an overwhelmed antioxidant defence system initiates and propagates processes involved in the pathogenesis of many diseases (Block *et al*., 2002) such as cancer (Liou and Storz, 2010; Reuter *et al*., 2010; Essick and Sam, 2010; Matés *et al*., 2012), cardiovascular disorders (Tsutsui *et al*., 2011; Li *et al*., 2014), neurodegenerative diseases (Melo *et al*., 2011; Federico *et al*., 2012), Alzheimer (Wang *et al*., 2014) and diabetes (Rains and Jain, 2011; Bullon *et al*., 2014).

2.2.3.3. Implication of ROS in immune suppression

The uncontrolled severe increase in ROS accompanied by an incompetent antioxidant defence system in immune cells results in cell death as the immune cell components are targeted by free radical action (Correa *et al*., 1999; De la Fuente and Victor, 2000). As immune cells are lost, the immune system is generally weakened, leading to an immunocompromised state that predisposes humans to a variety of ailments including infectious diseases. The relationship between ROS, oxidative stress and immune cell function was appreciated in the early years due to the identified association of diseases to antioxidant deficient diets and also the antioxidants immunity stimulating potential. Administration of antioxidants e.g. Vitamin C, E and glutathione has improved several immune functions (Knight, 2000; De la Fuente and Victor, 2000).

2.2.4 Antioxidants

Cell viability, activation, proliferation and function are critically dependent on the regulation of the reducing and oxidizing (redox) state of the cell, specifically maintenance of normal ROS concentrations. Aerobic organisms possess antioxidant systems that function to effectively eliminate the harmful effects of ROS. Antioxidants were defined as “any substance that delays, prevents or removes oxidative damage to a target molecule (Halliwell, 2007). Antioxidant activity efficiently occurs in different ways: inhibit oxidation of free radicals, eliminate singlet oxygen, interfere with autoxidation chain reactions, convert metal pro-oxidants and hydroperoxides into stable compounds and inhibit pro-oxidative enzymes.

The endogenous antioxidant systems consist of both enzymatic, which are further divided into primary and secondary enzymatic defences and non-enzymatic components. Despite its exceptional efficiency, the endogenous antioxidant system is not sufficient to counteract all kinds of oxidative damage *in vivo* and hence humans depend also on the diet as a supplementary source of antioxidants; termed the exogenous antioxidant system (Table 2.2).

Table 2.2: Components of the antioxidant systems

Endogenous		Exogenous
Enzymatic		Vitamins (A, C, E, K)
Primary enzymes	**Secondary enzymes**	Minerals (Zinc, Selenium)
Catalase	Glutathione reductase	
Glutathione peroxidise	Glutathione-6- phosphate-dehydrogenase	Metals (copper, manganese)
Superoxide dismutase		Plant polyphenols (flavonoids)
Glutathione S-transferase		
Non- enzymatic		
Billirubin		
Transferrin		
Albumin		
Coenzyme Q10		
Uric acid		
Thiols (Glutathione)		

2.2.4.1 Dietary antioxidants

The diet is the main source of exogenous antioxidants and plays a crucial role in supplying the body with additional antioxidants in an attempt to manage the extent of *in vivo* oxidative damage. Supplementing the diet with anti-oxidative compounds contained in natural medicinal plants and animal sources can help control this problem. A number of agricultural products which are considered important commodities worldwide have been shown to be reliable sources of antioxidants. These include fruits and vegetables, oils, nuts, spices, cereals and animal protein. It has been accepted that a plant based diet may reduce the risk of oxidative stress-related diseases. In fact, natural antioxidants have an important function in preventive medicine approaches (Jaberian *et al*., 2013).

2.2.5 Medicinal plants

Medicinal plants have been used to treat human diseases for centuries. They were defined by the World Health Organisation (WHO) (2001) as herbal preparations produced by subjecting plant materials to chemical, physical or biological processes which may be produced for immediate consumption or as a basis for herbal products. Many medicinal plants have demonstrated significant contributions to the treatment of diseases such as HIV/AIDS, malaria, diabetes, mental disorders (Elujoba *et al*., 2005; Okigbo and Mmeka*,* 2006) and microbial infections (Iwu *et al.*, 1999; Okigbo *et al.*, 2005). Their good therapeutic performance, affordability, general acceptance because of a long history of use and low toxicity, are the main contributing factors to their popularity (Lin *et al*., 2008; Vermani and Garg, 2002; Masevhe *et al*., 2015). According to Maroyi (2013), 80% of the population in developing countries uses medicinal plants in the treatment of diseases, whilst 60-80% of the world's entire population rely on medicinal plants for their medical care (Piljac- Žegarac *et al*., 2013). This is because the majority of the populace have no access to modern medicine and rely on traditional healers with whom they have close relations. Medicinal plants are the richest resource of biological material used in modern medicine, in the pharmaceutical and neutraceutical industries, and in traditional medicine systems (Das *et al.*, 2010). Medicinal plant substances such as polyphenols, saponins, tannins, essential oils and flavonoids are biologically active chemicals with curative properties (Harborne, 1973; Sofowora, 1993).

2.2.5.1 Plant polyphenols as antimicrobial and antioxidant agents

Polyphenols are catechins that are potent antimicrobial, anti-inflammatory, anti-allergy, anti-cancer and antioxidant agents, with positive effects on human health (Almajano *et al.*, 2008). Green forms of teas are very rich in catechins such as epicatechin, epigallocatechin, epicatechin-3-gallate and epigallocatechin-3-gallate (Anesini *et al.*, 2008) and usually contain more of the catechins in comparison to fermented tea forms (Dufresne and Farnworth, 2001). Polyphenols act as antioxidants by sequestering metal ions and scavenging reactive oxygen and nitrogen species (Frei and Higdon, 2003). The antioxidant capacity of polyphenols present in teas has been reported to be both chemo-protective and therapeutic (Rietveld and Wiseman, 2003). They have the ability to modify the growth of susceptible bacteria and suppress the virulent factors of bacteria by inhibiting biofilm production, reducing the bacteria adhesion potential to host and also neutralizing bacterial toxins (Daglia, 2012). Polyphenol toxicity to microorganisms is also contributed to by the compounds ability to inhibit bacterial enzymes (Cowan, 1999). The sensitivity of the bacteria to polyphenols is dependent on the polyphenols structure and the bacterial species (Almajano *et al*., 2008). They have been shown to inhibit the growth of clostridia and *Helicobacter pylori* (Gramza and Korczak, 2005). Different plant polyphenols exist, namely phenolic acids, flavonoids and many others, with flavonoids being the most abundant in foods (Daglia, 2012).

2.2.5.2 Flavonoids as antimicrobial and antioxidant agents

Flavonoids are aromatic compounds which are major constituents of human diets, found in fruits, stems, nuts, honey and flowers. They have two substituted benzene rings, connected by a chain of three carbon atoms and an oxygen bridge. Flavonoids, many of which are plant pigments, are abundant with almost 6500 different known structures (Hendrich, 2006). They have powerful antioxidant activities *in vitro*, being able to scavenge a wide range of reactive oxygen, nitrogen and chlorine species, such as superoxide, hydroxyl radical, peroxyl radical, hypochlorous acid and peroxynitrous acid (Haliwell, 2006). They show strong anti-inflammatory, anti-tumour and antimicrobial activity (Cushnie and Lamb, 2011). Their antimicrobial mode of action includes:

- Inhibition of fungal spore germination (Cushnie and Lamb, 2005)
- Damage or inhibition of synthesis of the cytoplasmic membrane of bacteria (Zhang *et al*., 2008(a, b); Jeong *et al*., 2009)
- Inhibition of bacterial nucleic acid (Deoxyribonucleic acid (DNA) and Ribonucleic acid (RNA)) formation (Gradisor *et al*., 2007)
- Inhibition of bacterial cell wall synthesis (Wu *et al*., 2008)

2.2.5.3 Antioxidant and Antimicrobial activity of teas

Teas are also classified as medicinal plants consumed as infusions. Numerous studies have associated different teas with diverse medicinal benefits, specifically antimicrobial and antioxidant activity (Table 2.3). They have the intrinsic ability to resist pathogenic microorganisms (antimicrobial) and also to scavenge free radicals (antioxidant) (Lin *et al*., 2008). Their unique chemical composition gives them an advantage over the synthetic drugs, as microorganisms have not yet been able to gain resistance to them.

Table 2.3: Antimicrobial and antioxidant activity of teas

Tea	Antimicrobial action	Antioxidant action	Reference
Green (Unfermented *Camellia sinensis*)	Inhibits *Staphylococcus* epidermis, *Micrococcus luteus, Brevibacterium linens*, *Pseudomonas fluorescens, Bacillus subtillis*, *Salmonella typhi*, *Vibrio cholera* , *Pseudomonas aeruginosa*, *Streptococcus mutans*	Positive	Sharma *et al*., 2012; Archana and Abraham, 2011; Kumar *et al*., 2012; Araghizadeh *et al*., 2013.
Black (Fermented *Camellia sinensis*)	Inhibits *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Escherichia coli*, *Vibrio cholerae*	Positive	Turkmen *et al*., 2007; Orak *et al*., 2013; Neyestani *et al*., 2007; Olusunde *et al*., 2012; Mandal *et al*., 2011; Bhuyan *et al*., 2013
Oolong (Semi-fermented *Camellia sinensis*)	Inhibits Mutans streptococci, *Staphylococcus aureus*	Positive	Villaño *et al.,* 2012; Chen *et al.*, 2009; Friedman *et al.,* 2006
Mountain tea (*Sideritis syriaca)*	Inhibits *S. aureus*	Positive	Goulas *et al*., 2014

2.2.5.4 Relationship between antioxidant and antimicrobial activity of medicinal plants

The relationship between the antimicrobial and antioxidant activity possessed by medicinal plants, more specifically teas is a complex phenomenon, with a few contradicting experimental studies published. Yildirim and colleagues (2000) illustrated that a weak antimicrobial activity can be expected when a high antioxidant activity is reported in medicinal plants, including *Camellia sinensis* (black tea). On the contrary results obtained by Chan. *et al* (2011) showed that the higher the antioxidant activity of the *Camellia sinensis* derived teas (green, black and herbal) the stronger the antimicrobial activity.

2.3 Indigenous herbal teas

Rooibos (*Aspalathus linearis)* and honeybush (*Cyclopia intermedia)* are two indigenous South African plants, with a long history of medicinal use. The beverages produced from these two medicinal plants may not be referred to as "tea", as that terminology only applies to beverages produced from the plant *Camellia sinensis* (Hodgson, 2006). Thus the beverages derived from these two plants are commonly referred to as "herbal teas" or tisanes.

2.3.1 *Aspalathus linearis* (Rooibos)

Figure 2.7: Rooibos plant and processed plant material used for tea-making purposes
(Adapted from http://www.plantzafrica.com/plantab/aspallinearis.htm)

2.3.1.1 History and epidemiology

Rooibos herbal tea is produced from *Aspalathus linearis* (Burm.f.) Dahlg (Fig. 2.7), which is a member of the genus *Aspalathus* (Fabaceae, Tribe Crotalarieae), that comprises 270 species (Joubert and de Beer, 2011; Dahlgren, 1968; Joubert *et al.*, 2006). It is an indigenous fynbos plant grown in the arid Western Cape of South Africa mainly in the mountains of Cederberg near Cape Town (Raynolds and Ngcwangu, 2010; Kawakami *et al.,* 1993). Rooibos is currently sold and enjoyed in more than 37 countries worldwide (Kawakami *et al.*, 1993). Its popularity is

greatly attributed to its potential health-promoting properties. Joubert and de Beer (2011) described Benjamin Ginsberg as the first person to realize the commercial potential of rooibos as a herbal tea in 1904. However, the agricultural significance of rooibos herbal tea was recognized by P. Le Fras Nortier of Clanwilliam, a medical practitioner and nature lover in the 1930s, laying the industry's foundation (Anon, 1985). With time, different types of rooibos herbal teas were produced and sold commercially. Today the red type, which is divided into the Nortier type and the Cederberg type, is the only one with significant commercial importance (Joubert and de Beer, 2011).

2.3.1.2 Ethnopharmacology

During pregnancy, African women take rooibos to ease heartburn, nausea, and for its iron content. The women also give it to their babies for colic relief (Gruenwald, 2009). Rooibos has also been consumed to relieve symptoms associated with digestive disorders, skin allergies, insomnia, nervous tension and mild depression (Morton, 1983).

2.3.1.3 Chemical composition

Rooibos is known for its caffeine-free and low tannin nature, though traces of the alkaloid sparteine have been reported (Joubert and de Beer, 2011). The unique phenolic metabolites of rooibos herbal tea act as potent antioxidants (Joubert and Ferreira, 1996). It contains two unique phenolic compounds, namely *aspalathin* which is a dihydrochalcone*C*-glucoside and *aspalalinin*, a cyclic dihydrochalcone (Breiter *et al.,* 2011). Nothofagin is a rare cyclic dihydrochalcone also present in rooibos (Koeppen and Roux, 1965). Aspalathin and nothofagin are the major flavonoids found in rooibos and have displayed good anti-mutagenic properties (Van Wyk and Verdoorn, 1989; Marnewick *et al.,* 2000). Other flavonoids found in rooibos namely quercetin, luteolin, rutin, isoquercitrin and iso-vitexin have displayed antioxidant activity (Pratt and Hudson, 1990). Other phenolic compounds present in rooibos include flavones, flavanones and flavonols (Joubert and de Beer, 2011). Phenolic acids, lignans, the flavone diglycosides, (+)-catechin a phenylpyruvic acid glycoside, the flavonol quercetin-3-*O*-robinobioside, and the coumarins, esculetin and esculin have also been identified in rooibos (Shimamura *et al.,* 2006; Beltrán-Debón *et al.,* 2011; Breiter *et al.,* 2011; Krafczyk, 2008).

2.3.1.4 Bioactivity

i. Antioxidant activity

The popularity of rooibos is strongly attributed to its significant antioxidant capacity shown by both *in vitro* and *in vivo* evidence (Hong *et al*., 2014; Marnewick, 2014). A study carried out to assess the effect of drinking rooibos herbal tea on total antioxidant capacity in humans, accumulated data supporting rooibos as a source of dietary antioxidants in humans (Villaño *et al*., 2010). The natural antioxidant potential of rooibos was evaluated in a study that involved the consumption of a rooibos tea extract by streptozotocin (STZ) diabetic-induced rats to assess whether it had the ability to prevent and treat oxidative stress in the test subjects. It was observed that the consumption of rooibos tea extracts by the rats partially prevented oxidative stress (Ulična *et al.*, 2006). Marnewick *et al* (2011) showed that the consumption of the traditional/fermented rooibos herbal tea has the ability of improving the redox status of adults at risk of developing cardiovascular disease. An *in vivo* study also showed that rooibos decreased oxidative damage by the superoxide anion radical generator (juglone) in the model organism *Caenorhabditis elegans,* significantly improving the survival rate of the free-living nematode (Chen *et al*., 2013).

ii. Antimicrobial activity

Coetzee *et al* (2008) proved that rooibos extracts have the ability to decrease the spore viability of the fungus *Botrytis cinerea*by 33.3%. A phosphate buffer saline extract of rooibos demonstrated antimicrobial activity against *Campylobacter jejuni*, *Escherichia coli*, *Salmonella enteritidis*, methicillin-resistant *Staphylococcus aureus, Pseudomonas aeruginosa* and *Pseudomonas putid*a 36 hours after incubation (Murali *et al*., 2012). When the antibacterial activity of natural rooibos infusions were compared to that of artificial infusions, it was shown that the natural infusions demonstrated bacterostatic/-cidal activity against Gram positive *Staphylococcus epidermidis* and *Staphylococcus aureus* and Gram negative *Escherichia coli* (Simpson *et al*., 2013; Schepers, 2001). Boyanova (2014) demonstrated that aqueous rooibos extracts exhibit anti-*Helicobacter pylori* activity, suggesting that it may be included in the therapeutic or prophylactic schedules for *H. pylori* infections. However, the minimum inhibitory concentration (MIC) experimental study by Hübsch *et al*. (2014) reported no noteworthy antimicrobial activity was observed when Gram-positive *Staphylococcus aureus, Enterococcus faecalis, Bacillus cereus;* Gram-negative *Klebsiella pneumonia, Escherichia coli, Pseudomonas aeruginosa* and fungi *Candida albicans*and *Cyptococcus neoformans* were exposed to both aqueous and organic extracts of *Aspalathus linearis*.

iii. Anti-diabetic activity

Son *et al* (2013) demonstrated that a rooibos herbal tea extract possesses anti-diabetic activity and further on showed that aspalathin, the major polyphenol of the extract, played a crucial role in this effect. They concluded that aspalathin increased glucose uptake by L6 myotubes, promoted AMP- activated protein kinase (AMPK) phosphorylation, and decreased expression of hepatic genes related to gluconeogenesis and lipogenesis. It was also shown that the rare enolicphenylpyruvic acid-2-O-glucoside (PPAG), one of the major constituents of fermented rooibos, increased *in vitro* glucose uptake and improved glucose tolerance in obese insulin-resistant rat models, suggesting its potential in contributing towards rooibos herbal tea antidiabetic effects (Muller *et al*., 2013).

iv. Anti-obesity activity

Continuous consumption of aqueous rooibos herbal tea extract by diet-induced obese mice resulted in the following changes: decreased serum cholesterol, triglycerides and free fatty acid levels and changes in adipocyte size and number (Beltrán-Debón *et al*., 2011). When the effects of fermented rooibos hot water soluble solids on *in vitro* adipocyte differentiation using differentiating 3T3-L1 adipocyteswas examined, it was shown that the hot water soluble solids of fermented rooibos inhibited adipogenesis and affected adipocyte metabolism, suggesting its potential in preventing obesity (Sanderson *et al*., 2014).

v. Anti-cancer activity

Marnewick *et al* (2005) carried out a study that involved the inhibition of mouse skin tumour promotion, supporting the role of topical application of rooibos extract in preventing skin cancer. In a related study, Magcwebeba (2013), reported methanol rooibos extracts strongly inhibited the proliferation of cancer cells in the skin. Sissing and others (2011) demonstrated that rooibos herbal tea possesses modulating effects on the development of methylbenzylnitrosamine (MBN) induced esophageal squamous cell carcinogenesis. They showed that unfermented rooibos significantly reduced the mean total papilloma size significantly by 87% but inhibited tumor multiplicity by less than 30%.

2.3.2 Cyclopia species *(Honeybush)*

Figure 2.8: Honeybush plant and processed plant material used for tea-making purposes
(Adapted from http://organicjar.com/2009/1156/)

2.3.2.1 History and epidemiology

Honeybush herbal tea, brewed from the leaves and stems of various *Cyclopia species* (Fig. 2.8), is a popular South African indigenous herbal tea enjoyed for its aroma and taste (Kamara *et al.,* 2003; Joubert *et al.,* 2008). *C. intermedia* grow in diverse conditions and are currently the major species of commercial significance (Joubert *et al*., 2011). It is produced for the herbal tea market and together with rooibos represents two spectrums of the South African herbal tea industry; from an established rooibos industry to a developing honeybush industry (Joubert *et al*., 2008). The earliest mention of *Cyclopia* was in 1705 (Kies, 1951), and since then, 23 species have been identified (Schutte, 1995; 1997). However, regardless of its long history, the popularity of honeybush herbal tea was not as clear as that of rooibos. After World War 2, its processing and production was almost discontinued up until 1960, when the branded product packaged under the name “Caspa Cyclopia Tea” appeared through the involvement of Benjamin Ginsberg (Joubert *et al*., 2011).The commercial popularity of rooibos in the 1990s led to renewed interest in honeybush, when the National Botanical Institute (Kirstenbosch); the Agricultural Research Council (ARC) (ARC, 2008) and the Medical Research Council (MRC) of South Africa developed projects concerning the commercial cultivation, processing and health-promoting properties of honeybush (Joubert *et al*., 2008; Marnewick *et al*., 2000; 2001; 2004; 2009). The honeybush industry is growing, with Germany and the Netherlands being the major market of the 25 countries currently commercializing it (Joubert *et al*., 2011).

2.3.2.2 Ethnopharmacology

Traditionally, honeybush has been used as a restorative, an expectorant in chronic catarrh and pulmonary tuberculosis, a stimulator of milk production in lactating women and to treat various

digestive disorders (Van Wyk *et al.,* 1997; Joubert *et al*., 2011; Rood, 1994; McKay and Blumberg, 2007).

2.3.2.3 Chemical composition

Honeybush is a caffeine-free (Greenish, 1881), low tannin (Marloth, 1925; Terblance, 1982) aromatic herbal tea with many polyphenols that are linked to its health-promoting properties (Joubert *et al*., 2011). The phenolic composition of the cyclopia species differ qualitatively and quantitatively from each other (Joubert *et al*., 2008). The major compounds present in *C. intermedia* are the xanthones, mangiferin and isomangiferin, and the flavanone hesperidin and hesperetin which is an *O*-glycoside (Coetzee *et al*., 2008; Joubert *et al.,* 2003) together with flavones, isoflavones, flavonols and coumestans which are the major flavanones in honeybush herbal tea (Joubert *et al*., 2011).

2.3.2.4 Bioactivity

i. Antioxidant activity

Currently the antioxidant activity and other biochemical properties of honeybush tea have not been vastly investigated considering its growing worldwide market, providing a scientific gap which needs to be addressed. However, *in vitro* studies demonstrating the antioxidant effects of mangiferin have been documented (Leiro *et al*., 2003). On monitoring the superoxide radical scavenging ability of aqueous extracts from five different *Cyclopia species*, Hubbe and Joubert (2000) reported that all possess a significant scavenging ability of the radical. Joubert *et al* (2008) showed that both the fermented and unfermented forms of honey bush herbal tea displayed significant antioxidant activity.

ii. Antimicrobial activity

Coetzee *et al* (2008) reported that honeybush has a bacteriostatic effect on *E. coli*, and decreases the spore germination of *Botrytis cinerea*. Hesperidin and mangiferin are the two compounds suspected of exerting the antimicrobial activity of honeybush, providing a foundation for confirmatory experimental studies.

iii. Anti-diabetic activity

Muller and collegues (2011) demonstrated that the oral ingestion of hot water *Cyclopia intermedia* (honeybush) extract effectively decreased the plasma glucose levels in the STZ-

induced diabetic rat models. When the same rats were fed a high-fat diet for a prolonged period of time, then treated with honey bush extracts, it was shown that the extracts improved other metabolic entities. Pre-treatment of STZ-induced diabetic Wistar rats with aqueous extracts of unfermented *Cyclopia maculata* ameliorated the diabetic effect of the STZ in the test rats. This effect was evident by the protection of the pancreatic *β-cells* from cytotoxicity, due to the high levels of antioxidants in the extract (Chellan *et al*., 2014).

iv. Anti-obesity activity

The *Cyclopia* species investigated by Dudhia *et al* (2013) significantly inhibited adipogenesis in 3T3-L1 pre-adipocytes, suggesting their potential use as anti-obesity agents. A related study showed that *Cyclopia maculate* stimulated lipolysis in mature 3T3-L1 adipocytes, further supporting the anti-obesity activity of honey bush herbal tea (Pheiffer *et al*., 2013).

v. Anti-cancer activity

Fermented and unfermented honeybush herbal tea displayed a level of protection against fumonisin B_1 (FB_1) induced cancer in rats (Marnewick *et al.,* 2009). Sissing *et al* (2011) reported that unfermented honeybush significantly inhibited tumor multiplicity by 45.5% and reduced the mean total esophageal papilloma size by 94%, whilst fermented honeybush reduced it by 74%.The topical application of green honeybush and fermented honeybush on mouse skin significantly suppressed tumor initiation by 90% and 84.2% respectively (Marnewick *et al*., 2005). Aqueous honeybush extracts displayed a weak inhibitory effect against skin carcinogenesis, with *Cyclopia genistoides* being the most effective (Magcwebeba, 2013).

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CHAPTER 3: RESEARCH ARTICLE ONE

The antimicrobial and associated antioxidant activity of various Rooibos (*Aspalathus linearis*) herbal tea extracts

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Abstract

The increase in the popularity of the South African plant *Aspalathus linearis*, commonly referred to as rooibos, as a health beverage has been attributed to the numerous studies done on its bioactivities. These include the numerous antioxidant studies and few antimicrobial investigations. However the relation of these two bioactivities within the rooibos herbal plant has never been documented. This is an important approach in assessing whether the easy accessible, affordable and non-toxic herbal beverage can be classified as a therapeutic of oxidative stress and infections with highly pathogenic microbes. The antimicrobial activity against nosocomial pathogens (*P. aeruginosa, S. aureus, S. pyogenes* and *C. albicans*), total antioxidant capacity, flavonoid and polyphenolic quantification of green/unfermented and fermented water, methanol, chloroform and ethyl acetate rooibos extracts were investigated. Methanol and water exhibited antimicrobial activity against *S. aureus* and *C. albicans* whilst the chloroform extracts displayed selective activity against *S. pyogenes.* However the high MIC values reported did not categorize all the rooibos extracts as noteworthy antimicrobial agents (MIC values >1 mg/ml). The Gram-negative *P. aeruginosa* was resistant to all 8 rooibos extracts. Thin layer chromatography-bioautography allowed the observation of the active antimicrobial components of the most potent extracts, which could possibly have higher antimicrobial activity than the whole crude extract. The total antioxidant capacity was assessed using ORAC, FRAP and TEAC. The green methanol extracts exhibited the strongest antioxidant activity. Generally the green incomparison to fermented extracts displayed the highest antioxidant capacity, total polyphenols and flavonoid content. These data suggested that a direct relation exists between the antimicrobial and antioxidant activities of the green and fermented rooibos extracts.

Keywords: Rooibos, antimicrobial, antioxidant

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3.1 Introduction

The relationship between the antioxidant and antimicrobial activities of some medicinal plants has been studied with contradicting results. Chan and colleagues (2011) observed that a high antioxidant activity is related to a strong antimicrobial activity in *Camellia sinensis* derived teas; black, green and herbal. However Yildirim *et al* (2000) had previously shown that in medicinal plants a weak antimicrobial activity is expected if a high antioxidant activity is reported. The outcomes of such studies have necessitated a subtle approach in linking these two fundamental bioactivities in plants, specifically rooibos herbal tea.

The popularity of rooibos herbal tea has greatly increased worldwide due to its potential health-promoting properties. A fynbos plant indigenous to the Western Cape Province of South Africa, rooibos herbal tea is now enjoyed in more than 37 countries worldwide (Kawakami *et al.*, 1993). With a unique caffeine- free and low tannin chemical nature, rooibos herbal tea has been associated with numerous bioactivities including anti-diabetic (Son *et al*., 2013; Muller *et al*., 2013), anti-obesity (Beltrán-Debón *et al*., 2011; Sanderson *et al*., 2014) and anti-cancer (Marnewick *et al*., 2005, 2009; Magcwebeba, 2013; Sissing *et al*., 2011). Strong antioxidant activity of rooibos herbal tea has been previously reported (Ulična *et al*., 2006; Marnewick *et al*., 2011; Hong *et al*., 2014) and the popularity of antimicrobial studies of the herbal tea has increased in recent years (Coetzee, 2008; Murali *et al*., 2012; Boyanova, 2014; Hübsch *et al*., 2014). This is linked to the search for sustainable, socially accepted therapeutic strategies to control oxidative stress and its detrimental effects and nosocomial infections. However to our knowledge these two bioactivities have not been studied concurrently allowing for the assessment of their correlation.

3.2 Methods and Materials

3.2.1 Chemicals and apparatus

Ampicillin, blood agar, chloroform, ciprofloxacin, cooked blood agar, dichloromethane (DCM), dimethyl sulfoxide (DMSO), ethyl acetate, fluconazole, hydrochloric acid (HCl), methanol, Mueller hinton agar, Mueller hinton broth, sabouraud dextrose 4% agar, sodium acetate, yeast peptose broth and vanillin were all purchased from Merck (Johannesburg, SA). 2,2′-azobis (2-methylpropionamidine) dihydrochloride (AAPH), 2,2'- azinobis-(3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) ferric chloride ($FeCl_3$), fluorescein sodium salt, folin ciocalteu's reagent, gallic acid, $K_2S_2O_8$ (potassium- peroxodisulfate), L-ascorbic acid, nitrotetrazolium blue chloride (NBT), potassium phosphate (KH_2PO_4), sodium carbonate, sulphuric acid (H_2SO_4), 2,4,6-tri[2-pyridyl]-s-triazine (TPTZ) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased for Sigma-Aldrich (Johannesburg, SA). All solvents purchased and used through the study were of analytical reagent grade. Sterilized water was used throughout the study. Greiner crystal clear 96-well flat bottom, Costar 96-well UV flat bottom and Nunclon black 96-well flat bottom microplates were supplied by Sigma-Aldrich (Johannesburg, SA).

3.2.2 Plant Material

Fermented and green (unfermented) rooibos plant material was obtained from the Oxidative Stress Research Centre (Cape Peninsula University of Tecnology, Symphony Way, Bellville, 7535, South Africa), stored in sealed plastic containers at room temperature in the dark. The plant material was similar to the form in which the plants are sold for commercial tea making purposes.

3.2.3 Extraction

Different extracts were prepared by macerating the plant material in solvents of differing polarity, namely water, methanol, chloroform and ethyl acetate.

Water extracts of the fermented and green/unfermented rooibos were prepared by adding 1000 ml of boiling distilled water to 100 g samples of the fine rooibos leaves, whilst the organic (ethyl acetate, methanol and chloroform) extracts were prepared by adding 1000 ml of the respective solvent to 100 g sample at room temperature. The mixture was left for 24 hours at room temperature on a magnetic stirrer, after which the extract was filtered through cotton wool, to

remove the residual tea leaves. The extracts were then filtered using Whatman no. 4 mm paper. The water extract filtrate was frozen and lyophilized using the freeze-drier. Ethyl acetate, methanol and chloroform extract filtrates were concentrated to a fifth of the initial volume using the rotary evaporator. These organic extracts were finally dried under a fume hood to yield crude extracts, after which the samples were weighed and stored at 4 ^{o}C in dark sterile sealed containers for further use.

3.2.4 Antimicrobial studies

3.2.4.1 Microorganisms

The microorganisms selected; Gram-positive *Staphylococcus aureus* (ATCC 25923) and *Streptococcus pyogenes* (ATCC 19615), Gram-negative *Pseudomonas aeruginosa* (ATCC 27853) and yeast *Candida albicans* (ATCC 10231); represent the three main groups of microorganisms. These microorganisms are associated with nosocomial infections and severe infections affecting the skin, respiratory and gastrointestinal tract traditionally treated by the selected plant (Hübsch *et al.*, 2014). *Staphylococcus aureus* and *Pseudomonas aeruginosa* were cultured on prepared 40 g/l blood agar (BA), *Steptococcus pyogenes* on 42 g/l cooked blood agar (CBA) whilst *Candida albicans* on Sabouraud dextrose agar (SDA) of 47 g/l concentration and all were kept viable by subculturing.

3.2.4.2 Agar disk diffusion assay

Agar diffusion techniques are preliminary screening methods that have been widely used to assay plant extracts for antimicrobial activity (Ncube *et al*., 2008). Sterile 9 mm disks were impregnated with 50 µl of dissolved plant extracts (at increasing concentrations) and incubated at 37 ^{o}C for 24 hours to dry. The positive control substances used for *Streptococcus pyogenes* and *Staphylococcus aureus* was the broad spectrum antibiotic ampicillin (10 µg), *Pseudomonas aeruginosa* was ciprofloxacin (5 µg) and that for *Candida albicans* was fluconazole (25 µg) and the respective extraction solvents were used as the negative controls. A suitable solid agar medium was inoculated with the respective test organisms (inoculum size: 10 µl of 1×10^{8} CFU/ml microorganisms) (Baris *et al*., 2006). The media utilised in this assay for the bacteria was Mueller Hinton agar, described as the medium of choice by Ncube and collegues (2008), whilst SDA was used for the fungus. The dried paper disks, saturated with the plant extracts at desired concentrations, together with the positive and negative controls were then placed

carefully onto the surface of the pre-inoculated agar. The plates were then incubated at 37 ^{o}C for 24 hours (bacteria) and 48 hours (fungus). The inhibition zones were measured in millimetres (mm) from the circumference of the disk to that of the growth-free zones around the disk and recorded (Salie *et al*., 1996). All samples were tested in triplicate (*n*=3).

3.2.4.3 Broth micro-dilution assay- Minimum Inhibitory Concentration

The antimicrobial activity of the extracts was further analyzed using the broth micro-dilution (or micro-titre plate) method, a useful technique for determining the minimum inhibitory concentration (MIC) of test samples, based on the contact of test microorganisms to a series of test sample dilutions (van Vuuren, 2008). MIC is the lowest concentration of the antimicrobial (in this case plant extracts) that inhibits microbial growth after incubation (Das *et al*., 2010). Its advantages include increased sensitivity for small quantities of extract; ability to distinguish between bacteriostatic and bactericidal effects and quantitative determination of the MIC (Langfield *et al*., 2004), delivering reproducible results.

The MIC for each sample was determined using the micro-titre plate method (Eloff, 1998). The samples were chosen according to the results obtained from the disk diffusion assay. Microbial suspensions in Mueller-Hinton (bacteria) and Yeast Peptone (fungus) broths were prepared and incubated for 24 hours at 37^{o}C, from which a McFarland No. 0.5 standard (approximately 1 × 10^{8} CFU/ml) were prepared. The plant extracts were dissolved in 10% dimethylsulfoxides (DMSO) (Baris *et al*., 2006; Salie *et al*., 1996; Langfield *et al*., 2004). The organic dried extracts, chloroform and methanol, were dissolved to starting concentrations of 200mg/ml and 300mg/ml, respectively and the aqueous extracts to a starting concentration of 600mg/ml. Those extracts that were difficult to dissolve were agitated using a vortex. The positive control substances used (to confirm microbial susceptibility) for *Streptococcus pyogenes* and *Staphylococcus aureus* were antibiotic ampicillin, and that for *Candida albicans* was fluconazole and the respective extraction solvents and 10% DMSO were used as the negative controls.

The 96-well micro-titre plates were aseptically prepared in a horizontal laminar air flow cabinet. In each well 100µl of 10% DMSO was added, and then 200µl of the extracts were added in the first wells from which serial dilutions (two-folds) were made down the plates to the desired minimum concentration. The microbial cultures (100µl) were added to the wells, which were then

placed on a shaker (500rpm) for 30 seconds. Soon after shaking, the plates were read at 620 nm wavelength with a multiplate reader to obtain the first reading (t=0hours), a sterile film was used to cover the plates to eliminate evaporation then incubated at 37^{o}C for 24 hours. After incubation the absorbance reading at the same wavelength of 620nm were recorded for each plate. The MIC determined by the spectrophotometric method was defined as the concentration at which there was a sharp decline in the absorbance value after incubation (Salie *et al*., 1996; Devienne and Raddi, 2002). All MIC values were tested in triplicates.

3.2.4.4 Thin Layer Chromatography (TLC) –Bioautography

Bioautography is a variation of the agar diffusion method where the analyte (extract) is adsorbed onto a thin layer chromatography (TLC) plate. It is also employed as a preliminary phytochemical screening technique, by bioassay-guided fractionation, to detect active components of the extracts under study (Nostro *et al*., 2000; Schmourlo *et al.,* 2004).

In the procedure, 20 µl of the extracts (aqueous and organic) were applied to pre-coated plates of silica gel 60 F_{254} (Merck, Germany). Development of the TLC plates was done followed by the visualization of the bands through observing under UV at λ254 nm and at λ366 nm using UV lamp (CAMAG, Switzerland). Finally the detection agent vanillin - sulphuric acid was sprayed on the plates. Chemical profiling of the bands was done based on the colour produced after viewing. The solvent system used for the TLC development of the *A. linearis* extracts fractions was dichloromethane- methanol (95:5).

The duplicate plates were used for the bioautographic agar overlay assay. A suspension of the test microorganisms (approximately 1x10^{8} CFU/ml) in growth medium was sprayed onto the developed TLC plates. The bioautograms were then incubated at 37^{o}C for 24 hours in humid conditions. A microbial indicator, nitrotetrazolium blue chloride (NBT), was used as a growth detector (Silva *et al*., 2005). It was sprayed onto the plates, which were re-incubated at 37 ^{o}C for 3-4 hours (Dilika *et al*., 1996; Runyoro *et al*., 2006). Clear zones on the bioautogram indicated inhibition of growth i.e. antimicrobial activity of the extract components.

3.2.5 Antioxidant capacity studies

3.2.5.1 Oxygen Radical Absorbance Capacity (ORAC) assay

The ORAC assay provides a direct measure of the antioxidant capacity of a substance (Ou *et al*., 2001). The thermal decomposition of 2, 2'-azobis (2-methyl-propanamide) dihydrochloride (AAPH) to a peroxyl radical which oxidizes a fluorescent probe, results in a loss of fluorescence intensity. The decrease in fluorescence intensity reflects on the concentration of free radicals and a delay of this loss signifies the presence of antioxidants in the test sample counteracting the activity of the oxidative species. These antioxidants tend to inhibit the oxidative degradation of the fluorescence. In this study, fluorescein (FL) was used as the probe and the assay was based on measuring the inhibition of fluorescein decay by antioxidants. The azo compound AAPH was thermally decomposed at 37 ^{o}C to form the peroxyl radical. The antioxidant capacity of the extracts (at different concentrations) was measured by comparing the areas under the fluorescene decay curve (AUC) produced by the extracts to the areas produced by the controls prepared from the Trolox solution. Trolox (6-Hydroxy-2,5,7,8- tetramethylchroman-2-carboxylic acid) is a water-soluble vitamin E analog with known antioxidant activity. ORAC results were expressed as micromoles of Trolox® equivalents (TE) per gram of sample (µmol of TE/g). The procedure of this assay was that modified by Rautenbach and co-workers (2010).

The reagents and standards (FL, AAPH, Trolox) were prepared using the ORAC phosphate buffer (75mM at pH 7.4). One hundred and thirty eight microlitres of fluorescein solution (14 µM concentration) and 12 µl of sample (controls and extracts) were dispensed and mixed in a 96-well black plate. Trolox controls were prepared within a range of 0-417 µM. Fifty microlitres of AAPH solution (500 µM conentration) were added to each well prior to reading the plates. The fluorescence readings were measured with 485 and 538 nm as excitation and emission wavelengths, respectively using a fluoroskan ascent plate reader (Thermo Fisher Scientific, Waltham, Mass, U.S.A). Fluorescence readings were taken at every minute thereafter (*f* 1, *f* 2, *f* 3, …) for a duration of 2hours. The ORAC value was calculated by dividing the area under the sample curve by the area under the Trolox (control) curve with both areas being corrected by eliminating the area under the blank curve. A single ORAC unit was assigned as being the net protection area provided by 1 µM Trolox in the final concentration. The final antioxidant activity results were given in Trolox equivalents. All samples were assessed in triplicates.

3.2.5.2 Ferric Reducing Ability of Plasma (FRAP) assay

The FRAP assay is used as a novel method for assessing "antioxidant power" of biological and pure samples (Benzie and Strain, 1996). The principle of this spectrophotometric method is based on the antioxidants ability to reduce the ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}), at low pH.

The antioxidant capacity of the samples is evaluated by the ability of the antioxidants to donate electrons during a redox (reduction/oxidation) reaction. At acidic pH, the oxidant ferric chloride hexahydrate $Fe_3(TPTZ)_2Cl_3$ (TPTZ= 2,4,6, Tripyridyl-s-triazine) is reduced by antioxidants in the test sample to give an intense blue coloured ferrous tripyridyltriazine complex. The colour develops in the presence of electron donating antioxidants and is monitored by a spectrophotometer measuring the change in absorption maximum at 593 nm (Benzie and Strain, 1996; Gupta *et al*., 2009).

The FRAP reagent was prepared by mixing acetate buffer (300 mM, pH 3.6), TPTZ (10 mM), $FeCl_3$ (20 mM) and distilled water. The TPTZ reagent was prepared in hydrochloric acid (40 mM HCl), whilst the rest of the reagents were prepared using distilled water. L-Ascorbic acid was used in the preparation of the antioxidant standard solutions ranging between 0-1000 µM. Ten micolitres of the standards/extracts followed by 300 µl of the FRAP reagent were added to a 96-well plate and incubated at 37 ^{0}C for 30min before reading. Results were obtained by comparison to the calibration standard curve using the regression equation (y = mx + b, where m=slope, b=y-axis intercept). Each sample was assayed in triplicate.

3.2.5.3 Trolox Equivalent Antioxidant Capacity (TEAC) assay

The TEAC assay measures the total antioxidant activity of a given substance. The procedure was previously described by Re and collegues (1999). The ABTS reagent (2,2'- azinobis-(3-ethyl-benzothiazoline-6-sulfonic acid)) was prepared by mixing 5 ml 7 mM) ABTS and 88 µl (140 mM) $K_2S_2O_8$ (potassium- peroxodisulfate) and allowed to react for 24 hours in the dark at room temperature. Trolox (6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as the standard with concentrations ranging from 0 to 500 µM. After 24 hours, the ABTS reagent was diluted with absolute ethanol to read an initial absorbance of approximately 2.0 (± 0.1), which is the control value. Twenty five microlitres of standards/extracts and 300 µl of ABTS mix were added and mixed in 96-well plates and were allowed to react at room temperature in the dark for 30 min. The plates were then read at 734 nm at 25 ^{0}C using the multiskan plate reader (Thermo Fisher Scientific, Waltham, Mass, U.S.A) and results were expressed as micromole Trolox equivalents per milligram dry weight of sample (µM TE/g). All samples were run in triplicate.

3.2.6 Phytochemical assays

3.2.6.1 Total Polyphenols

The total polyphenol content (TPC) was determined by spectrophotometry, using Folin-Ciocalteu reagent (Sigma-Aldrich, Johannesburg, SA) with gallic acid as a standard. The reagents (10% ethanol, Folin-Ciocalteu (0.1 g/ml), Sodium carbonate (0.075 g/ml) were all prepared using distilled water except the gallic acid standards (give an absorbance of 0.509 ± 0.010 at 280 nm) which were diluted in 10% ethanol. The gallic acid stock solutions were prepared in varying concentrations, ranging between 0-500 mg/l. Twenty five microlitres of standards/extracts (of varying concentrations) were added with 125 µl of Folin-Ciocalteu in the crystal clear 96-well plates. After 5 min 100 µl of Sodium carbonate was then pipetted into each well. The plates were incubated at room temperature for 2 hours before being read with the multiskan plate reader (Thermo Fisher Scientific, Waltham, Mass, U.S.A) at an absorbance of 765 nm. Total polyphenols results were expressed as milligram Gallic acid equivalents per gram of extract (mg Gallic acid/g). The concentration of polyphenols in extracts were derived from a standard curve of gallic acid standards ranging from 10-50 µg/mL (Pearson's correlation coefficient: r^2 = 0.9996). Samples were analysed in triplicate.

3.2.6.2 High Performance Liquid Chromatography (HPLC)

The HPLC is a quantitative analysis, detecting and characterizing known and unknown sample components. The Spectra HPLC system P2000 pump was equipped with HPLC column C18 (150 x 4.6mm), 5 µm particle size (Agilent Zorbax, South Africa) and a Spectra system FL3000 fluorescence detector. The chromatographic conditions included flow rate 1ml/min, 15 min run time, sample injection volume of 20µl and the mobile phases A (KH_2PO_4 (50mM)) and B (methanol). The temperature of the column and detector array was maintained at room temperature (25 ± 1 °C). Crude extracts were diluted using DMSO to a concentration of 1 mg/ml. The measurements were made at 360 and 287 nm for aspalathin against the pure compound. The analytical signals were monitored at 2-20 mV potentials applied.

3.2.7 Statistical analysis

All antimicrobial data are expressed as mean ± standard error of the mean (SEM) and antioxidant data is expressed as mean ± standard deviation. One-way analysis of variance (ANOVA) was used to test for significance of the antioxidant activity of the extracts. Bonferroni

Multiple Comparison analysis was used to compare the antioxidant activity of the different extracts. Statistical analysis of antimicrobial activity of the different rooibos extracts was achieved using the unpaired Student's *t*-test. Comparisons were considered significant at $P<0.05$. GraphPad™ PRISM5 software package was used for all statistical evaluations and graphical representations.

3.3 Results

3.3.1 The antimicrobial activity of rooibos extracts against *Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus pyogenes* and *Candida albicans*

3.3.1.1 Agar disk diffusion assay

The agar disk diffusion assay allowed the identification of the microbes that were sensitive to the rooibos extracts whilst assessing effects of fermentation on these sensitivities by statistically comparing the inhibition of the fermented extracts with the inhibitory activity of the green extracts. The rooibos extract concentrations were linearly proportional to their inhibitory activity, observed by the increased zones of inhibition with an increment of the extract concentrations. The positive controls ciprofloxacin, ampicillin and fluconazole inhibited the growth of specific microorganisms. Extraction solvents which served as the negative control did not have any effect on *P. aeruginosa, S. aureus, S. pyogenes* and *C. albicans*, thus no zones of inhibition were observed around the impregnated disks.

The rooibos extracts were inactive against *P. aeruginosa* as no inhibition zones were observed (Table 3.1). The polar extracts (methanol and water) of both green and fermented rooibos exhibited some degree of antimicrobial activity against *S. aureus* whilst ethyl acetate and lipophilic chloroform rooibos extracts were inactive against this Gram- positive bacterium. Green and fermented methanol rooibos extracts proved to be most potent, with the fermented form being more active. The rooibos fermented methanol extract was 5 fold more active than the less active rooibos green water extract against *S. aureus*. At 600 mg/ml the most significant ($P<0.05$) comparison of green and fermented methanol rooibos extracts was observed, whilst for the aqueous extracts the most significant ($P<0.05$) comparison of the two forms of rooibos was recorded at 200 mg/ml.

S. pyogenes was sensitive to the green and fermented water, methanol and chloroform rooibos extracts. However the ethyl acetate extracts of the two forms of rooibos did not possess any inhibitory effect against the microorganism. The green rooibos water and fermented rooibos chloroform extracts possessed the greatest anti- Streptococal activity. The activity of the stronger rooibos fermented chloroform extract was 6 fold greater than the weaker rooibos fermented water extract. The most significant (P<0.05) comparison between the green and fermented forms of rooibos tea against *S. pyogenes* was observed at 900mg/ml for water, 600 mg/ml methanol extracts and at 200 mg/ml for the chloroform extracts.

C. albicans exhibited degrees of sensitivity to methanol and water extracts of both green and fermented rooibos whilst ethyl acetate and chloroform rooibos extracts were inactive against the fungus. Green methanol rooibos extract was the most active against *C. albicans*. When comparing the activity of green and fermented rooibos extracts against *C. albicans*, the methanol extracts proved most potent. The antimicrobial activity of green methanol extract was two folds greater than that of the weaker fermented water extract. At 300 mg/ml the most significant (P<0.05) comparison of green and fermented methanol rooibos extracts was observed, whilst for the aqueous extracts the most significant (P<0.05) comparison of the two forms of rooibos was recorded at 100 mg/ml and 900 mg/ml. Generally the methanol extracts displayed greater antimicrobial activity in comparison to the water and chloroform (displayed selective activity) extracts. The fermented form extracted by the appropriate solvent (*S. aureus*- methanol and *S. pyogenes*- chloroform) resulted in increased inhibitory activity.

Table 3.1: Inhibition of the bacteria *Psedomonas aeruginosa, Staphylococcus aureus*, *Streptococcus pyogenes* and fungus *Candida albicans* by varying concentrations of Rooibos extracts

Microorganisms	Extraction solvent	Conc (mg/ml)	Average zones of inhibition (mm±SEM)		Statistical significance (P)
			RG [a]	RF [b]	ab
Pseudomonas aeruginosa	Water		-		
	Methanol		-		
	Chloroform		-		

	Ethyl acetate		**-**		
Positive control	**Ciprofloxacin**	**5 µg/ml**	**12 mm**		
Staphylococcus aureus	**Water**	**200**	**0.2±0.17**	**1.2±0.17**	**0.0132 ***
		300	1.2±0.17	1.7±0.17	0.1012
		600	1.8±0.17	2.2±0.17	0.2302
		900	3.7±0.17	4.2±0.17	0.1012
	Methanol	50	0.3±0.17	0.8±0.17	0.1012
		100	0.8±0.17	1.5±0.29	0.1161
		150	**1.2±0.17**	**2.2±0.17**	**0.0132 ***
		200	**2.2±0.17**	**3.2±0.17**	**0.0132 ***
		300	**3.7±0.33**	**4.8±0.17**	**0.0352 ***
		500	**4.7±0.33**	**7.3±0.33**	**0.0048 ****
		600	**6.3±0.33**	**9.3±0.33**	**0.0031 ****
	Chloroform		**-**		
	Ethyl acetate		**-**		
Positive control	**Ampicillin**	**10 µg/ml**	**13 mm**		
Streptococcus pyogenes	**Water**	**100**	**2.2±0.17**	**0.2±0.17**	**0.0011****
		150	**2.7±0.33**	**0.7±0.17**	**0.0058****
		200	**4.3±0.33**	**1.3±0.33**	**0.0031****
		300	**4.8±0.17**	**1.7±0.33**	**0.0011****
		600	**6.3±0.33**	**2.5±0.29**	**0.001*****
		900	**7.7±0.33**	**3.8±0.17**	**0.0005*****
	Methanol	50	1.2±0.17	0.7±0.33	0.2508
		100	2.3±0.33	1.3±0.17	0.055
		150	**3.2±0.17**	**2.2±0.17**	**0.0132***
		200	3.5±0.29	3.2±0.17	0.3739
		300	4.2±0.17	4.2±0.17	1
		500	**4.6±0.33**	**6.3±0.33**	**0.0241***
		600	**6.8±0.17**	**8.2±0.17**	**0.0048****

		50	**2.2±0.17**	**1.2±0.17**	**0.0132***
	Chloroform	100	3.2±0.17	3.2±0.17	1
		150	**3.7±0.17**	**5.2±0.17**	**0.0031****
		200	**5.2±0.17**	**7.2±0.17**	**0.0011****
	Ethyl acetate			**-**	
Positive control	**Ampicillin**	**13 µg/ml**		**15 mm**	
Candida albicans	**Water**	50	1.3±0.33	0.5±0.29	0.1318
		100	**2.2±0.17**	**1.2±0.17**	**0.0132***
		150	2.3±0.33	1.7±0.33	0.2302
		200	2.8±0.17	2.3±0.33	0.2508
		300	3.3±0.33	3.2±0.17	0.6779
		600	5.3±0.33	4.3±0.33	0.1012
		900	**7.3±0.33**	**5.3±0.33**	**0.0132***
	Methanol	50	0.2±0.17	0.5±0	0.1049
		100	0.3±0.17	0.8±0.17	0.1012
		150	1.2±0.17	1±0	0.4211
		200	2.3±0.33	1.7±0.33	0.2302
		300	**4±0**	**2.3±0.33**	**0.0073****
		500	4.7±0.33	3.7±0.33	0.1012
		600	6.7±0.33	5.7±0.33	0.1012
	Chloroform			**-**	
	Ethyl acetate			**-**	
Positive control	**Fluconazole**	**25 µg/ml**		**10 mm**	

***Abbreviations*:** Conc: concentration; mg/ml: milligram per millilitre; mm: millimeter; µg/ml: microgram per milliliter; SEM: standard error of the mean (for *n*=3); RG: rooibos green; RF: rooibos fermented. * *P*<0.05; ***P*<0.01; ****P*<0.001. **-**: No inhibition of specific microorganisms by the rooibos extracts.

3.3.1.2 Broth micro-dilution assay- Minimum Inhibitory Concentration

The lowest concentration of the extracts that inhibited the growth of the microbes was determined using the broth micro-dilution assay. Water and methanol rooibos (green and fermented) extracts exhibited activity against *S. aureus, S. pyogenes* and *C. albicans.* The green and fermented forms of chloroform extracts selectively inhibited *S. pyogenes*. Water was observed to weakly extract antimicrobial active components from both green/unfermented and fermented rooibos. Fermented rooibos methanol, fermented rooibos chloroform and green rooibos methanol were most active against *S. aureus* (MIC- 37.5 mg/ml), *S. pyogenes* (MIC- 12.5 mg/ml) and *C. albicans* (MIC- 18.75 mg/ml) respectively (Table 3.2). According to the study, fermentation increased the antibacterial activity of the rooibos extract whilst the unfermented form of rooibos possessed significant antifungal activity. *P. aeruginosa* continued to exhibit resistant characteristics in the presence of varying concentrations of the rooibos extracts.

Table 3.2: MIC (mg/ml) values of extracts derived from *A. linearis* (Rooibos)

Test microorganism	Rooibos extracts	MIC (mg/ml)
Pseudomonas aeruginosa	-	
Staphylococcus aureus	**RGW**	**150**
	RFW	**150**
	RGM	**75**
	RFM	**37.5**
Streptococcus pyogenes	**RGW**	**75**
	RFW	**150**
	RGM	**75**
	RFM	**50**
	RGC	**25**
	RFC	**12.5**
Candida albicans	**RGW**	**37.5**
	RFW	**75**
	RGM	**18.75**
	RFM	**37.5**

***Abbreviations**:* mg/ml: milligrams per millilitre; MIC: minimum inhibitory concentration; RGW: rooibos green water; RFW: rooibos fermented water; RGM: rooibos green methanol; RFM: rooibos fermented methanol; RGC: rooibos green chloroform; RFC: rooibos fermented chloroform.

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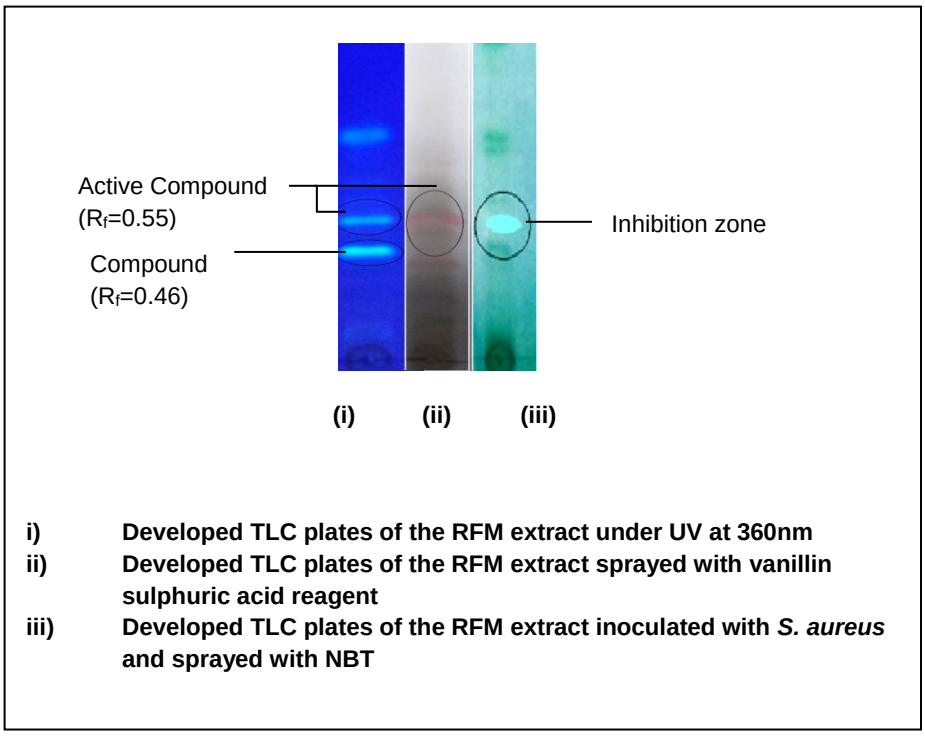
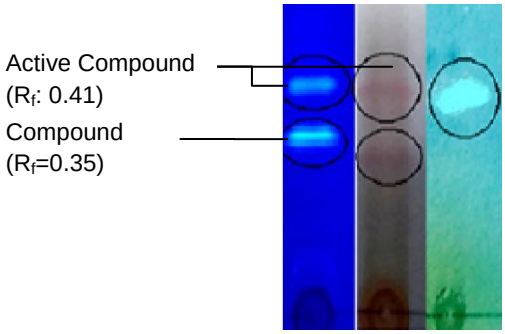
3.3.1.3 Thin Layer Chromatography (TLC) – Bioautography

The thin layer chromatography profiles of the most potent extracts against *S. aureus, S. pyogenes* and *C. albicans* were analyzed (Figure 3.1) under UV at 360 nm and sprayed with vanillin- sulphuric acid reagent then heated (noted as (i) and (ii) respectively). The bioautogram of the fermented rooibos methanol, fermented rooibos chloroform and green rooibos extract against *S. aureus*, *S. pyogenes* and *C. albicans* respectively were also reported (noted as (iii)) and a zone of inhibition caused by the active compound within the specific extract were observed. The detected active compound against *S. aureus* within fermented rooibos methanol had a retardation factor (R_f) value of 0.55 corresponding with a reddish-pink compound on the TLC plate. The active antimicrobial compound in fermented rooibos chloroform extract against *S. pyogenes* corresponded with a dark pink band on the TLC plate had a R_f of 0.41. Rooibos green methanol extract possessed an active compound against *C. albicans* with a R_f of 0.50, corresponding with a yellow-brown band on the TLC plate. This assay revealed the existence of two distinct antimicrobial compounds present within the fermented form of rooibos and a single compound within the green, evident by the different R_f values possessed by the compounds.

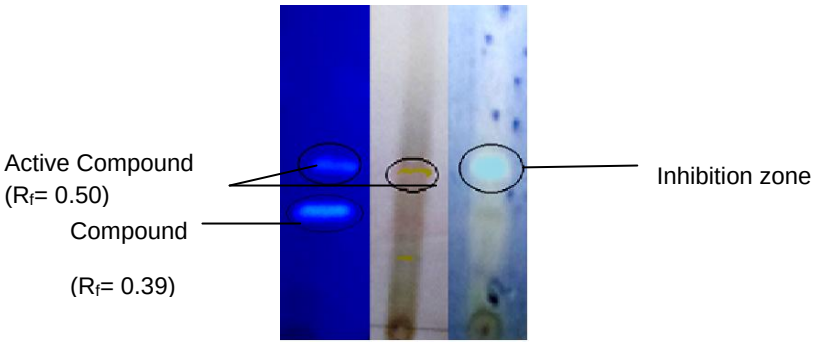


i) **Developed TLC plates of the RFM extract under UV at 360nm**
ii) **Developed TLC plates of the RFM extract sprayed with vanillin sulphuric acid reagent**
iii) **Developed TLC plates of the RFM extract inoculated with *S. aureus* and sprayed with NBT**



(i) (ii) (iii)

i) Developed TLC plates of the RFC extract under UV at 360nm.
ii) Developed TLC plates of the RFC extract sprayed with vanillin sulphuric acid reagent.
iii) Developed TLC plates of the RFC extract inoculated with *S. pyogenes* and sprayed with NBT.



(i) (ii) (iii)

i) Developed TLC plates of the RGM extract under UV at 360nm
ii) Developed TLC plates of the RGM extract sprayed with vanillin sulphuric acid reagent
iii) Developed TLC plates of the RGM extract inoculated with *C. albicans* and sprayed with NBT

Figure 3.1: The antimicrobial activity of RFM, RFC and RGM extract against *S. aureus, S.pyogenes* and *C. albicans* respectively

***Abbreviations*:** TLC: thin layer chromatography; RFM: rooibos fermented methanol; RFC: rooibos fermented chloroform; RGM: rooibos green methanol; R_f: retardation factor; NBT: nitrotetrazolium blue chloride.

3.3.2 The antioxidant activity of rooibos extracts

3.3.2.1 Oxygen Radical Absorbance Capacity

The total antioxidant capacity of green and fermented rooibos extracts were analyzed and reported. The significant (P<0.05) difference when comparing the antioxidant levels present in each extract at specific concentrations was also reported (Table 3.3 and 3.4). The most significant rooibos green extract comparisons were noted at both 5 mg/ml and 15 mg/ml extract concentrations. However two exceptions existed whereby no significant difference (P>0.05) existed between rooibos green chloroform and ethyl acetate (5 mg/ml and 15 mg/ml extract concentration). At 0.5 mg/ml extract concentration, the difference between the different extracts was statistically not significant. The highest antioxidant activity was observed in the green rooibos water extract and the least in the green ethyl acetate extracts.

Fermented rooibos extracts generally had lower total antioxidant capacity in comparison to those of green rooibos as shown in Figure 3.2. Statistically significant (P<0.05) comparisons between the various rooibos fermented extracts existed at 5mg/ml and 15mg/ml except when rooibos fermented water and rooibos fermented chloroform where compared (Table 3.4). Rooibos fermented methanol and chloroform were respectively observed as the extracts with the highest and lowest total antioxidant capacity.

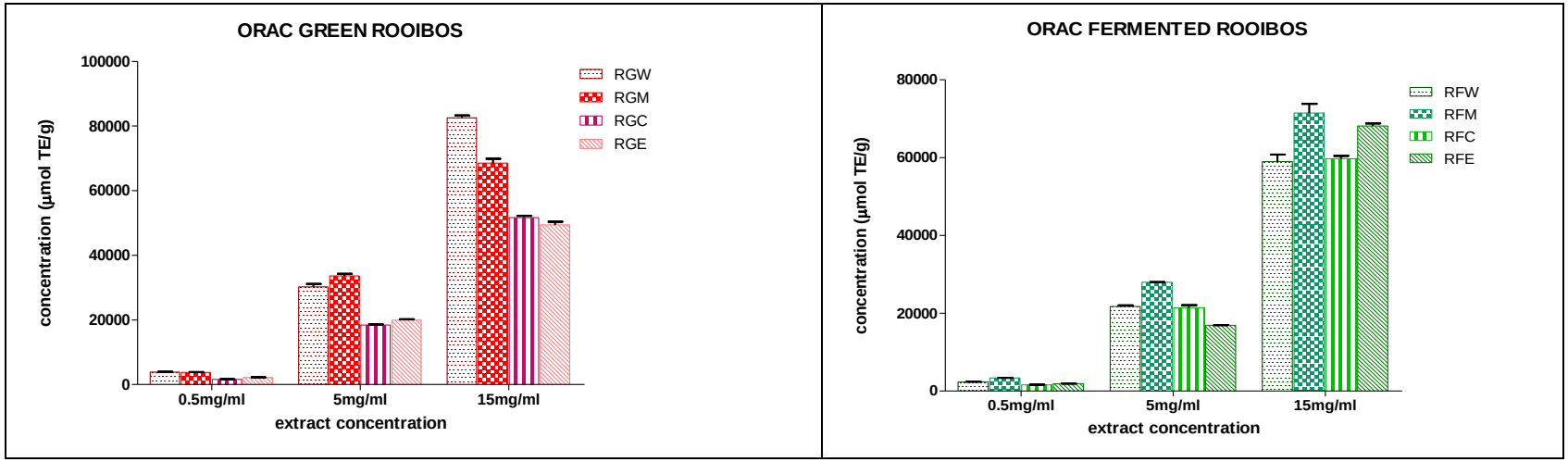


Figure 3.2: Direct measurement of the antioxidant capacity of green and fermented rooibos extracts using the ORAC assay

***Abbreviations*:** µmol TE/g: micromoles of Trolox® equivalents per gram; ORAC: oxygen radical absorbance capacity; mg/ml: milligram per milliliter.

Extracts

RGW: Green rooibos water

RGM: Green rooibos methanol

RGC: Green rooibos chloroform

RGE: Green rooibos ethyl acetate

RFW: Fermented rooibos water

RFM: Fermented rooibos methanol

RFC: Fermented rooibos chloroform

RFE: Fermented rooibos ethyl acetate

Table 3.3: Statistical comparison of the ORAC values of green rooibos extracts

Green rooibos						
Extract Conc (mg/ml)	**Average ORAC conc (µmol TE/g)±SD**				**Statistical comparison**	**Statistical significance (*P*)**
	RGW	**RGM**	**RGC**	**RGE**		
0.5	3831±208	3757±111	1597±115	2151±145	RGW: RGM	ns
					RGW: RGC	ns
					RGW: RGE	ns
					RGM: RGC	ns
					RGM: RGE	ns
					RGC: RGE	ns
5	30250±1476	33610±1149	18400±363	19940±452	**RGW: RGM**	**< 0.01****
					RGW: RGC	**<0.001*****
					RGW: RGE	**<0.001*****
					RGM: RGC	**<0.001*****
					RGM: RGE	**<0.001*****
					RGC: RGE	ns
15	82510±1248	68540±2283	51620±1080	49410±1660	**RGW: RGM**	**<0.001*****
					RGW: RGC	**<0.001*****
					RGW: RGE	**<0.001*****
					RGM: RGC	**<0.001*****
					RGM: RGE	**<0.001*****
					RGC: RGE	ns

Abbreviations: conc: concentration; mg/ml: milligram per milliliter; ns: not significant; SD: standard deviation. ** *P*<0.01; ****P*<0.001. Average for *n*=3.

Extract comparisons

RGW: RGM- green rooibos water versus green rooibos methanol

RGW: RGE- green rooibos water versus green rooibos ethyl acetate

RGW: RGE- green rooibos water versus green rooibos ethyl acetate

RGM: RGC- green rooibos methanol versus green rooibos chloroform

RGM: RGE- green rooibos methanol versus green rooibos ethyl acetate

RGC: RGE- green rooibos chloroform versus green rooibos ethyl acetate

Table 3.4: Statistical comparison of the ORAC values of fermented rooibos extracts

Fermented rooibos						
Extract conc (mg/ml)	**Average ORAC conc (µmol TE/g)±SD**				**Statistical comparison**	**Statistical significance (*P*)**
	RFW	**RFM**	**RFC**	**RFE**		
0.5	2324±157	3350±115	1582±114	1890±48	RFW: RFM	ns
					RFW: RFC	ns
					RFW: RFE	ns
					RFM: RFC	ns
					RFM: RFE	ns
					RFC: RFE	ns
5	21730±481	27970±37	21420±1081	16920±58	**RFW: RFM**	**<0.001*** **
					RFW: RFC	ns
					RFW: RFE	**<0.01** **
					RFM: RFC	**<0.001*** **
					RFM: RFE	**<0.001*** **
					RFC: RFE	**<0.01** **
15	58970±3118	71490±4032	59710±1254	68110±1187	**RFW: RFM**	**<0.001*** **
					RFW: RFC	ns
					RFW: RFE	**<0.001*** **
					RFM: RFC	**<0.001*** **
					RFM: RFE	**<0.05***
					RFC: RFE	**<0.001*** **

Abbreviations: conc: concentration; mg/ml: milligram per milliliter; ns: not significant; SD: standard deviation. * *P*<0.05; ** *P*<0.01; *** *P*<0.001. Average for *n*=3.

Extract comparisons

RFW: RFM- fermented rooibos water versus fermented rooibos methanol

RFW: RFC- fermented rooibos water versus fermented rooibos chloroform

RFW: RFE- fermented rooibos water versus fermented rooibos ethyl acetate

RFM: RFC- fermented rooibos methanol versus fermented rooibos chloroform

RFM: RFE- fermented rooibos methanol versus fermented rooibos ethyl acetate

RFC: RFE- fermented rooibos chloroform versus fermented rooibos ethyl acetate

3.3.2.2 Ferric Reducing Ability of Plasma

The reducing ability of antioxidants in green and fermented rooibos extracts were measured and reported (Figure 3.3). The statistical significance ($P<0.05$) of comparing the antioxidant levels present in each extract at specific concentrations was also reported (Table 3.5 and 3.6). The antioxidant ferric reducing ability was linearly proportional to the extract concentrations, the higher the extract concentration the greater the reducing ability. The highest reducing potential for green rooibos extracts was observed in rooibos green methanol extracts and the least in rooibos green chloroform extracts. At 0.5 mg/ml, 5 mg/ml and 15 mg/ml all differences between the different rooibos green extracts were significant ($P<0.05$) as shown in Table 3.10. According to our observation the rooibos green extracts generally possessed a stronger ability to reduce ferric ions than the rooibos fermented extracts (Figure 3.3).

Rooibos fermented methanol extracts were reported in Table 3.6 to have a higher ferric reducing ability whilst rooibos fermented ethyl acetate extracts possed the least ferric reducing ability among the rooibos fermented extracts. All rooibos fermented extract comparisons (0.5-15mg/ml) were significant ($P<0.05$) except for the difference between fermented rooibos chloroform and ethyl acetate which was statistically not significant ($P>0.05$), as shown in Table 3.6.

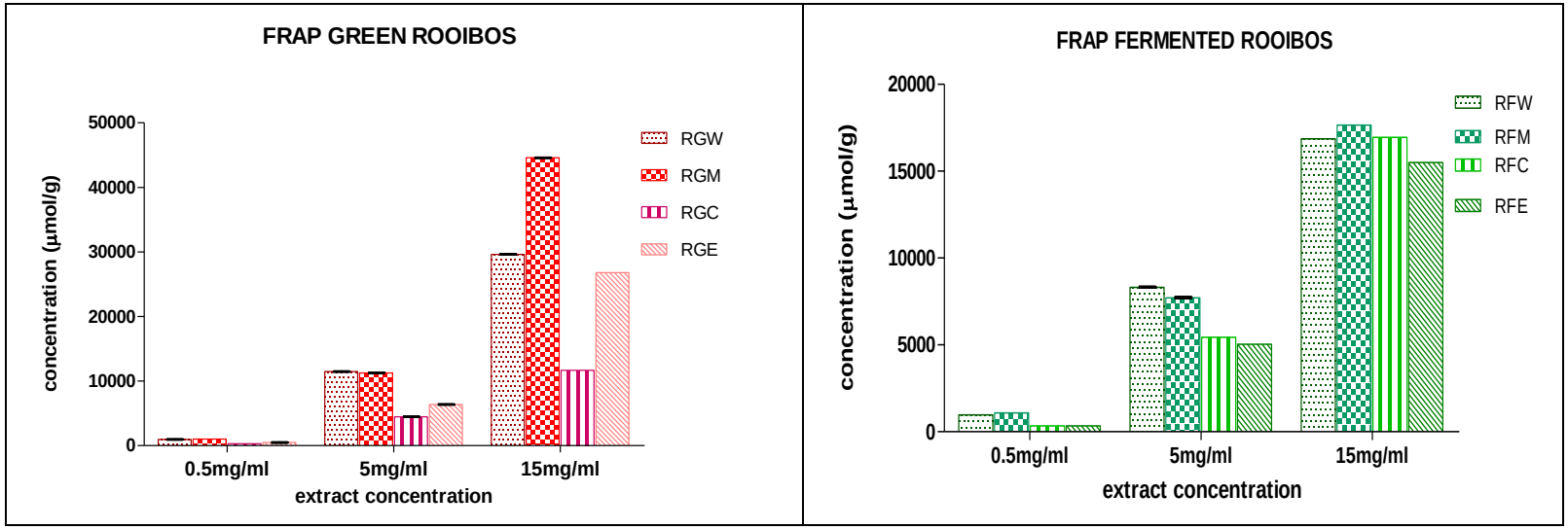


Figure 3.3: Direct measurement of the antioxidant capacity of green and fermented rooibos extracts using the FRAP assay

***Abbreviations*:** µmol /g: micromoles per gram; FRAP: Ferric reducing ability of plasma; mg/ml: milligram per milliliter.

Extracts

RGW: Green rooibos water

RGM: Green rooibos methanol

RGC: Green rooibos chloroform

RGE: Green rooibos ethyl acetate

RFW: Fermented rooibos water

RFM: Fermented rooibos methanol

RFC: Fermented rooibos chloroform

RFE: Fermented rooibos ethyl acetate

Table 3.5: Statistical comparison of the FRAP values of green rooibos extracts

Green rooibos						
Extract conc (mg/ml)	**Average FRAP conc (µmol /g)±SD**				**Statistical comparison**	**Statistical significance (*P*)**
	RGW	**RGM**	**RGC**	**RGE**		
0.5	949.1±37	1024±5	297.7±7	478.3±17	**RGW: RGM**	**<0.001*** **
					RGW: RGC	**<0.001*** **
					RGW: GRE	**<0.001*** **
					RGM: RGC	**<0.001*** **
					RGM: GRE	**<0.001*** **
					RGC: GRE	**<0.001*** **
5	11480±27	11270±12	4458±23	6361±28	**RGW: RGM**	**<0.001*** **
					RGW: RGC	**<0.001*** **
					RGW: GRE	**<0.001*** **
					RGM: RGC	**<0.001*** **
					RGM: GRE	**<0.001*** **
					RGC: GRE	**<0.001*** **
15	29630±32	44580±11	11680±7	26800±11	**RGW: RGM**	**<0.001*** **
					RGW: RGC	**<0.001*** **
					RGW: GRE	**<0.001*** **
					RGM: RGC	**<0.001*** **
					RGM: GRE	**<0.001*** **
					RGC: GRE	**<0.001*** **

***Abbreviations*:** conc: concentration; mg/ml: milligram per milliliter; ns: not significant; SD: standard deviation. ****P*<0.001. Average for *n*=3.

Extract comparisons

RGW: RGM- green rooibos water versus green rooibos methanol

RGW: RGC- green rooibos water versus green rooibos chloroform

RGW: GRE- green rooibos water versus green rooibos ethyl acetate

RGM: RGC- green rooibos methanol versus green rooibos chloroform

RGM: RGE- green rooibos methanol versus green rooibos ethyl acetate

RGC: RGE- green rooibos chloroform versus green rooibos ethyl acetate

Table 3.6: Statistical comparison of the FRAP values of fermented rooibos extracts

Fermented rooibos						
Extract conc (mg/ml)	**Average FRAP conc (µmol /g)±SD**				**Statistical comparison**	**Statistical significance (*P*)**
	RFW	**RFM**	**RFC**	**RFE**		
0.5	971.7±1.3	1089±2.1	332.3±1.1	336.1±0.8	**RFW: RFM**	**<0.001*** **
					RFW: RFC	**<0.001*** **
					RFW: RFE	**<0.001*** **
					RFM: RFC	**<0.001*** **
					RFM: RFE	**<0.001*** **
					RFC: RFE	ns
5	8318±14.7	7714±2.1	5434±1.2	5037±0.6	**RFW: RFM**	**<0.001*** **
					RFW: RFC	**<0.001*** **
					RFW: RFE	**<0.001*** **
					RFM: RFC	**<0.001*** **
					RFM: RFE	**<0.001*** **
					RFC: RFE	**<0.001*** **
15	16860±1.6	17650±1	16950±0.6	15490±0.6	**RFW: RFM**	**<0.001*** **
					RFW: RFC	**<0.001*** **
					RFW: RFE	**<0.001*** **
					RFM: RFC	**<0.001*** **
					RFM: RFE	**<0.001*** **
					RFC: RFE	**<0.001*** **

Abbreviations: conc: concentration; mg/ml: milligram per milliliter; ns: not significant; SD: standard deviation. ****P*<0.001. Average for *n*=3.

Extract comparisons

RFW: RFM- fermented rooibos water versus fermented rooibos methanol

RFW: RFC- fermented rooibos water versus fermented rooibos chloroform

RFW: RFE- fermented rooibos water versus fermented rooibos ethyl acetate

RFM: RFC- fermented rooibos methanol versus fermented rooibos chloroform

RFM: RFE- fermented rooibos methanol versus fermented rooibos ethyl acetate

RFC: RFE- fermented rooibos chloroform versus fermented rooibos ethyl acetate

3.3.2.3 Trolox Equivalent Antioxidant Capacity

The radical scavenging ability of green and fermented rooibos extracts was measured using the TEAC assay. The statistical significance (P<0.05) of comparing the antioxidant levels with radical scavenging ability present in each extract at specific concentrations was also reported (Table 3.7 and 3.8). This ability was directly proportional to the extract concentrations, the higher the extract concentration the greater their radical scavenging ability.

The highest radical scavenging ability of the green rooibos extracts was observed in green water extract, and the lowest in green chloroform extracts. At all studied rooibos green extract concentrations (0.5-15 mg/ml), statistically significant (P<0.05) differences between all compared extracts (Table 3.12) were reported. In this instance, the rooibos green extracts had an overall higher radical scavenging ability compared to that possessed by the rooibos fermented extracts (Figure 3.4).

The statistical comparison trends of the radical scavenging ability of different rooibos fermented extracts at increasing extract concentrations (0.5-15mg/ml) were similar to those reported for rooibos green extracts. All compared extracts were significantly different (P<0.05) at 0.5-15mg/ml extract concentrations (Table 3.8). Rooibos fermented methanol and chloroform extracts were reported to posses the highest and lowest radical scavenging ability respectively (Figure 3.4 and Table 3.8).

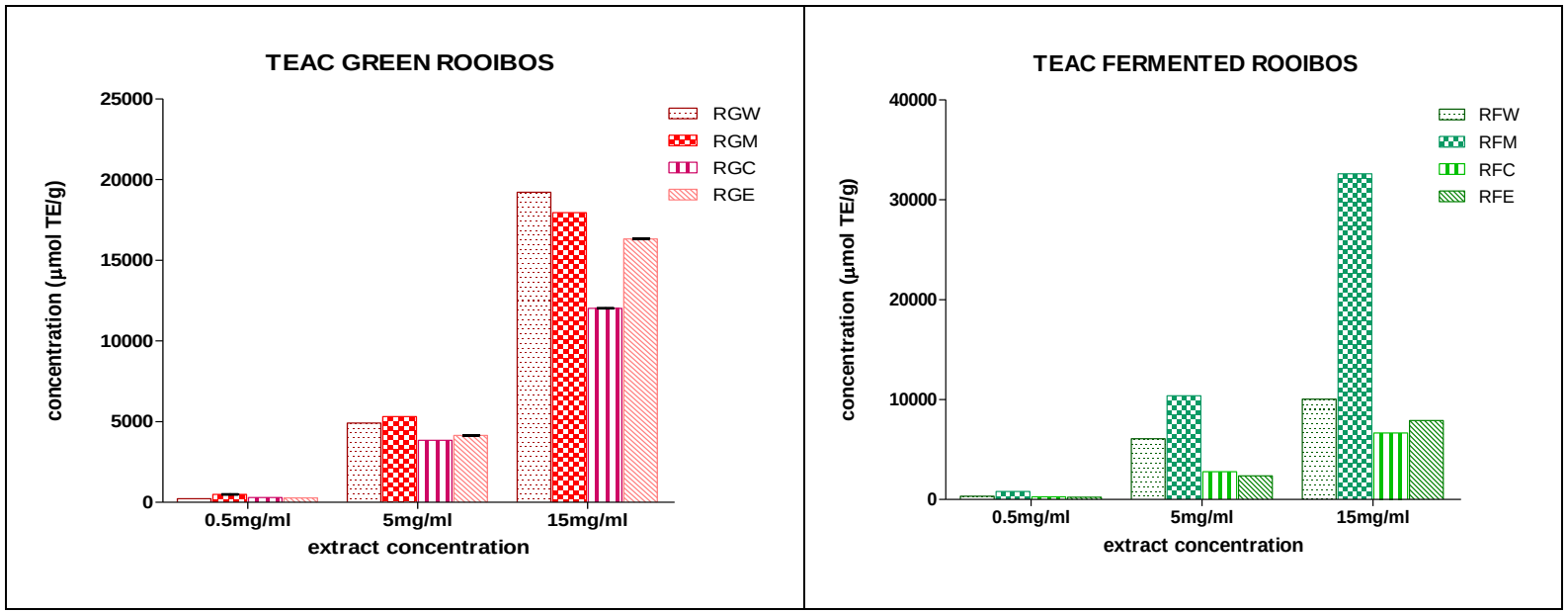


Figure 3.4: Direct measurement of the antioxidant activity of green and fermented rooibos extracts using the TEAC assay

***Abbreviations*:** µmol TE/g: micromoles of Trolox® equivalents per gram; TEAC: Trolox equivalent antioxidant capacity; mg/ml: milligram per milliliter.

Extracts

RGW: Green rooibos water

RGM: Green rooibos methanol

RGC: Green rooibos chloroform

RGE: Green rooibos ethyl acetate

RFW: Fermented rooibos water

RFM: Fermented rooibos methanol

RFC: Fermented rooibos chloroform

RFE: Fermented rooibos ethyl acetate

Table 3.7: Statistical comparison of the TEAC values of green rooibos extracts

Green rooibos						
Extract Conc (mg/ml)	**Average TEAC conc (µmol TE/g)±SD**				**Statistical comparison**	**Statistical significance (*P*)**
	RGW	**RGM**	**RGC**	**RGE**		
0.5	224.7±1	486.3±3.8	293.8±3.6	270.8±1.3	**RGW: RGM**	**<0.001*** **
					RGW: RGC	**<0.001*** **
					RGW: GRE	**<0.001*** **
					RGM: RGC	**<0.001*** **
					RGM: GRE	**<0.001*** **
					RGC: GRE	**<0.001*** **
5	4915±1.3	5314±0.3	3842±3.1	4144±13.9	**RGW: RGM**	**<0.001*** **
					RGW: RGC	**<0.001*** **
					RGW: GRE	**<0.001*** **
					RGM: RGC	**<0.001*** **
					RGM: GRE	**<0.001*** **
					RGC: GRE	**<0.001*** **
15	19210±1.9	17950±5.77	12010±3	16330±2	**RGW: RGM**	**<0.001*** **
					RGW: RGC	**<0.001*** **
					RGW: GRE	**<0.001*** **
					RGM: RGC	**<0.001*** **
					RGM: GRE	**<0.001*** **
					RGC: GRE	**<0.001*** **

Abbreviations: conc: concentration; mg/ml: milligram per milliliter; ns: not significant; SD: standard deviation. *** *P*<0.001. Average for *n*=3.

Extract comparisons

RGW: RGM- green rooibos water versus green rooibos methanol

RGW: RGC- green rooibos water versus green rooibos chloroform

RGW: GRE- green rooibos water versus green rooibos ethyl acetate

RGM: RGC- green rooibos methanol versus green rooibos chloroform

RGM: RGE- green rooibos methanol versus green rooibos ethyl acetate

RGC: RGE- green rooibos chloroform versus green rooibos ethyl acetate

Table 3.8: Statistical comparison of the TEAC values of fermented rooibos extracts

	Fermented rooibos					
Concentration (mg/ml)	**TEAC values (µmol TE/g)±SD**				**Statistical comparison**	**Statistical significance (*P*)**
	RFW	**RFM**	**RFC**	**RFE**		
0.5	323.5±1.3	792.9±1	262.8±3.6	224.4±1.4	**RFW: RFM**	**<0.001*** **
					RFW: RFC	**<0.001*** **
					RFW: RFE	**<0.001*** **
					RFM: RFC	**<0.001*** **
					RFM: RFE	**<0.001*** **
					RFC: RFE	**<0.001*** **
5	6 077±1.3	10380±1.4	2 773±1.6	2 372±2.4	**RFW: RFM**	**<0.001*** **
					RFW: RFC	**<0.001*** **
					RFW: RFE	**<0.001*** **
					RFM: RFC	**<0.001*** **
					RFM: RFE	**<0.001*** **
					RFC: RFE	**<0.001*** **
15	10040±2.5	32600±2.1	6657±3.7	7908±1.5	**RFW: RFM**	**<0.001*** **
					RFW: RFC	**<0.001*** **
					RFW: RFE	**<0.001*** **
					RFM: RFC	**<0.001*** **
					RFM: RFE	**<0.001*** **
					RFC: RFE	**<0.001*** **

Abbreviations: conc: concentration; mg/ml: milligram per milliliter; ns: not significant. *** *P*<0.001. Average for *n*= 3.

Extract comparisons

RFW: RFM- fermented rooibos water versus fermented rooibos methanol

RFW: RFC- fermented rooibos water versus fermented rooibos chloroform

RFW: RFE- fermented rooibos water versus fermented rooibos ethyl acetate

RFM: RFC- fermented rooibos methanol versus fermented rooibos chloroform

RFM: RFE- fermented rooibos methanol versus fermented rooibos ethyl acetate

RFC: RFE- fermented rooibos chloroform versus fermented rooibos ethyl acetate

3.3.3 Phytochemical assays

3.3.3.1 Total Polyphenols

The total polyphenol content of green and fermented rooibos extracts was measured and reported (Figure 3.5 and Table 3.9, 3.10). The statistical significance ($P<0.05$) of comparing the total polyphenols present in each extract at specific concentrations was also reported (Table 3.9 and 3.10). The polyphenol content was directly related to the extract concentration analyzed, the higher the extract concentration the greater the total polyphenolic content observed. For green rooibos, the highest total polyphenol concentration was observed in methanol and the least in chloroform extracts. At 5 and 15 mg/ml the rooibos green extracts were significantly different from each other except for the comparison of rooibos green methanol and water and also rooibos green chloroform and ethyl acetate, both at 5 mg/ml (Table 3.9). Generally the rooibos green extracts had higher total polyphenol concentrations than fermented rooibos extracts (Figure 3.5).

The rooibos fermented water extract had high total polyphenol content in comparison to the remaining rooibos fermented extracts whilst the rooibos fermented ethyl acetate extract had the lowest polyphenolic content. All rooibos fermented extract comparisons (Table 3.10) revealed significant ($P<0.05$) differences at 5 and 15 mg/ml except for the comparison between rooibos fermented chloroform and rooibos fermented ethyl acetate at 15mg/ml.

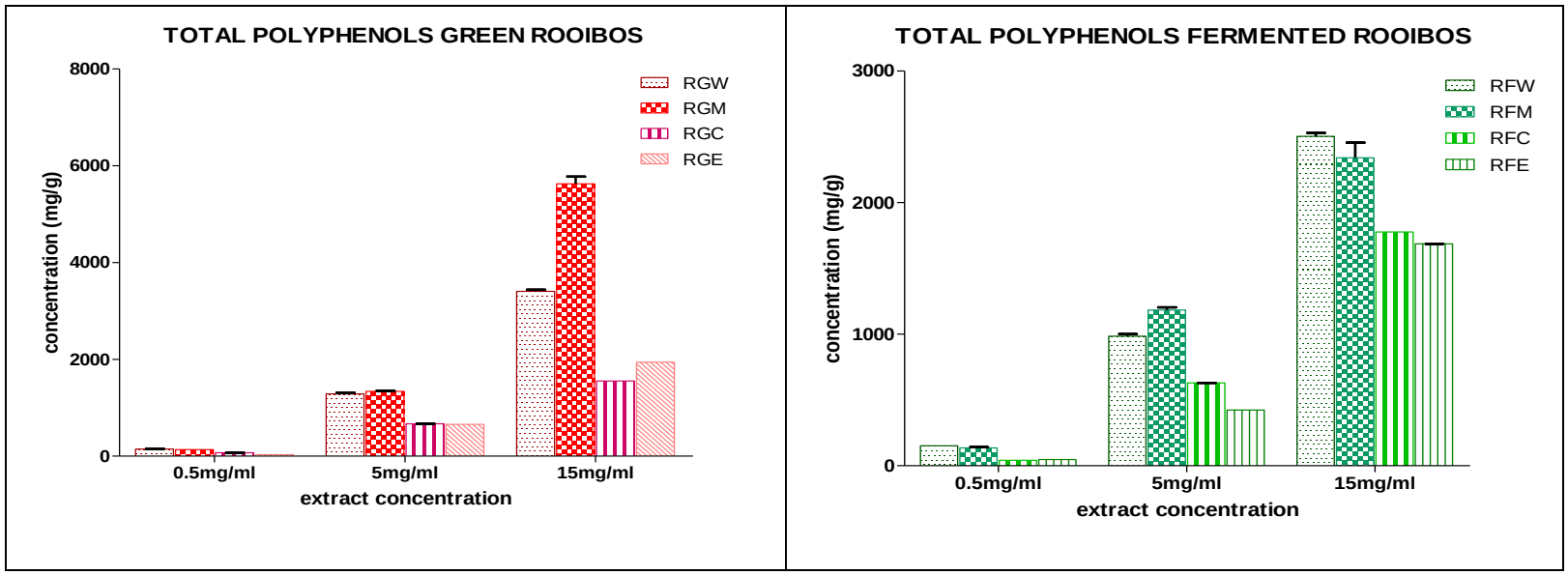


Figure 3.5: Quantifying the amount of polyphenols present in green and fermented rooibos extracts using the Total polyphenols assay.

***Abbreviations*:** mg/g: milligrams per gram; mg/ml: milligram per milliliter.

Extracts

RGW: Green rooibos water

RGM: Green rooibos methanol

RGC: Green rooibos chloroform

RGE: Green rooibos ethyl acetate

RFW: Fermented rooibos water

RFM: Fermented rooibos methanol

RFC: Fermented rooibos chloroform

RFE: Fermented rooibos ethyl acetate

Table 3.9: Statistical comparison of the total polyphenols values of green rooibos extracts

Green rooibos						
Extract conc (mg/ml)	**Average Total polyphenols conc (mg/g)**				**Statistical comparison**	**Statistical significance (*P*)**
	RGW	**RGM**	**RGC**	**RGE**		
0.5	146±5	136.1±0	68.74±0.6	30.98±0	RGW: RGM	ns
					RGW: RGC	ns
					RGW: GRE	ns
					RGM: RGC	ns
					RGM: GRE	ns
					RGC: GRE	ns
5	1284±45	1340±20	670.1±0.6	657.6±0.6	RGW: RGM	ns
					RGW: RGC	**<0.001*** **
					RGW: GRE	**<0.001*** **
					RGM: RGC	**<0.001*** **
					RGM: GRE	**<0.001*** **
					RGC: GRE	ns
15	3403±62	5624±257	1554±0.6	1942±0.6	**RGW: RGM**	**<0.001*** **
					RGW: RGC	**<0.001*** **
					RGW: GRE	**<0.001*** **
					RGM: RGC	**<0.001*** **
					RGM: GRE	**<0.001*** **
					RGC: GRE	**<0.001*** **

Abbreviations: mg/ml: milligram per milliliter; ns: not significant. ****P*<0.001. Average for *n*=3.

Extract comparisons

RGW: RGM- green rooibos water versus green rooibos methanol

RGW: RGC- green rooibos water versus green rooibos chloroform

RGW: GRE- green rooibos water versus green rooibos ethyl acetate

RGM: RGC- green rooibos methanol versus green rooibos chloroform

RGM: RGE- green rooibos methanol versus green rooibos ethyl acetate

RGC: RGE- green rooibos chloroform versus green rooibos ethyl acetate

Table 3.10: Statistical comparison of the total polyphenols values of fermented rooibos extracts

Fermented rooibos						
Concentration (mg/ml)	**Total polyphenol content**				**Statistical comparison**	**Statistical significance (*P*)**
	RFW	**RFM**	**RFC**	**RFE**		
0.5	151.8±0.9	135.5±9.8	41.79±0.6	47.25±0.6	RFW: RFM	ns
					RFW: RFC	ns
					RFW: RFE	ns
					RFM: RFC	ns
					RFM: RFE	ns
					RFC: RFE	ns
5	984±29	1184±35	629.6±0.6	423.8±0.6	**RFW: RFM**	**<0.01****
					RFW: RFC	**<0.001*****
					RFW: RFE	**<0.001*****
					RFM: RFC	**<0.001*****
					RFM: RFE	**<0.001*****
					RFC: RFE	**<0.01****
15	2504±46	2341±119	1777±0.6	1686±0.6	**RFW: RFM**	**<0.01****
					RFW: RFC	**<0.001*****
					RFW: RFE	**<0.001*****
					RFM: RFC	**<0.001*****
					RFM: RFE	**<0.001*****
					RFC: RFE	ns

Abbreviations: conc: concentration; mg/ml: milligram per milliliter; ns: not significant. ** *P*<0.01; ****P*<0.001. Average for *n*=3.

Extract comparisons

RFW: RFM- fermented rooibos water versus fermented rooibos methanol

RFW: RFC- fermented rooibos water versus fermented rooibos chloroform

RFW: RFE- fermented rooibos water versus fermented rooibos ethyl acetate

RFM: RFC- fermented rooibos methanol versus fermented rooibos chloroform

RFM: RFE- fermented rooibos methanol versus fermented rooibos ethyl acetate

RFC: RFE- fermented rooibos chloroform versus fermented rooibos ethyl acetate

3.3.3.2 High Performance Liquid Chromatography (HPLC)

The quantity of the flavonoids aspalathin, orientin, isoorientin, isovetexin, vetexin, hyperroside, quercetin and luteolin present in the green and fermented rooibos extracts was measured (appendix) and the concentrations were reported (Table 3.11). The water, methanol and ethyl acetate extracts of green rooibos were reported to contain high concentrations of aspalathin, orientin and isoorientin, with rooibos green water extract containing the highest levels. Rooibos green chloroform was the only extract in which all the flavonoids tested were absent. In addition rooibos fermented ethyl acetate and chloroform were reported to have the least concentrations of the flavonoids tested.

Table 3.11: Quantification of flavonoids (mg/g) in rooibos extracts

	Flavonoid concentrations (mg/g)							
Extract	**Aspalathin**	**Orientin**	**Isoorientin**	**Isovetixin**	**Vetexin**	**Hyperoside**	**Quercetin**	**Luteolin**
RGW	98.73	10.38	11.99	1.78	1.99	10.40	0.00	0.00
RFW	4.18	3.41	6.53	1.24	1.28	3.15	0.15	0.064
RGM	64.36	5.23	4.43	0.52	1.49	7.39	0.00	0.00
RFM	3.63	2.79	3.72	4.53	1.51	2.81	0.77	0.33
RGC	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
RFC	0.00	0.00	0.00	0.00	0.00	0.00	0.92	0.83
RGE	20.64	1.90	1.70	0.37	0.82	2.84	0.21	1.26
RFE	0.00	0.00	0.00	0.00	0.00	0.00	0.47	0.44

Abbreviations: mg/g: milligram per gram.

Extracts

RGW: Green rooibos water

RFW: Fermented rooibos water

RGM: Green rooibos methanol

RFM: Fermented rooibos methanol

RGC: Green rooibos chloroform

RFC: Fermented rooibos chloroform

RGE: Green rooibos ethyl acetate

RFE: Fermented rooibos ethyl acetate

3.4 Discussion

The unique chemical composition of rooibos herbal tea has attributed to its long history of use. Numerous studies have been done on the antioxidant activities of rooibos extracts and its role in oxidative stress-associated diseases and conditions (Son *et al*., 2013; Muller *et al*., 2013; Beltrán-Debón *et al*., 2011; Sanderson *et al*., 2014; Marnewick *et al*., 2005; Magcwebeba, 2013; Sissing *et al*., 2011). However, very few studies have investigated the antimicrobial effects of this indigenous tea (Coetzee *et al.,* 2008; Murali *et al*., 2012; Simpson *et al*., 2013; Schepers, 2001; Hübsch *et al*., 2014). Therefore, little is known about the connection of these two bioactivities of rooibos. The current investigation took a step further in an attempt not only to confirm the level of antimicrobial and antioxidant capacity possessed by the different rooibos extracts, but also to investigate the relationship of these bioactivities in the rooibos extracts.

The antimicrobial activity of rooibos extracts against the bacteria *P. aeruginosa*, *S. aureus*, *S. pyogenes* and the fungus *C. albicans* was evaluated using the agar disk diffusion assay, minimum inhibitory concentration and thin layer chromatography-bioautography. The general principle of these assays is to provide the qualitative assessment of either the sensitivity or resistance of the microorganisms to the plant extracts. The minimum inhibitory concentration broth micro-dilution assay allows for further quantitative antimicrobial activity analysis.

P. aeruginosa commonly causes severe blood-stream infections and pneumonia (Driscoll *et al*., 2007). The unique structure of the Gram-negative *P. aeruginosa* cell wall has been identified as the main contributory factor to the microorganisms' resistant nature. Gram-negative bacteria unlike Gram-positive bacteria have an outer membrane that decreases the permeability of the cell wall to different agents; in this case the rooibos extracts (McGaw *et al*., 2013). In this study, *P. aeruginosa* was resistant to all the rooibos extracts. Hübsch and colleagues (2014) observed an MIC value of about 8 mg/ml when *P. aeruginosa* was exposed to a green rooibos water extract. This discrepancy could have been due to the extraction process utilized in their study which involved the grounding of the leaves to a fine powder, a step that possibly increased the efficient extraction of antimicrobially active components of the herbal tea. The growing conditions, storage time and processing of the plants could also affect the concentration of the bioactive compounds, further explaining these discrepancies observed (Piljac- Žegarac *et al*., 2013).

S. aureus is the causative agent of a variety of infections including pneumonia and central nervous system infections (Tong *et al*., 2015). Two (water and methanol) of the four extracts exhibited activity against *S. aureus* and *C. albicans. S. aureus* was most sensitive to the fermented rooibos methanol (MIC-37.5 mg/ml); whilst *C. albicans* was sensitive to the green rooibos methanol extract (MIC-18.75 mg/ml) in comparison to the other extracts. Methanol is the most commonly used solvent for preliminary investigations of antimicrobial activity in plants, and is also known for its ability to extract polyphenols (Parekh *et al.*, 2006; Lourens *et al*., 2004; Salie *et al*., 1996; Rojas *et al*., 2006; Anwar and Przybylski, 2012). Polyphenolic compounds have been associated with strong antimicrobial activity (Daglia, 2012; Cowan, 1999). According to Tian and collegues (2009), the more polar the extracting solvent the weaker the antimicrobial activity of the extract. This was made evident by the weak inhibition of the study microorganisms possessed by the greatly polar water extracts. Chloroform on the other hand is the ideal solvent for the extraction of non-polar lipophillic biologically active compounds (Harmala *et al*., 1992). In this study, green and fermented chloroform extracts exhibited selective activity, proving very active against *S. pyogenes* (green chloroform- MIC 37.5 mg/ml; fermented chloroform- MIC 12.5 mg/ml) whilst inactive against *S. aureus* and *C. albicans.* Ethyl acetate extracts proved to be inactive against all the specific microorganisms. The selective sensitivity of the microorganisms to specific rooibos extracts was due to the type of extracting solvent utilized and microbial strain as each possesses unique features and characteristics.

A noteworthy antimicrobial agent is defined as an agent with an MIC reading below 1.00 mg/ml (Ncube *et al*., 2008, Rìos and Recio, 2005). The observed antimicrobial activity in this study however only occurred at much higher concentrations; therefore rooibos cannot be classified as a noteworthy antimicrobial agent. This lack of noteworthy antimicrobial activity of rooibos is in line with other published studies. Schepers (2001) and Coetzee *et al* (2008) reported antimicrobial activity of rooibos against selected microorganisms at extract concentrations ranging between 500 mg/ml and 5000 mg/ml (Hübsch *et al*., 2014). With specific consideration to the extraction process used in this study, the single solvent extraction could have resulted in the poor extraction of the rooibos antimicrobial active components. The widely used extraction procedure is the soxhlet extraction method which is a hot continuous extraction process (Handa, 2008). Regardless of this fact, the existence of some degree of antimicrobial activity of the rooibos extracts is to be appreciated.

Bioautography is a method that provides information about the antimicrobial active components of plants, facilitating the localization of these active components on a chromatogram. Green methanol and the fermented methanol and chloroform extracts of rooibos showed a single major inhibiting fraction against *C. albicans*, *S. aureaus* and *S. pyogenes* respectively. These were indicated as a zone of inhibition on the bioautograms. The TLC analysis further showed that the fraction present in the specific extracts under UV-366 nm was the same as the compound exhibiting the antimicrobial activity, which was visible on the bioautograms (Figure 3.1). Furthermore, the bioautogram confirmed the existence of three distinct antibiotic compounds within the extracts due to the different polarity of the extraction solvents and the fermentation state of the different plantmaterial.

For further investigation of the inhibitory compound, nuclear magnetic resonance (NMR) and mass spectroscopy (MS) could be utilized to identify the active compounds found in the rooibos extracts (McGaw *et al*., 2013). These active compounds could well have a much higher degree of antimicrobial activity than that observed in the whole plant extracts. This could indicate the presence of a mixture of compounds in the whole plant that work antagonistically with each other.

The existing strong association of oxidative stress with symptoms of various diseases and conditions that cause morbidity and mortality worldwide is a great concern. These include cardiovascular disorders, cancers, diabetes and neurodegenerative disorders (Finkel and Holbrook, 2000; Madhavi *et al.*, 1996; Jomova and Valko, 2011). The severity of these conditions on human health has increased the global interest in identifying antioxidant compounds that are pharmacologically efficient with little or no side effects (Jomova and Valko, 2011). With its increasing worldwide market, the scientific interest on rooibos has also elevated. Establishing the regular intake of rooibos as an approach to maintaining the internal redox environment of humans is important considering its easy availability, low toxicity and affordability.

The antioxidant capacity of different rooibos extracts was evaluated using a variety of assays; ORAC, FRAP, and TEAC whilst the total polyphenol content of the extracts was analyzed using spectrophotometry. Furthermore the quantification of specific flavonoids present in rooibos was

accomplished using HPLC. The ORAC assay revealed that the rooibos green water extract contained the highest antioxidant capacity in comparison to other rooibos extracts, whilst the lowest levels were reported in green rooibos ethyl acetate. The FRAP and TEAC assays on the other hand both revealed that rooibos green methanol extracts had the highest ferric reducing ability and free radical scavenging ability respectively and green chloroform the lowest. This discrepancy is strongly attributed to the different solvents utilized in this study and also to the key aspects of these individual assays. According to Pérez-Jiménez and Saura-Calixto (2006) these solvents possess different properties including polarity and extraction potential. Polar solvents (water and methanol) enable the efficient extraction of flavonoids and polyphenols (Anwar and Przybylski, 2012). ORAC assay correlates both inhibition degree and inhibition time of oxidative damage with the antioxidant activity of the extract. The FRAP assay relates the ferric ion reduction rate to the antioxidant activity through electron donation by the antioxidant, whilst TEAC correlates radical scavenging potential with antioxidant capacity analyzing both hydrophilic and lipophilic antioxidants (Piljac- Žegarac *et al*., 2013). Therefore, all these assays revealed that the water and methanol extracts of both green and fermented rooibos generally had the highest antioxidant activity. The statistical comparison of the extracts allowed for the enumeration of the effects of the extracting solvent on the antioxidant activity. The strong correlation between the polyphenolic content and antioxidant activity of medicinal plant extracts has been reported in numerous studies (Chan *et* al., 2010; Elfalleh *et al*., 2012; Khan *et al*., 2012). Simpson and collegues (2013) reported that the dihydrochalcone aspalathin is the main controlling compound of the antioxidant activity of rooibos infusions. The current study revealed the existence of this correlation, confirmed by the high polyphenolic content observed in rooibos green methanol and water extracts. Futhermore the HPLC allowed for the flavonoid profiling of all the extracts studied. These revealed high levels of aspalathin, orientin and isoorientin in water and methanol extracts of both green and fermented rooibos extracts, which could be a probable explanation for their significant antioxidant activity. However rooibos green ethyl acetate extract displayed high levels of these flavonoids, but was coupled with average, although significant, antioxidant activity.

Overall, our study has shown the existence of a direct relationship between the antimicrobial activity and antioxidant activity of the green and fermented rooibos extracts evident by increased antioxidant activity associated with an increased antimicrobial activity. Regardless of this, weak (though significant) antimicrobial activity of the rooibos extracts was coupled with a strong antioxidant activity and polyphenolic content.

3.5 Conclusion

The result of this study indicates that the antioxidant effects of rooibos green and fermented extracts may serve as a mechanism involved in their antimicrobial properties and suggests that green and fermented rooibos may be beneficial in reducing the risk of infectious diseases in immunocompromised patients. Additionally, a recent study by Park and colleagues (2014) showed that rooibos inhibited the growth and spore germination of *Clostridium perfringes*, a food borne pathogen. Together with our findings we conclude rooibos to be an effective health-promoter as well as a probable organic food preservative and it is recommended that future studies are aimed to include these aspects.

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CHAPTER 4: RESEARCH ARTICLE TWO

Antimicrobial and antioxidant activity of different solvent Honeybush (*Cyclopia intermedia*) herbal tea extracts

Phumuzile Dube, Samantha Meyer and Jeanine L. Marnewick

Abstract

Honeybush herbal tea has been associated with positive effects on human health. The inhibitory effect on the growth of nosocomial microorganisms, total antioxidant capacity, total polyphenols and flavonoids content of green/unfermented and fermented honeybush (*Cyclopia intermedia*) water, methanol, chloroform and ethyl acetate extracts were dertemined. Six of the eight extracts showed antimicrobial activity, with the fermented and green methanol extracts being most effective against *S. aureus* and *C. albicans,* respectively whilst the green chloroform extract was most potent against *S. pyogenes*. Thin layer chromatography-bioautography acknowledged the existence of highly active antimicrobial fractions within these active honeybush extracts. Total antioxidant capacities were determined by ORAC, FRAP and TEAC assays. Regardless of the assay, the green extracts generally exhibited the highest antioxidant capacity. The total polyphenols and flavonoid content was also observed to be highest in the green extracts. A directrelation between the antimicrobial and antioxidant activity of the herbal tea extracts wasobserved.The current data suggest that honeybush herbal tea can be recommended for its good antioxidant properties and also for its antimicrobial activity.

Keywords: Honeybush, antimicrobial, *Cyclopia*, antioxidant

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4.1 Introduction

The Honeybush plant is endemic to the South African fynbos region and the herbal tea brewed from the plant material has been consumed widely because of its unique aroma and taste (Kamara *et al.,* 2003; Joubert *et al.,* 2008, Joubert *et al*., 2011). It has been associated with a variety of bioactivities which include anti-diabetic (Muller *et al*., 2013; Chellan *et al*., 2014), anti-cancer (Marnewick *et al.,* 2009; Sissing *et al*., 2011; Magcwebeba, 2013), anti-obesity (Dudhia *et al*., 2013; Pheiffer *et al*., 2013), antioxidant (Hubbe and Joubert, 2000; Joubert *et al*., 2008) and antimicrobial (Coetzee *et al.,* 2008) activities. However, considering its steady increasing popularity as a health beverage, studies of its possible bioactivities are currently lagging. The antimicrobial activity of honeybush is one of the activities that have been overlooked by the research community. Furthermore, to our knowledge the relationship, if any exists, between the antioxidant and antimicrobial bioactivities has never been investigated. These two bioactivities have been concurrently examined in some medicinal plants including *Camellia sinensis*, the traditional tea plant. These studies revealed contradictory observations; one reporting a direct relation meaning high antioxidant activity is associated with strong antimicrobial effects (Chan *et al*., 2011), whilst the other observed an inverse relation entailing the expectation of a weak antimicrobial activity if a high antioxidant capacity has been displayed (Yildirim *et al*., 2000). The current study aims at not only contributing novel data to the existing knowledge pool pertaining to the antimicrobial and antioxidant activity of the honeybush herbal tea but also to confirm whether a relationship exists between the two activities.

The rising morbidity and mortality of oxidative stress-related conditions like cancer and drug resistant *P*. *aeruginosa*, *S. aureus*, *S. pyogenes* and *C. albicans* infections in Africa including South Africa is alarming. As it has been said ‘prevention is better than cure’, preventative measures of these conditions are still being discovered. Ideally these should be affordable, non toxic, easily accessible and efficient. Honeybush herbal tea fits the criteria and hence makes a suitable candidate. With the outcomes of this study, the community will acknowledge the health benefits of a consistent daily consumption of honeybush herbal tea. It is important to note that the phytochemical constituents present in honeybush herbal teas are different to those present in the other indigenous herbal tea, rooibos. For the current study, it is therefore crucial to assess the antimicrobial and related antioxidant activities of the various honeybush extracts and not to assume it will be similar to that of rooibos.

4.2 Methods and Materials

4.2.1 Chemicals and apparatus

Ampicillin, blood agar, chloroform, ciprofloxacin, cooked blood agar, dichloromethane (DCM), dimethyl sulfoxide (DMSO), ethyl acetate, fluconazole, hydrochloric acid (HCl), methanol, Mueller hinton agar, Mueller hinton broth, sabouraud dextrose 4% agar, sodium acetate, yeast peptose broth and vanillin were all purchased from Merck (Johannesburg, SA). 2,2′-azobis (2-methylpropionamidine) dihydrochloride (AAPH), 2,2'- azinobis-(3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) ferric chloride ($FeCl_3$), fluorescein sodium salt, folin ciocalteu's reagent, gallic acid, $K_2S_2O_8$ (potassium- peroxodisulfate), L-ascorbic acid, nitrotetrazolium blue chloride (NBT), potassium phosphate (KH_2PO_4), sodium carbonate, sulphuric acid (H_2SO_4), 2,4,6-tri[2-pyridyl]-s-triazine (TPTZ) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased for Sigma-Aldrich (Johannesburg, SA). All solvents purchased and used through the study were of analytical reagent grade. Sterilized water was used throughout the study. Greiner crystal clear 96-well flat bottom, Costar 96-well UV flat bottom and Nunclon black 96-well flat bottom microplates were supplied by Sigma-Aldrich (Johannesburg, SA).

4.2.2 Plant Material

Fermented and green/unfermented honeybush (*Cyclopia intermedia*) (Family: Fabaceae; Tribe: Podalyrieae), plant material (of superior grade) was obtained from the Oxidative Stress Research Centre (CPUT) supplied by Rooibos Ltd. (Clanwilliam), stored in sealed plastic containers at room temperature in the dark. The plant material is similar to the form in which it is being used commercially to brew a herbal tea from.

4.2.3 Extraction

The different extracts were prepared by macerating the plant material in solvents of differing polarity. Water extracts (as it is traditionally consumed) of the fermented and green honeybush were prepared by adding 1000 ml of boiling distilled water to 100 g samples of the fine honeybush leaves. The ethyl acetate, methanol and chloroform extracts were prepared by adding 1000 ml of the respective solvent to 100 g sample at room temperature. The mixtures were left for 24 hours at room temperature on a magnetic stirrer, after which the various extracts

were filtered through cotton wool, to remove the residual plant material. The extracts were then filtered using Whatman no. 4 mm paper. The water extract filtrate was frozen and lyophilized using a freeze-drier. The organic (ethyl acetate, methanol and chloroform) extract filtrates were concentrated to a fifth of the initial volume using a rotary evaporator. These were finally dried under a fume hood to yield crude extracts. All dried extracts were weighed and stored at 4 ^{o}C in dark, sterile sealed containers for further use.

4.2.4 Antimicrobial studies

4.2.4.1 Microorganisms

The microorganisms selected; Gram-positive *Staphylococcus aureus* (ATCC 25923) and *Streptococcus pyogenes* (ATCC 19615), Gram-negative *Pseudomonas aeruginosa* (ATCC 27853) and yeast *Candida albicans* (ATCC 10231); represent the three main groups of microorganisms. *Staphylococcus aureus* and *Pseudomonas aeruginosa* were cultured on prepared Blood agar (BA), *Steptococcus pyogenes* on Cooked Blood agar (CBA) whilst *Candida albicans* on (SDA) and all were kept viable by subculturing.

4.2.4.2 Agar disk diffusion assay

This assay has been used as a preliminary screening for antimicrobial activity of plant extracts (Salie *et al*., 1996; Ncube *et al*., 2008). The dried extracts were dissolved in their respective extracting solvents, yielding different extracts of increasing concentrations. Sterile 9 mm disks were impregnated with 50 µl of plant extracts and incubated at 37 ^{o}C for 24 hours to dry. Each extract was tested in triplicate. Negative control discs contained 50 µl of sterile extracting solvent. The positive control substances used for *Streptococcus pyogenes* and *Staphylococcus aureus* was the broad spectrum antibiotic ampicillin, for *Pseudomonas aeruginosa* was ciprofloxacin and that for *Candida albicans* was fluconazole. A suitable solid agar medium was inoculated with the respective test organisms (inoculum size: 1×10^{8} CFU/ml of bacteria) (Baris *et al*., 2006 form ncube). The media utilised in this assay for the bacteria was Mueller Hinton agar, described as the medium of choice by Ncube and collegues (2008), whilst sabouraud dextrose 4% agar (SDA) was used for the fungus. The dried paper disks, saturated with the plant extracts at desired concentrations, together with the positive and negative controls were then placed carefully onto the surface of the preinoculated agar. The plates were then incubated at 37 ^{o}C for 24 hours (bacteria) and 48 hours (fungus). The inhibition zones were measured in

millimetres (mm) from the circumference of the disk to that of the growth-free zones around the disk and recorded (Salie *et al*., 1996).

4.2.4.3 Broth micro-dilution: Minimum Inhibitory Concentration (MIC)

The antimicrobial activity of the extracts were further analyzed using the broth micro-dilution (or micro-titre plate) method, a useful technique for determining the minimum inhibitory concentration (MIC) of test samples, based on the contact of test microorganisms to a series of dilution of test samples (van Vuuren, 2008). MIC is the lowest concentration of the antimicrobial (in this case plant extracts) that inhibits microbial growth after incubation (Das *et al*., 2010). Its advantages include increased sensitivity for small quantities of extract; ability to distinguish between bacteriostatic and bactericidal effects, and quantitative determination of the MIC (Langfield *et al*., 2004), delivering reproducible results.

The MIC for each sample was determined using the micro-titre plate method (Eloff, 1998). The samples were chosen according to the results obtained from the disk diffusion assay. Microbial suspensions in Mueller-Hinton (bacteria) and Yeast Peptose (fungus) broths were prepared and incubated for 24 hours at 37 ^{o}C, from which a McFarland No. 0.5 standard (approximately 1 × 10^{8} CFU/ml) was prepared. The plant extracts were dissolved in 10% dimethyl sulfoxides (DMSO) (Salie *et al*., 1996; Langfield *et al*., 2004). The organic dried honeybush extracts, chloroform and methanol, were dissolved to starting concentrations of 200 mg/ml and 300 mg/ml, respectively, and the aqueous extracts to a starting concentration of 600mg/ml. Those extracts that were difficult to dissolve were agitated using a vortex. The positive control substances used (to confirm microbial susceptibility) for *Streptococcus pyogenes* and *Staphylococcus aureus* was the antibiotic ampicillin, and that for *Candida albicans* was fluconazole and the respective extraction solvents and 10% DMSO were used as the negative controls.

The 96-well micro-titre plates were aseptically prepared in a horizontal laminar air flow cabinet. In each well 100 µl of 10% DMSO was added, and then 200 µl of the extracts were added in the first wells from which serial dilutions (two-folds) were made to the desired minimum concentration. The microbial cultures (100 µl) were added to the wells, then the whole plate was placed on a shaker (500rpm) for 30 seconds. Soon after shaking, the plates were read at 620

nm wavelength with a multiplate reader to obtain the first reading (t = 0hours), a sterile film was used to cover the plates to prevent evaporation then incubated at 37 ^{o}C for 24 hours. After incubation the absorbance reading at the same wavelength of 620 nm were recorded for each plate. The MIC determined by the spectrophotometric method was defined as the concentration at which there was a sharp decline in the absorbance value after incubation (Salie *et al*., 1996; Devienne and Raddi, 2002). All MIC values were tested in triplicate.

4.2.4.4 Thin Layer Chromatography (TLC) –Bioatography

Bioautography is employed as a preliminary phytochemical screening technique, by bioassay-guided fractionation, to detect active antimicrobial components of the extracts under study (Nostro *et al*., 2000; Schmourlo *et al.,* 2004).

Twenty micromoles of the extracts (aqueous and organic) were applied onto pre-coated duplicate plates of silica gel 60 F_{254} (Merck, Germvny). The TLC plates were developed and visualization was done by observing the bands under UV at λ254 nm and at λ366 nm using UV lamp (CAMAG, Switzerland), then finally spraying them with the detection agent vanillin-sulphuric acid. Chemical profiling of the bands was done based on the colour produced after viewing. The solvent system used for the TLC development of the honeybush (*C. intermedia*) extracts fractions is dichloromethane- methanol (95:5). The duplicate plates were used for the bioautographic agar overlay assay. A suspension of the test microorganisms (approximately 1x10^{8} CFU/ml) was sprayed onto the developed TLC plates. The bioautograms were then incubated at 37 ^{o}C for 24 hours in humid conditions. Nitrotetrazolium blue chloride (NBT), the microbial indicator was used as a growth detector (Silva *et al*., 2005). It was sprayed onto the plates, which were re-incubated at 37 ^{o}C for 3-4 hours (Dilika *et al*., 1996; Runyoro *et al*., 2006). Clear zones on the bioautogram indicated inhibition of growth i.e. antimicrobial activity of the extract components.

4.2.5 Antioxidant Capacity studies

4.2.5.1 Oxygen Radical Absorbance Capacity (ORAC) assay

Antioxidant capacity of the various solvent honeybush extracts was measured using the ORAC assay (Ou *et al*., 2001). It involves the thermal decomposition of 2, 2'-azobis (2-methyl-propanamide) dihydrochloride (AAPH) to a peroxyl radical which oxidizes a fluorescent probe,

resulting in a loss of fluorescence intensity. The decrease in fluorescence intensity reflects on the concentration of free radicals and a delay of this loss signifies the presence of antioxidants in the test sample, counteracting the activity of the oxidative species. The antioxidants tend to inhibit the oxidative degradation of the fluorescence. In this study, fluorescein (FL) was used as the probe and the assay was based on measuring the inhibition of fluorescein decay by antioxidants. The azo compound AAPH was thermally decomposed at 37 ^{o}C to form the peroxyl radical and the ORAC results were expressed as micromoles of Trolox® equivalents (TE) per gram of sample (µmol of TE/g). The procedure used for this assay was as described by Rautenbach and co-workers (2010), a slightly modified version of Ou *et al* (2001).

The reagents and standards (FL, AAPH, Trolox) were prepared using the ORAC phosphate buffer (75mM at pH 7.4). One hundred and thirty eight litre of fluorescein solution (14 µM concentration), 12 µl of sample (controls and extracts) and 50 µl of AAPH solution (500 µM) were dispensed and mixed in a 96-well black plate prior to reading the plates. Trolox controls were prepared within a range of 0-417 µM. The fluorescence readings were measured with 485 and 538 nm as excitation and emission wavelengths, respectively using a fluoroskan ascent plate reader (Thermo Fisher Scientific, Waltham, Mass, U.S.A). Fluorescence readings were taken at every minute thereafter for 2 hours and the ORAC value was calculated by dividing the area under the sample curve by the area under the Trolox (control) curve with both areas being corrected by eliminating the area under the blank curve. A single ORAC unit was assigned as being the net protection area provided by 1 µM Trolox in the final concentration. The final antioxidant activity results were given in Trolox equivalents. All samples were assessed in triplicate.

4.2.5.2 Ferric Reducing Ability of Plasma (FRAP) assay

The FRAP assay is used as a novel method for assessing “antioxidant power” of biological and pure samples (Benzie and Strain, 1996). The principle of this spectrophotometric method is based on the antioxidants’ ability to reduce the ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}), in acidic environment. The antioxidant capacity of the samples is evaluated by the ability of the antioxidants to donate electrons during a redox (reduction/oxidation) reaction. At acidic pH, the oxidant ferric chloride hexahydrate $Fe_3(TPTZ)_2Cl_3$ (TPTZ= 2,4,6, Tripyridyl-s-triazine) is reduced by antioxidants in samples to give an intense blue coloured ferrous tripyridyltriazine complex. The colour develops in the presence of electron donating antioxidants and is monitored by a spectrophotometer measuring the change in absorption maximum at 593nm (Benzie and Strain, 1996; Gupta *et al*., 2009).

The FRAP reagent was prepared by mixing acetate buffer (300 mM, pH 3.6), TPTZ (10 mM), $FeCl_3$ (20 mM) and distilled water. The TPTZ reagent was prepared in hydrochloric acid (40 mM HCl), whilst the rest of the reagents were prepared using distilled water. L-Ascorbic acid was used in the preparation of the antioxidant standard solutions ranging between 0-1000 µM. 10 µl of the standards/extracts followed by 300 µl of the FRAP reagent were added to a 96-well plate and incubated at 37 ^{0}C for 30min before reading. Results were obtained by comparison to the calibration standard curve using the regression equation (y = mx + b). Samples were each assayed in triplicates.

4.2.5.3 Trolox Equivalent Antioxidant Capacity (TEAC) assay

The TEAC assay measures the total antioxidant capacity of a given substance. The procedure was previously described by Re and collegues (1999). The ABTS reagent (2,2'- azinobis-(3-ethyl-benzothiazoline-6-sulfonic acid)) was prepared by mixing 5 ml (7 mM) ABTS and 88 µl (140 mM) $K_2S_2O_8$ (potassium- peroxodisulfate) and allowed to react for 24 hours in the dark at room temperature. Trolox (6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as the standard with concentrations ranging from 0 to 500 µM. After 24 hours, the ABTS reagent was diluted with absolute ethanol to read an initial absorbance of approximately 2.0(± 0.1), which is the control value. Twenty five microlitres of standards/extracts and 300 µl of ABTS mix were added and mixed in 96-well plates and were allowed to react at room temperature in the dark for 30 min. The plates were then read at 734 nm at 25 ^{0}C using the plate readerand results were expressed as micromoles Trolox equivalents per milligram dry weight of sample (µM TE/g). All samples were assayed in triplicate.

4.2.6 Phytochemical assays

4.2.6.1 Total Polyphenols

Total polyphenols content (TPC) of the honeybush extracts was determined by spectrophotometry, using Folin-Ciocalteu reagent (Sigma- Aldrich, Johanesburg SA) with gallic acid as a standard. The reagents (10% ethanol, Folin-Ciocalteu (0.1 g/ml), sodium carbonate (0.075 g/ml) were all prepared using distilled water except the gallic acid standards (give an absorbance of 0.509±0.010 at 280 nm) which were diluted in 10% ethanol. The gallic acid stock solutions were prepared in varying concentrations, ranging between 0-500 mg/l. Twenty five

microlitres of standards/extracts (of varying concentrations) were added to 125 µl of Folin-Ciocalteu in the 96-well plates. After 5 min, 100 µl of sodium carbonate was then pipetted into each well. The plates were incubated at room temperature for 2 hours before being read with the multiskan plate reader at an absorbance of 765 nm. Total polyphenol results were expressed as milligram Gallic acid equivalents per gram of extract (mg Gallic acid/g).The concentration of polyphenols in extracts were derived from a standard curve of gallic acid standards ranging from 10-50 µg/mL (Pearson's correlation coefficient: r^2 = 0.9996). Samples were analysed in triplicate.

4.2.6.2 High Performance Liquid Chromatography (HPLC)

The HPLC is a quantitative analysis, detecting and characterizing known and unknown sample components. The Spectra HPLC system P2000 pump was equipped with HPLC column C18 (150 x 4.6mm), 5 µm particle size (Agilent Zorbax, South Africa) and a Spectra system FL3000 fluorescence detector. The chromatographic conditions included a flow rate of 1 ml/min, 15 min run time, sample injection volume of 20 µl and the mobile phases A (KH_2PO_4 (50 mM)) and B (methanol (pH 5.8)). The temperature of the column and detector array was maintained at room temperature (25±1 °C). Crude extracts were diluted using DMSO to a concentration of 1mg/ml. The measurements were made at 280 nm for mangiferin and hesperidin against the pure compounds. The analytical signals were monitored at 2-20 mV potentials applied.

4.2.7 Statistical analysis

Antimicrobial experimental data are expressed as mean ± standard error of the mean (SEM) whilst the antioxidant data are expressed as mean ± standard deviation (SD). Statistical analysis of antimicrobial activity of the different honeybush extracts was achieved using the unpaired Student's *t*-test. One-way analysis of variance (ANOVA) was used to test for significance of the antioxidant activity of the extracts. Bonferroni Multiple Comparison analysis was used to compare the antioxidant activity of the different extracts. Comparisons were considered significant at $P<0.05$. GraphPad™ PRISM5 software package was used for all statistical evaluations and graphical representations.

4.3 Results

4.3.1 The antimicrobial activity of honeybush extracts against *Pseudomonas aeruginosa, Staphylococcus aureus*, *Streptococcus pyogenes* and *Candida albicans*

The positive control ciprofloxacin (5 µg) inhibited the growth of *P. aeruginosa* with a zone of 12 mm. No zones of inhibition were observed around the negative control (extraction solvent) disks. *P. aeruginosa* was resistant to all honeybush extracts because no inhibition was observed when the agar disk diffusion, minimum inhibitory concentration (MIC) and thin layer chromatography (TLC) were performed.

4.3.2 The antimicrobial activity of honeybush extracts against *Staphylococcus aureus* (*S. aureus*)

4.3.2.1 Agar disk diffusion assay

The antimicrobial activity of the various honeybush extracts was analyzed and the effect of fermentation on these activities was assessed by statistically comparing the inhibition of the fermented extracts with the activity of the green extracts. The inhibitory activity of the extracts against specific microorganisms was observed to be linearly proportional to the extract concentration. Inhibition zones caused by honeybush extracts were compared using an unpaired *t*-test.

P. aeruginosa was resistant to all honeybush extracts because no zones of inhibition were observed. Methanol and water extracts of both green and fermented honeybush displayed some antimicrobial activity against *S. aureus* (Table 4.1) whilst ethyl acetate and lipophilic chloroform honeybush extracts were inactive. The most potent honeybush extract against the Gram-positive microorganism was fermented methanol which was 7 fold more active than the least effective fermented water extract. When comparing the activity of green and fermented honeybush extracts against *S. aureus*, the methanol extracts proved most antimicrobially active. At 600 mg/ml the most significant ($P<0.05$) comparison of green and fermented methanol honeybush extracts was observed, whilst for the aqueous extracts the most significant ($P<0.05$) comparison of the two forms of honeybush was recorded at 300 mg/ml.

The ethyl acetate extracts of the two forms of honeybush did not display any activity against *S.* pyogenes, whilst the green and fermented water, methanol and chloroform honeybush extracts exhibited some degree antimicrobial activity against the microorganisms. The green chloroform extracts was 7 fold more potent than the weakly active green chloroform extract. The most significant (P<0.05) comparison between the green and fermented forms of honeybush tea against *S. pyogenes* was observed at 200 mg/ml (water); 100 mg/ml and 200 mg/ml (methanol); 100 mg/ml and 150 mg/l (chloroform). As the concentrations of the honeybush extracts increased so did the inhibitory activity of *S. pyogenes* by the extracts.

The methanol and water extracts of both green and fermented honeybush exhibited some degree of antifungal activity against *C.albicans* whilst the ethyl acetate and chloroform honeybush extracts were inactive against the fungus. Green methanol honeybush extract was two fold more potent than the weaker fermented water extracts. When comparing the activity of green and fermented honeybush extracts against *C. albicans*, the differences were not as significant (P>0.05), with 300 and 500 mg/ml methanol extracts as exceptions (P<0.05). Generally the polar methanol and water extracts displayed a greater inhibitory potential in comparison to the ethyl acetate and chloroform extracts (selective inhibitory activity against *S. pyogenes*). The negative controls, which were the respective extracting solvents, did not inhibit any of the test microorganisms as expected.

Table 4.1: Inhibition of *Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus pyogenes* and *Candida albicans* by varying concentrations of honeybush extracts

Microorganisms	Extraction solvent	Conc (mg/ml)	Average zones of inhibition (mm±SEM)		Statistical significance (*P*)
			HG[a]	HF [b]	ab
Pseudomonas aeruginosa	Water			-	
	Methanol			-	
	Chloroform			-	
	Ethyl acetate			-	

Positive control	**Ciprofloxacin**	**5 µg/ml**	**12 mm**		
Staphylococcus aureus	**Water**	**200**	**1.2±0.17**	**0.2±0.17**	**0.0132 ***
		300	**1.8±0.17**	**0.7±0.17**	**0.0078 ****
		600	**2.2±0.17**	**1.2±0.17**	**0.0132 ***
		900	3.6±0.17	2.7±0.33	0.055
	Methanol	50	0.2±0.17	0.3±0.17	0.5185
		100	0.7±0.17	0.8±0.17	0.5185
		150	**1.2±0.17**	**1.8±0.17**	**0.0474 ***
		200	**1.2±0.17**	**2.8±0.17**	**0.0078 ****
		300	**1.8±0.17**	**4.2±0.17**	**0.0006 *****
		500	**2.2±0.17**	**5.7±0.33**	**0.0007 *****
		600	**3.7±0.33**	**8.2±0.17**	**0.0003 *****
	Chloroform		**-**		
	Ethyl acetate		**-**		
Positive control	**Ampicillin**	**10 µg/ml**	**13 mm**		
Streptococcus pyogenes	**Water**	50	0.2±0.17	0.8±0.17	0.0474
		100	0.7±0.17	1±0	0.1049
		150	**0.8±0.17**	**3±0**	**0.0002*****
		200	**1±0**	**4±0**	**<0.0001*****
		300	**1.3±0.17**	**4.7±0.33**	**0.0009*****
		600	**1.5±0.29**	**5±0.29**	**0.001****
		900	**2±0**	**5.7±0.33**	**0.0004*****
	Methanol	**50**	**0.7±0.33**	**2±0**	**0.0155***
		100	**1±0**	**3±0**	**<0.0001*****
		150	**1.7±0.33**	**3.7±0.33**	**0.0132***

		200	**2±0**	**4±0**	**<0.0001*** **
		300	**3±0**	**4.8±0.17**	**0.0004*** **
		500	**4±0**	**5.3±0.33**	**0.0169***
		600	**5.3±0.33**	**7±0**	**0.0073****
		50	0.7±0.33	0±0	0.1233
	Chloroform	**100**	**2±0**	**1±0**	**<0.0001*** **
		150	**4±0**	**2±0**	**<0.0001*** **
		200	**6.7±0.33**	**3.7±0.33**	**0.0031****
	Ethyl acetate	**-**			
Positive control	**Ampicillin**	**10 µg/ml**	**15 mm**		
Candida albicans	**Water**	100	1±0	1.2±0.17	0.4211
		150	2.2±0.17	1.7±0.33	0.2508
		200	2.7±0.33	2.2±0.17	0.2508
		300	3±0	3.2±0.17	0.4211
		600	3.7±0.33	3.7±0.33	1
		900	5±0	4.3±0.33	0.1100
	Methanol	50	0±0	0±0	1
		100	1.3±0.33	0.7±0.33	0.2302
		150	1.7±0.33	1.3±0.17	0.4216
		200	2.7±0.33	2±0	0.1233
		300	**3.7±0.33**	**2.3±0.33**	**0.0474***
		500	**4±0**	**2.7±0.33**	**0.0155***
		600	5.3±0.33	4.3±0.33	0.1012
	Chloroform		**-**		
	Ethyl acetate		**-**		
Positive control	**Fluconazole**	**25 µg/ml**		**10 mm**	

***Abbreviations*:** Conc: concentration; mg/ml: milligram per millilitre; mm: millimeter; SEM: standard error of the mean (for *n*=3); HG: honeybush green; HF: honeybush fermented. * *P*<0.05; ***P*<0.01; ****P*<0.001. **-**: No zones of inhibition observed with varying extract concentrations.

4.3.2.2 Broth micro-dilution: Minimum Inhibitory Concentration

The minimum extract concentration that posseses inhibitory activity against specific microorganisms was assessed (Table 4.2). The methanol extracts of fermented honeybush was the most active against *S. aureus*, with an MIC of 18.75 mg/ml. *S. pyogenes* was most sensitive to green honeybush chloroform extract with an MIC value of 12.5 mg/ml whilst *C. albicans* was greatly sensitive to the green honeybush methanol extract, with MIC value of 37.5 mg/ml. *P. aeruginosa* exhibited resistant traits in the presence of various honeybush extracts. According to this study, the water solvent inefficiently extracted antimicrobially active components from the green and fermented forms of honeybush, evident by the high MIC values recorded for most water extracts.

Table 4.2: MIC (mg/ml) values of extracts derived from *C. intermedia* (Honeybush)

Test microorganism	Extract	MIC (mg/ml)
Pseudomonas aeruginosa	**-**	
Staphylococcus aureus	HGW	150
	HGM	75
	HFW	300
	HFM	**18.75**
Streptococcus pyogenes	HGW	150
	HGM	75
	HGC	**12.5**
	HFW	75
	HFM	37.5
	HFC	50
Candida albicans	HGW	150
	HGM	**37.5**
	HFW	150
	HFM	75

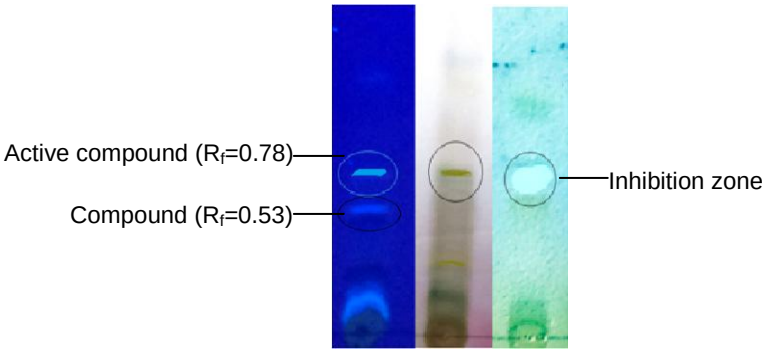
Abbreviations: HGW:honeybush green water; HGM: honeybush green methanol; HGC: honeybush green chloroform; HE: honeybush green ethyl acetate; HFW: honeybush fermented water; HFM: honeybush fermented

methanol; HFC: honeybush fermented chloroform; HFE: honeybush fermented ethyl acetate; MIC: minimum inhibitory concentration in milligrams per milliliter (mg/ml). *n*=3.

4.3.2.3 Thin Layer Chromatography (TLC) – Bioautography

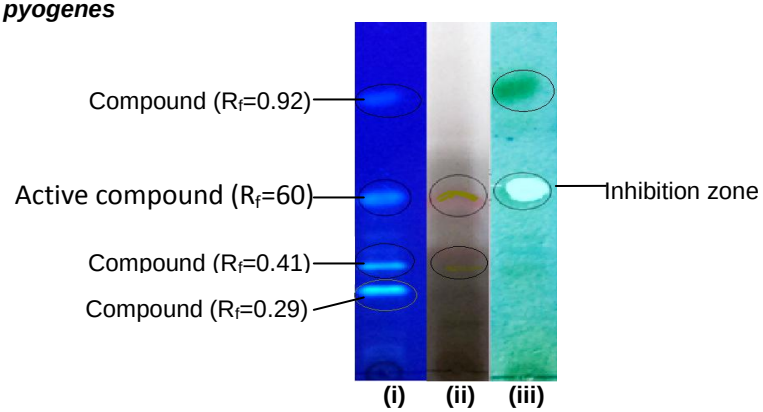
The thin layer chromatographic profile of the most potent extracts against *S. aureus, S. pyogenes* and *C. albicans* were analyzed to enumerate antimicrobially active components present within the plant extracts (Figure 4.1). The TLC plates were viewed under UV at 360 nm and sprayed with H_2SO_4/vanillin then heated (noted as (i) and (ii) respectively). The bioautograms of the honeybush fermented methanol (HFM), green chloroform (HGC) and green methanol (HGM) extracts against *S. aureus, S. pyogenes* and *C. albicans* were also analyzed (noted as (iii)) and zones of inhibition were observed in the presence of the active antimicrobial fraction. The HFM zone of inhibition corresponded with a greenish- yellow compound on the TLC plate and had a retardation factor (R_f) value of 0.78. The HGC zone of inhibition corresponded with a yellowish band on the TLC plate and had a R_f of 0.60 whilst the HGM zone of inhibition had a R_f of 0.57 corresponding with a yellowish compound on the TLC plate. The green forms of honeybush extracted with the appropriate solvent resulted in an efficient extraction of antimicrobially active components against the specific microorganisms. Generally the assay revealed the existence of three distinct antimicrobial compounds present within honeybush herbal tea, evident by the different R_f values possessed by the compounds.

Staphylococcus aureus



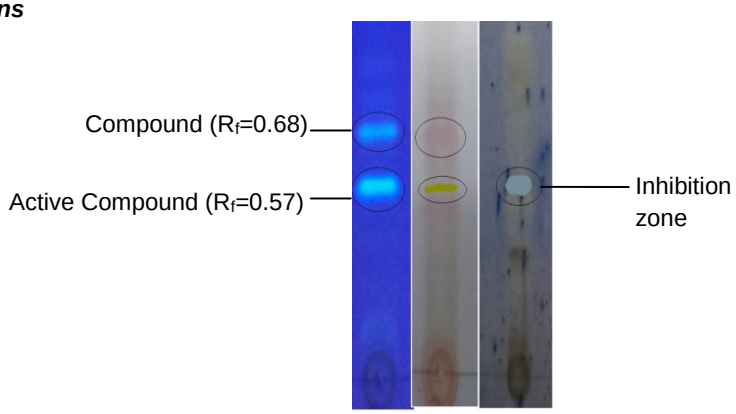
i) Developed TLC plates of the HFM extract under UV at 360nm.
ii) Developed TLC plates of the HFM extract sprayed with vanillin sulphuric acid reagent.
iii) Developed TLC plates of the HFM extract inoculated with *S. aureus* and sprayed with NBT.

Streptococcus pyogenes



i) Developed TLC plates of the HGC extract under UV at 360nm
ii) Developed TLC plates of the HGC extract sprayed with vanillin sulphuric acid reagent
iii) Developed TLC plates of the HGC extract inoculated with *S. pyogenes* and sprayed with NBT

Candida albicans



i) Developed TLC plates of the HGM extract under UV at 360nm
ii) Developed TLC plates of the HGM extract sprayed with vanillin sulphuric acid reagent.
iii) Developed TLC plates of the HGM extract inoculated with *C. albicans* and sprayed with NBT

Figure 4.1: Antimicrobial activity of HFM, HGC and HGM extracts against *S. aureus, S. pyogenes* and *C. albicans* respectively

***Abbreviations*:** TLC: thin layer chromatography; HFM: honeybush fermented methanol; HGC: honeybush green chloroform; HGM: honeybush green methanol; R_f: retardation factor; NBT: nitrotetrazolium blue chloride.

4.3.2 The antioxidant capacity of honeybush extracts

4.3.2.1 Oxygen Radical Absorbance Capacity

The antioxidant capacity of green/unfermented and fermented honeybush extracts were analyzed and reported. The statistical significance ($P<0.05$) of comparing the antioxidant levels present in each extract at specific concentrations was also reported (Table 4.3 and 4.4). The most significant honeybush extract comparisons were noted at 5 mg/ml and 15 mg/ml of both the green and fermented forms, with a few exceptions. For the green extracts these exceptions were at extract concentration 5 mg/ml between honeybush water and honeybush methanol; honeybush water and honebush ethyl acetate; honeybush methanol and honeybush chloroform; honeybush chloroform and honeybush ethyl acetate; honeybush methanol and honeybush ethyl acetate. With the fermented extracts these exceptions existed between honeybush water and honeybush methanol; honeybush methanol and honeybush ethyl acetate; honeybush water and honeybush ethyl acetate, all at extract concentration 5 mg/ml and honeybush water and honeybush ethyl acetate at 15mg/ml extract concentration. At 0.5 mg/ml extract concentration, the difference between each of the extracts were not statistically significant ($P>0.05$). The antioxidant activity was directly related to the extract concentrations, the higher the extract concentration the greater the antioxidant activity.

The highest antioxidant activity was observed in green and fermented honeybush methanol extracts and the least in green and fermented chloroform extracts. When considering the ORAC assay, the green extracts of honeybush generally contained a higher antioxidant activity compared to that of fermented honeybush extracts (Figure 4.2).

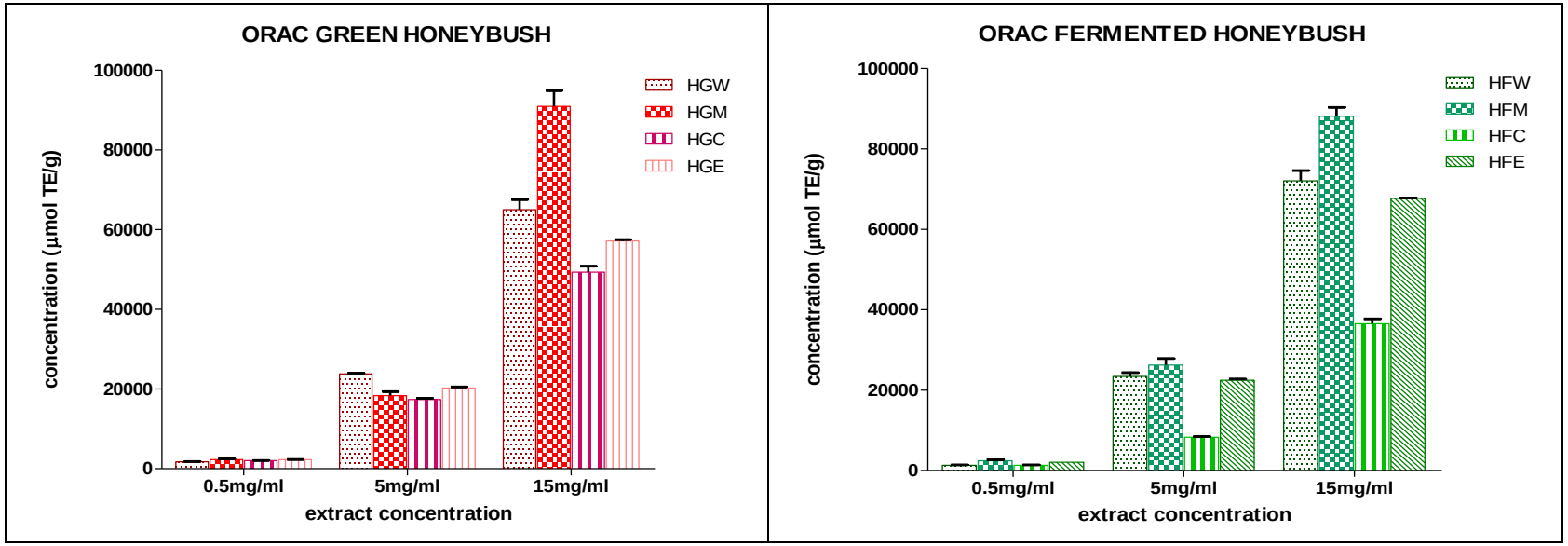


Figure 4.2: Direct measurement of the antioxidant capacity of green and fermented honeybush extracts using the ORAC assay.

***Abbreviations*:** µmol TE/g: micromoles of Trolox® equivalents per gram; ORAC: oxygen radical absorbance capacity; mg/ml: milligram per milliliter.

Extracts

HGW: honeybush green water

HGM: honeybush green methanol

HGC: honeybush green chloroform

HGE: honeybush green ethyl acetate

HFW: honeybush fermented water

HFM: honeybush fermented methanol

HFC: honeybush fermented chloroform

HFE: honeybush fermented ethyl acetate

Table 4.3: Statistical comparison of the ORAC values of green honeybush extracts

Green honeybush						
Extract conc (mg/ml)	**Average ORAC conc (µmol TE/g)±SD**				**Statistical comparison**	**Statistical significance (*P*)**
	HGW	**HGM**	**HGC**	**HGE**		
0.5	1759±102	2283±295	2029±101	2297±23	HGW: HGM	ns
					HGW: HGC	ns
					HGW: HGE	ns
					HGM: HGC	ns
					HGM: HGE	ns
					HGC: HGE	ns
5	23820±350	18330±1822	17360±533	20280±502	HGW: HGM	ns
					HGW: HGC	**<0.05***
					HGW: HGE	ns
					HGM: HGC	ns
					HGM:HGE	ns
					HGC: HGE	ns
15	65050±4379	90970±6876	49330±2550	57190±601	**HGW: HGM**	**<0.001*****
					HGW: HGC	**<0.001*****
					HGW: HGE	**<0.01****
					HGM: HGC	**<0.001*****
					HGM: HGE	**<0.001*****
					HGC: HGE	**<0.01****

Abbreviations: conc: concentration; mg/ml: milligram per milliliter; ns: not significant; SD: standard deviation. *P<0.05; **P<0.01**; P<0.001***.

Extract comparisons

HGW: HGM- honeybush green water versus honeybush green methanol

HGW: HGC- honeybush green water versus honeybush green chloroform

HGW: HGE- honeybush green water versus honeybush green ethyl acetate

HGM: HGC- honeybush green methanol versus honeybush green chloroform

HGM: HGE- honeybush green methanol versus honeybush green ethyl acetate

HGC: HGE- honeybush green chloroform versus honeybush green ethyl acetate

Table 4.4: Statistical comparison of the ORAC values of fermented honeybush extracts

Fermented honeybush						
Concentration (mg/ml)	**Average ORAC conc (µmol TE/g)±SD**				**Statistical comparison**	**Statistical significance (*P*)**
	HFW	**HFM**	**HFC**	**HFE**		
0.5	1317±99	2413±520	1318±107	2047±5.11	HFW: HFM	ns
					HFW: HFC	ns
					HFW: HFE	ns
					HFM: HFC	ns
					HFM: HFE	ns
					HFC: HFE	ns
5	23410±1565	26240±2770	8294±207	22470±432	HFW: HFM	ns
					HFW: HFC	**<0.001*** **
					HFW: HFE	ns
					HFM: HFC	**<0.001*** **
					HFM: HFE	ns
					HFC: HFE	**<0.001*** **
15	72090±4379	88140±3795	36530±2073	67700±168.3	**HFW: HFM**	**<0.001*** **
					HFW: HFC	**<0.001*** **
					HFW: HFE	ns
					HFM: HFC	**<0.001*** **
					HFM: HFE	**<0.001*** **
					HFC: HFE	**<0.05***

Abbreviations: conc: concentration; mg/ml: milligram per milliliter; ns: not significant; SD: standard deviation.
**P*<0.05*; ***P*<0.001.

Extract comparisons

HFW: HFM- honeybush fermented water versus honeybush fermented methanol

HFW: HFC- honeybush fermented water versus honeybush fermented chloroform

HFW: HFE- honeybush fermented water versus honeybush fermented ethyl acetate

HFM: HFC- honeybush fermented methanol versus honeybush fermented chloroform

HFM: HFE- honeybush fermented methanol versus honeybush fermented ethyl acetate

HFC: HFE- honeybush fermented chloroform versus honeybush fermented ethyl acetate

4.3.3.2 Ferric Reducing Ability of Plasma

The ferric reducing ability of all green and fermented honeybush extracts was measured and reported (Figure 4.3 and Table 4.5, 4.6). The antioxidant activity was directly related to the extract concentrations, the higher the extract concentration the greater the total antioxidant activity. The highest reducing ability was observed in green and fermented methanol extracts and the least in green ethyl acetate and fermented chloroform extracts. Generally the green extracts of honeybush displayed a higher total antioxidant capacity when compared to the fermented honeybush extracts (Figure 4.3) when considering the FRAP assay.

The statistical significance ($P<0.05$) of comparing the antioxidant levels present in each extract at specific concentrations was noted (Table 4.5 and 4.6). All green and fermented extract comparisons at 0.5mg/ml, 5mg/ml and 15mg/ml were statistically significant ($P<0.05$).

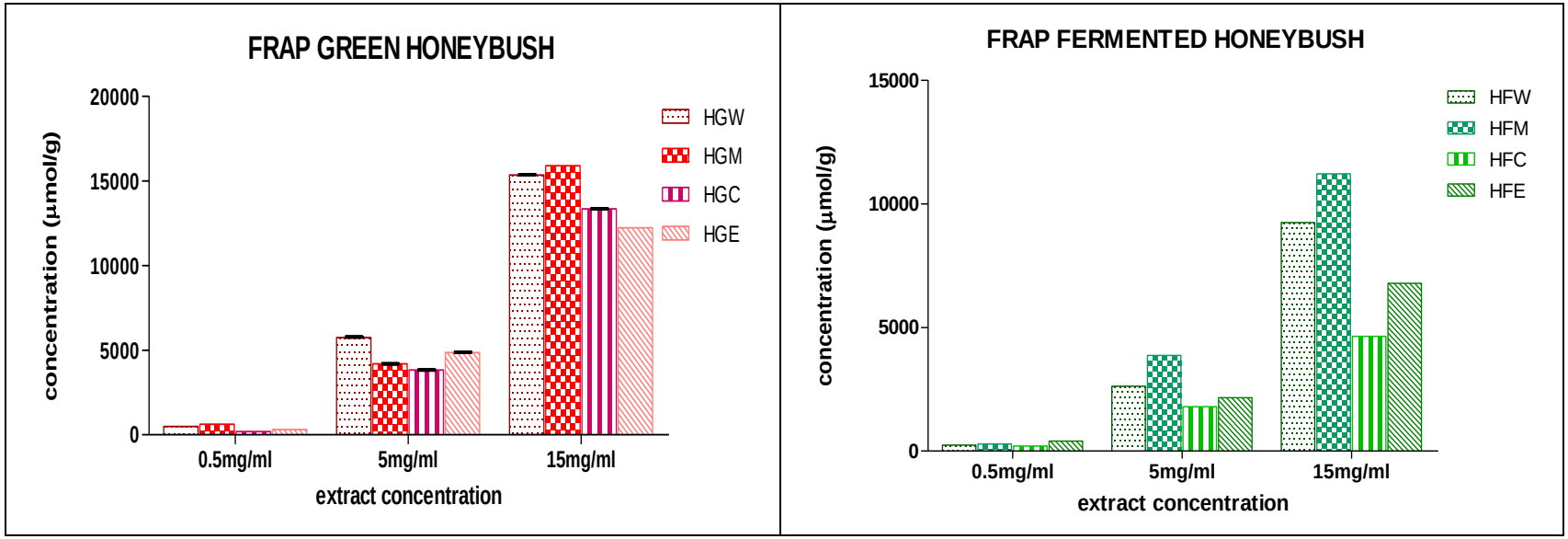


Figure 4.3: Direct measurement of the antioxidant capacity of green and fermented honeybush extracts using the FRAP assay

***Abbreviations*:** µmol /g: micromoles per gram; FRAP: Ferric reducing ability of plasma; mg/ml: milligram per milliliter.

Extracts

HGW: honeybush green water

HGM: honeybush green methanol

HGC: honeybush green chloroform

HGE: honeybush green ethyl acetate

HFW: honeybush fermented water

HFM: honeybush fermented methanol

HFC: honeybush fermented chloroform

HFE: honeybush fermented ethyl acetat

Table 4.5: Statistical comparison of the FRAP values of green honeybush extracts

Green honeybush						
Extract conc (mg/ml)	**Average FRAP conc (µmol /g)±SD**				**Statistical comparison**	**Statistical significance (*P*)**
	HGW	**HGM**	**HGC**	**HGE**		
0.5	486.4±3.2	620.7±1.2	188.8±1.1	309.7±1.5	**HGW: HGM**	**<0.001*** **
					HGW: HGC	**<0.001*** **
					HGW: HGE	**<0.001*** **
					HGM: HGC	**<0.001*** **
					HGM: HGE	**<0.001*** **
					HGC: HGE	**<0.001*** **
5	5745±46.6	4184±6.1	3820±22	4864±11	**HGW: HGM**	**<0.001*** **
					HGW: HGC	**<0.001*** **
					HGW: HGE	**<0.001*** **
					HGM: HGC	**<0.001*** **
					HGM:HGE	**<0.001*** **
					HGC: HGE	**<0.001*** **
15	15350±2.1	15900±0.7	13330±6.6	12230±3.6	**HGW: HGM**	**<0.001*** **
					HGW: HGC	**<0.001*** **
					HGW: HGE	**<0.001*** **
					HGM: HGC	**<0.001*** **
					HGM: HGE	**<0.001*** **
					HGC: HGE	**<0.001*** **

Abbreviations: conc: concentration; mg/ml: milligram per milliliter; ns: not significant; SD: standard deviation. ****P*<0.001. Average for *n*=3.

Extract comparisons

HGW: HGM- honeybush green water versus honeybush green methanol

HGW: HGC- honeybush green water versus honeybush green chloroform

HGW: HGE- honeybush green water versus honeybush green ethyl acetate

HGM: HGC- honeybush green methanol versus honeybush green chloroform

HGM: HGE- honeybush green methanol versus honeybush green ethyl acetate

HGC: HGE- honeybush green chloroform versus honeybush green ethyl acetate

Table 4.6: Statistical comparison of the FRAP values of fermented honeybush extracts

Fermented honeybush						
Extracts Conc (mg/ml)	**Average FRAP conc (µmol /g)±SD**				**Statistical comparison**	**Statistical significance (*P*)**
	HFW	**HFM**	**HFC**	**HFE**		
0.5	238.7±1.1	288.3±1.1	203.7±0.6	407.5±0.4	**HFW: HFM**	**<0.001*** **
					HFW: HFC	**<0.001*** **
					HFW: HFE	**<0.001*** **
					HFM: HFC	**<0.001*** **
					HFM: HFE	**<0.001*** **
					HFC: HFE	**<0.001*** **
5	2624±1.1	3871±1.4	1794±0.1	2160±0.8	**HFW: HFM**	**<0.001*** **
					HFW: HFC	**<0.001*** **
					HFW: HFE	**<0.001*** **
					HFM: HFC	**<0.001*** **
					HFM: HFE	**<0.001*** **
					HFC: HFE	**<0.001*** **
15	9243±0.8	11210±1.7	4644±0.3	6790±0.4	**HFW: HFM**	**<0.001*** **
					HFW: HFC	**<0.001*** **
					HFW: HFE	**<0.001*** **
					HFM: HFC	**<0.001*** **
					HFM: HFE	**<0.001*** **
					HFC: HFE	**<0.001*** **

Abbreviations: conc: concentration; mg/ml: milligram per milliliter; SD: standard deviation. ****P*<0.001.

Extract comparisons

HFW: HFM- honeybush fermented water versus honeybush fermented methanol

HFW: HFC- honeybush fermented water versus honeybush fermented chloroform

HFW: HFE- honeybush fermented water versus honeybush fermented ethyl acetate

HFM: HFC- honeybush fermented methanol versus honeybush fermented chloroform

HFM: HFE- honeybush fermented methanol versus honeybush fermented ethyl acetate

HFC: HFE- honeybush fermented chloroform versus honeybush fermented ethyl acetate

4.3.3.3 Trolox Equivalent Antioxidant Capacity

The radical scavenging ability of green and fermented honeybush extracts was measured using the TEAC assay. This ability was directly proportional to the extract concentrations, the higher the extract concentration the greater their radical scavenging ability. The highest radical scavenging ability was observed in green water and fermented methanol honeybush extracts and the least was in green ethyl acetate and fermented chloroform extracts. In this case, the green extracts of honeybush had a higher radical scavenging ability compared to the fermented honeybush extracts (Figure 4.4).

The statistical significance ($P<0.05$) of comparing the scavenging ability of the extracts at specific concentrations was also reported (Table 4.7 and 4.8). All the comparisons of the different green and fermented extracts at increasing concentrations (0.5-15 mg/ml) were significant ($P<0.05$) except when comparing the honeybush green chloroform and ethyl acetate extracts at 0.5 mg/ml extract concentration.

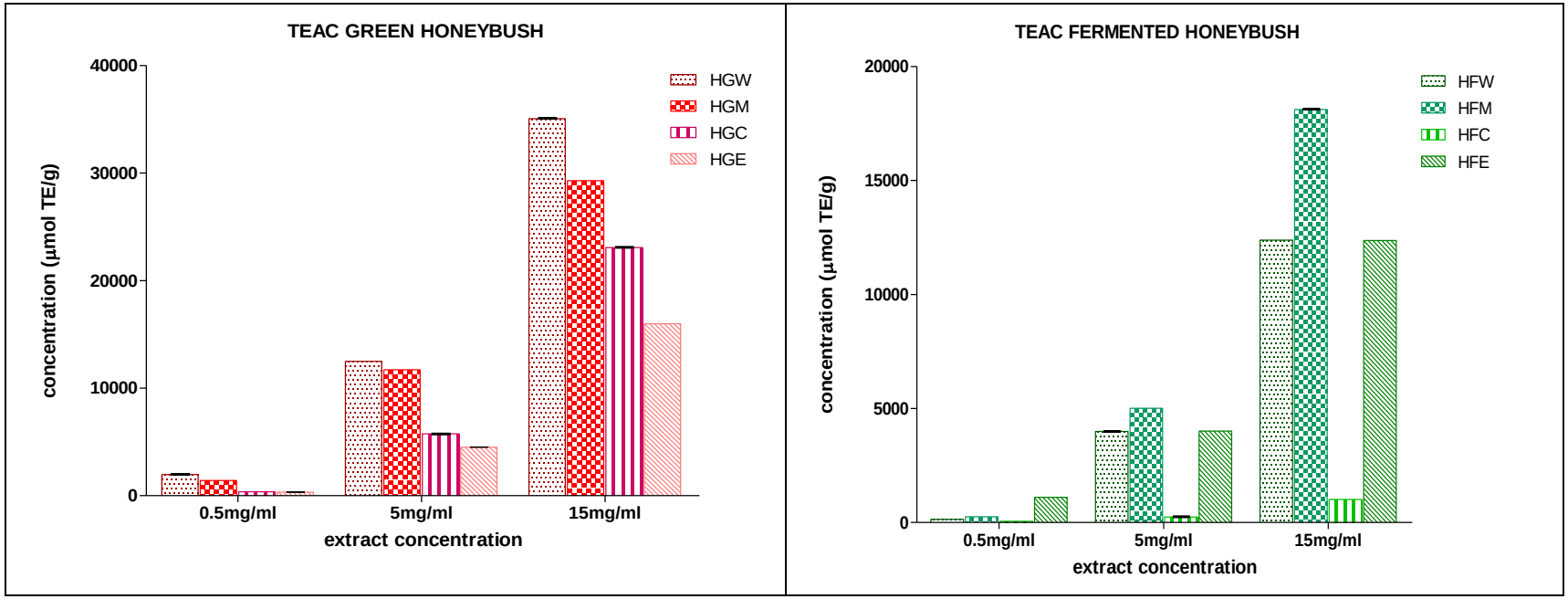


Figure 4.4: Direct measurement of the antioxidant activity of green and fermented honeybush extracts using the TEAC assay.

***Abbreviations*:** µmol TE/g: micromoles of Trolox® equivalents per gram; TEAC: Trolox equivalent antioxidant capacity; mg/ml: milligram per milliliter.

Extracts

HGW: honeybush green water

HGM: honeybush green methanol

HGC: honeybush green chloroform

HGE: honeybush green ethyl acetate

HFW: honeybush fermented water

HFM: honeybush fermented methanol

HFC: honeybush fermented chloroform

HFE: honeybush fermented ethyl acetate

Table 4.7: Statistical comparison of the TEAC values of green honeybush extracts

Green honeybush						
Extract Conc (mg/ml)	**Average TEAC conc (µmol TE/g)±SD**				**Statistical comparison**	**Statistical significance (*P*)**
	HGW	**HGM**	**HGC**	**HGE**		
0.5	1966±32	1406±0.1	360.5±2.2.	334.3±7.5	**HGW: HGM**	**<0.01****
					HGW: HGC	**<0.001*****
					HGW: HGE	**<0.001*****
					HGM: HGC	**<0.001*****
					HGM: HGE	**<0.001*****
					HGC: HGE	ns
5	12480±2.5	11710±5	5743±3	4514±3.2	**HGW: HGM**	**<0.001*****
					HGW: HGC	**<0.001*****
					HGW: HGE	**<0.001*****
					HGM: HGC	**<0.001*****
					HGM:HGE	**<0.001*****
					HGC: HGE	**<0.001*****
15	35040±34	29290±1.3	23070±4	16000±3	**HGW: HGM**	**<0.001*****
					HGW: HGC	**<0.001*****
					HGW: HGE	**<0.001*****
					HGM: HGC	**<0.001*****
					HGM: HGE	**<0.001*****
					HGC: HGE	**<0.001*****

Abbreviations: conc: concentration; mg/ml: milligram per milliliter; ns: not significant. ***P*<0.01; ****P*<0.001. Average for *n*=3.

Extract comparison

HGW: HGM- honeybush green water versus honeybush green methanol

HGW: HGC- honeybush green water versus honeybush green chloroform

HGW: HGE- honeybush green water versus honeybush green ethyl acetate

HGM: HGC- honeybush green methanol versus honeybush green chloroform

HGM: HGE- honeybush green methanol versus honeybush green ethyl acetate

HGC: HGE- honeybush green chloroform versus honeybush green ethyl acetate

Table 4.8: Statistical comparison of the TEAC values of fermented honeybush extracts

Fermented honeybush						
Extract conc (mg/ml)	**Average TEAC conc (µmol TE/g)±SD**				**Statistical comparison**	**Statistical significance (*P*)**
	HFW	**HFM**	**HFC**	**HFE**		
0.5	144±0.3	249.2±3.3	53.03±0.3	1103±1.4	**HFW: HFM**	**<0.001*** **
					HFW: HFC	**<0.001*** **
					HFW: HFE	**<0.001*** **
					HFM: HFC	**<0.001*** **
					HFM: HFE	**<0.001*** **
					HFC: HFE	**<0.001*** **
5	3993±6.5	5008±1.2	246.9±1.8	4010±0.5	**HFW: HFM**	**<0.001*** **
					HFW: HFC	**<0.001*** **
					HFW: HFE	**<0.001*** **
					HFM: HFC	**<0.001*** **
					HFM: HFE	**<0.001*** **
					HFC: HFE	**<0.001*** **
15	12380±3.4	18120±1.6	10090±1.6	12360±2.5	**HFW: HFM**	**<0.001*** **
					HFW: HFC	**<0.001*** **
					HFW: HFE	**<0.001*** **
					HFM: HFC	**<0.001*** **
					HFM: HFE	**<0.001*** **
					HFC: HFE	**<0.001*** **

Abbreviations: conc: concentration; mg/ml: milligram per milliliter; ns: not significant; SD: standard deviation. ****P*<0.001. Average for *n*=3.

Extract concentrations

HFW: HFM- honeybush fermented water versus honeybush fermented methanol

HFW: HFC- honeybush fermented water versus honeybush fermented chloroform

HFW: HFE- honeybush fermented water versus honeybush fermented ethyl acetate

HFM: HFC- honeybush fermented methanol versus honeybush fermented chloroform

HFM: HFE- honeybush fermented methanol versus honeybush fermented ethyl acetate

HFC: HFE- honeybush fermented chloroform versus honeybush fermented ethyl acetate

4.3.3 Phytochemical assays

4.3.3.1 Total Polyphenols

The total polyphenol content of green and fermented honeybush extracts was measured and reported (Figure 4.5 and Table 4.9, 4.10). The polyphenol content was directly related to the extract concentration analyzed; the higher the extract concentration the higher the total polyphenolic content. The highest total polyphenol concentration was observed in green water and fermented methanol extracts and the least in green and fermented chloroform. Generally the green extracts of honeybush contained higher concentrations of total polyphenols than fermented honeybush extracts (Figure 4.5).

The statistical significance ($P<0.05$) of comparing the total polyphenols present in each extract at specific concentrations was also reported (Table 4.9 and 4.10). The statistical comparison of the total polyphenol content in the green and fermented honeybush were all significant except for the green water and methanol; green water and ethyl acetate and also the fermented water and methanol; fermented methanol and chloroform; fermented methanol and ethyl acetate; fermented ethyl acetate and chloroform comparisons, all at 0.5mg/ml extract concentration. These differences were not significant ($P>0.05$).

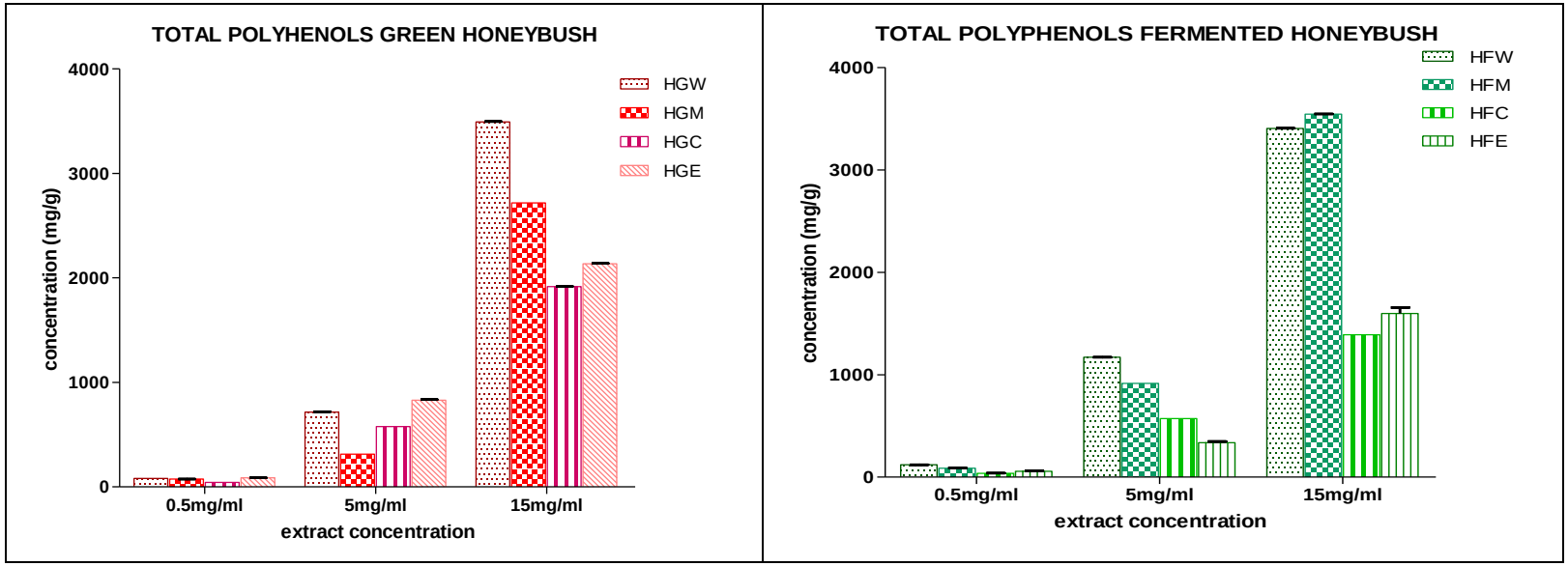


Figure 4.5: Quantifying the amount of polyphenols present in green and fermented honeybush extracts using the Total polyphenols assay

***Abbreviations*:** mg/g: milligrams per gram; mg/ml: milligram per milliliter.

Extracts

HGW: honeybush green water

HGM: honeybush green methanol

HGC: honeybush green chloroform

HGE: honeybush green ethyl acetate

HFW: honeybush fermented water

HFM: honeybush fermented methanol

HFC: honeybush fermented chloroform

HFE: honeybush fermented ethyl acetate

Table 4.9: Statistical comparison of the Total polyphenols values of green honeybush extracts

Green honeybush						
Extract Conc (mg/ml)	**Average Total polyphenols conc (mg/g)±SD**				**Statistical comparison**	**Statistical significance (*P*)**
	HGW	**HGM**	**HGC**	**HGE**		
0.5	81.03±0.5	74.12±0.8	43.51±0.6	86.4±2.6	HGW: HGM	ns
					HGW: HGC	**<0.001*** **
					HGW: HGE	ns
					HGM: HGC	**<0.001*** **
					HGM: HGE	**<0.01** **
					HGC: HGE	**<0.001*** **
5	716±5.3	313.2±0	578.2±0.6	829.5±9.8	**HGW: HGM**	**<0.001*** **
					HGW: HGC	**<0.001*** **
					HGW: HGE	**<0.001*** **
					HGM: HGC	**<0.001*** **
					HGM:HGE	**<0.001*** **
					HGC: HGE	**<0.001*** **
15	3493±6.1	2718±0	1918±0.6	2136±5.2	**HGW: HGM**	**<0.001*** **
					HGW: HGC	**<0.001*** **
					HGW: HGE	**<0.001*** **
					HGM: HGC	**<0.001*** **
					HGM: HGE	**<0.001*** **
					HGC: HGE	**<0.001*** **

Abbreviations: conc: concentration; mg/ml: milligram per milliliter; ns: not significant; SD: standard deviation. ***P*<0.01; ****P*<0.001. Average for *n*=3.

Extract comparisons

HGW: HGM- honeybush green water versus honeybush green methanol

HGW: HGC- honeybush green water versus honeybush green chloroform

HGW: HGE- honeybush green water versus honeybush green ethyl acetate

HGM: HGC- honeybush green methanol versus honeybush green chloroform

HGM: HGE- honeybush green methanol versus honeybush green ethyl acetate

HGC: HGE- honeybush green chloroform versus honeybush green ethyl acetate

Table 4.10: Statistical comparison of the Total polyphenols values of fermented honeybush extracts

Fermented honeybush						
Concentration (mg/ml)	**Average Total polyphenols conc (mg/g)±SD**				**Statistical comparison**	**Statistical significance (*P*)**
	HFW	**HFM**	**HFC**	**HFE**		
0.5	119.9±0.7	87.67±0.6	37.68±0.6	56.11±6.8	HFW: HFM	ns
					HFW: HFC	**<0.01****
					HFW: HFE	**<0.05***
					HFM: HFC	ns
					HFM: HFE	ns
					HFC: HFE	ns
5	1170±0.8	915.6±0.4	571.9±0.6	336.3±21.6	**HFW: HFM**	**<0.001*****
					HFW: HFC	**<0.001*****
					HFW: HFE	**<0.001*****
					HFM: HFC	**<0.001*****
					HFM: HFE	**<0.001*****
					HFC: HFE	**<0.001*****
15	3404±5.3	3545±0.6	1390±0.6	1598±98.9	**HFW: HFM**	**<0.001*****
					HFW: HFC	**<0.001*****
					HFW: HFE	**<0.001*****
					HFM: HFC	**<0.001*****
					HFM: HFE	**<0.001*****
					HFC: HFE	**<0.001*****

Abbreviations: conc: concentration; mg/ml: milligram per milliliter; ns: not significant; SD: standard deviation.*P<0.05; **P<0.01; ***P<0.001. Average for n=3.

Extract comparisons

HFW: HFM- honeybush fermented water versus honeybush fermented methanol

HFW: HFC- honeybush fermented water versus honeybush fermented chloroform

HFW: HFE- honeybush fermented water versus honeybush fermented ethyl acetate

HFM: HFC- honeybush fermented methanol versus honeybush fermented chloroform

HFM: HFE- honeybush fermented methanol versus honeybush fermented ethyl acetate

HFC: HFE- honeybush fermented chloroform versus honeybush fermented ethyl acetate

4.3.3.2 High Performance Liquid Chromatography (HPLC)

HPLC allowed for the quantification of the flavonoids mangiferin and hesperidin present in the green and fermented honeybush extracts (Table 4.11 and appendix). The green honeybush methanol extract was reported to contain the highest concentration (observed as the area under the graph) of mangiferin (306.60 mg/g) and hesperidin (243.22 mg/g). The honeybush green ethyl acetate generally contained the least concentration of the flavonoids; mangiferin (7.81 mg/g) and hesperidin (5.06 mg/g).

Table 4.11: The Quantification of mangiferin and hesperidin (mg/g) in honeybush extracts

Extracts	Mangiferin(mg/g)	Hesperidin(mg/g)
HGW	11.26	58.87
HFW	15.66	30.73
HGM	306.60	243.22
HFM	35.86	145.08
HGC	24.79	225.97
HFC	6.51	14.23
HGE	7.81	5.06
HFE	8.26	5.14

***Abbreviations*:** mg/m: milligram per gram.

Extracts

HGW: honeybush green water
HFW: honeybush fermented water
HGM: honeybush green methanol
HFM: honeybush fermented methanol
HGC: honeybush green chloroform
HFC: honeybush fermented chloroform
HGE: honeybush green ethyl acetate
HFE: honeybush fermented ethyl acetate

4.4 Discussion

Regardless of its long history of traditional use, the popularity of honeybush herbal tea is still emerging in the South African herbal tea industry. The scientific interest shown in this herbal tea is lacking, evident by the few experimental studies available. A handful of studies have evaluated the antioxidant (Leiro *et al*., 2003; Garcia *et al*., 2003; Hubbe and Joubert, 2000; Joubert *et al*. 2008; van der Merwe *et al.*, 2012; Malherbe *et al*., 2014) and antimicrobial (Coetzee *et al*., 2008) activity of some of the *Cyclopia spp*. This lack of scientific evidence was the main motivational drive for this current investigation. The current study did not only focus on exploring the antimicrobial and antioxidant activity of honeybush tea, but also investigated the connection, if any between these two bioactivities.

The agar disk diffusion assay, minimum inhibitory concentration and thin layer bioautography were used to analyze the antimicrobial activity of green/unfermented and fermented honeybush (*C. intermedia*) extracts against the bacteria *P. aeruginosa, S. aureus, S. pyogenes* and fungus *C. albicans*. *P. aeruginosa,* which is one of the main causes of nosocomial infections, exhibited resistance towards the green and fermented honeybush extracts. The basis for its resilience is its impermeable outer membrane surrounding the bacterial cell wall, making pseudomonal infections amongst the most difficult infections to treat (Levinson and Jawetz, 1992; Vardakas *et al*., 2013).

The growth of *S. aureus* and *C. albicans* were inhibited by methanol and water extracts of green and fermented honeybush, but was resistant to the chloroform and ethyl acetate extracts. *S. aureus* is the causative agent of a wide range of infections, from non-invasive to life threatening systemic infections (Iwamoto *et al*., 2013), whilst candidiasis (infections caused by *C. albicans*) are known for their increased frequency in HIV/AIDS patients (Conti *et al*., 2014). The methanol extracts of green and fermented honeybush proved more efficacious in inhibiting the growth of *C. albicans* (green methanol MIC- 37.5 mg/ml) and *S. aureus* (fermented methanol MIC- 18.75 mg/ml), respectively. The high levels of the flavonoids hesperidin and mangiferin detected in the methanol extracts; green methanol (hesperidin- 243.22 mg/g; mangiferin- 306.60 mg/g) and fermented methanol (hesperidin- 145.08 mg/g ; mangiferin- 35.86 mg/g); maybe the rationale for this observation. The water extracts on the other hand exhibited a weaker inhibitory activity against the *S. aureus* (green water MIC- 150 mg/ml; fermented water MIC- 300 mg/ml) and *C.*

albicans (green water MIC- 150 mg/ml; fermented water MIC- 150 mg/ml). Lower concentrations of hesperidin and mangiferin were observed in green (hesperidin- 58.87 mg/g; mangiferin- 11.26 mg/g) and fermented (hesperidin- 30.73 mg/g; mangiferin-15.66 mg/g) water extracts. Also, the high polarity of the water solvent is associated with a weaker antimicrobial activity (Tian *et al*., 2009). Both mangiferin (Stoilova *et al*., 2005; Singh *et al*., 2009) and hesperidin (Iranshahi *et al*., 2015) have been shown to play vital roles in the protection against pathogenic microorganisms, confirming the antimicrobial activity associated with these two major honeybush flavonoids.

S. pyogenes proved highly sensitive to the honeybush green chloroform (MIC- 18.75 mg/ml) extract as well as to the fermented chloroform, green and fermented methanol and water extracts. The antimicrobial potential of lipophilic compounds extracted by chloroform and other lipophilic solvents has been previously investigated (Kabara *et al*., 1977; Barros *et al*., 2013). In addition, the high levels of hesperidin (225.97 mg/g) and mangiferin (24.79 mg/g) present in honeybush green chloroform could further elucidate the antimicrobial capacity of this extract. With consideration to the hesperidin (14.23 mg/g) and mangiferin (6.51 mg/g) concentration of the honeybush fermented chloroform extract, its weaker activity against *S. pyogenes* (MIC-75 mg/ml) was not an alarming observation. In general, the higher the concentration of hesperidin and mangiferin present in the honeybush extract, the greater the antimicrobial activity of the extracts. The resistance of *P. aeruginosa*, *S. aureus* and *C. albicans* to the chloroform extracts could be as a result of the characteristic differences within the individual microbial strains (Chan *et al*., 2011).

Considering the high MIC values observed for the honeybush extracts against *S. aureus, S. pyogenes* and fungus *C. albicans*, honeybush cannot be considered a noteworthy antimicrobial agent. Noteworthy antimicrobial agent is that agent whose MIC value against a specific microorganism is less than 1.00 mg/ml (Ncube et al., 2008, Rìos and Recio, 2005; Van Vuuren, 2008). Despite this fact, the antimicrobial activity exhibited by the honeybush extracts should be acknowledged. Biautography allowed the localization of highly active antimicrobial compounds in each of the potent extracts on a silica gel plate. These were noted as zones of inhibition on the bioautograms (Figure 4.1). The TLC assay permitted the confirmation of the fractions observed in honeybush fermented methanol, honeybush green chloroform and honeybush green methanol under UV at 366 nm as those exhibiting antimicrobial activity against *S.* aureus, *S. pyogenes* and *C.* albicans respectively. The bioautogram confirmed the existence of three

distinct antibiotic compounds within the extracts due to the different polarity of the extraction solvents and the fermentation state of the plants. The identification and further isolation of these active compounds using nuclear magnetic resonance (NMR) and mass spectrometry (MS) will be ideal for future investigatory purposes, as these individual compounds are likely to be more active than the whole plant extracts (McGaw *et al*., 2013).

The insufficiency of studies performed investigating the antioxidant activity of honeybush herbal tea is startling, considering the teas steadily increasing popularity (McKay and Blumberg, 2007; Joubert *et al*., 2011). This study investigated the antioxidant activity of different honeybush extracts using Oxygen Radical Absorbance Capacity (ORAC), Ferric Reducing Ability of Plasma (FRAP) and Trolox Equivalent Antioxidant Capacity (TEAC). The quantification of the total polyphenols and major flavonoids; hesperidin and mangiferin; present in the extracts was accomplished using spectrophotometry and High Performance Liquid Chromatography (HPLC) respectively.

ORAC analyses the ability of the extract to reduce oxidative damage by oxygen species. This inhibition can therefore be utilized as a reflection of the extracts antioxidant capacity. FRAP, on the other hand, evaluates the ability of the antioxidants in the extract to reduce the ferric ion whilst TEAC focuses on the antioxidants ability to scavenge free radicals (Zulueta *et al*., 2009; Gorjanović *et al*., 2013; Morales-Soto *et al*., 2014). In general, the results obtained from these assays represent the concentration of antioxidants present that are capable of displaying the specific characteristics under study. The study revealed that the green and fermented methanol extracts generally had the highest ORAC values. This observation may be because methanol has the ability to extract both polar and non-polar compounds with potential antioxidant activities. The FRAP results displayed the green and fermented methanol extracts to possess the greatest potential to reduce the ferric ion. TEAC revealed that the green water and fermented methanol extracts exhibited the highest free radical scavenging ability. The polar nature of water and methanol allows them to extract polar compounds. According to this study, the polar compounds extracted from honeybush green and fermented leaves by water and methanol solvents exhibited higher free radical scavenging ability. An antioxidant is defined as a compound when present can significantly retard or prevent the oxidation of oxidizable molecules, scavenge oxygen derived compounds or prevent the formation of reactive oxygen species (Halliwell, 1995). By this definition generally all the honeybush extracts displayed antioxidant traits,

evaluated by the different assays ORAC, FRAP and TEAC, though at differing levels and rankings. The statistical comparison of the extracts allowed for the enumeration of the effects of the extracting solvent on the antioxidant activity.

The total polyphenolic content ranking of honeybush green extracts (15 mg/ml) in decreasing polyphenolic concentration was water (3 493mg/g), methanol (2 718 mg/g), ethyl acetate (2 136 mg/g), chloroform (1 918 mg/g) and that for honeybush fermented extracts was methanol (3 545 mg/g), water (3 40 9mg/g), ethyl acetate (1 598 mg/g), chloroform (1 390 mg/g). This observation was in agreement with studies that identified the polar methanol and water as the ideal solvents in the extraction of polyphenols from whole plants (Erol *et al*., 2009; Bonilla *et al*., 1999). Statistically the differences between the total polyphenolic content of the green and fermented extracts at 0.5 mg/ml, 5 mg/ml and 15 mg/ml were significant ($P<0.05$) with infrequent exceptions at 0.5mg/ml (Tables 4.9 and 4.10).

Ranking of the flavonoid content in honeybush extracts in decreasing concentrations of mangiferin, was green methanol (306.60 mg/g), fermented methanol (35.86 mg/g), green chloroform (24.79 mg/g), fermented water (15.66 mg/g), green water (11.26 mg/g), fermented ethyl acetate (8.26 mg/g), green ethyl acetate (7.81 mg/g), fermented chloroform (6.51 mg/g) and of hesperidin content in decreasing levels was green methanol green (243.22 mg/g), chloroform (225.97 mg/g), fermented methanol (145.08 mg/g), green water (58.87 mg/g), fermented water (30.73 mg/g), fermented chloroform (14.23 mg/g), fermented ethyl acetate (5.14 mg/g), green ethyl acetate (5.06 mg/g). These results demonstrated a relation between the total polyphenol content and individual flavonoid concentrations in the extracts, a relation that has been investigated and documented since flavonoids are classified as a group of phenolic compounds (Verzelloni *et al.*, 2007, Daglia, 2012).

The methanol and water extracts generally exhibited the highest ORAC, FRAP and TEAC readings. With consideration of their generally high antioxidant capacity coupled with the displayed antimicrobial activity against *S. aureus* and *C. albicans* together with the high flavonoids mangiferin and hesperidin, the existence of a relation between these two bioactivities should not be revoked. However in-depth correlation studies, including correlation coefficient

studies, where these two bioactivities are manipulated further in all these extracts should be performed in order for the relation to become more vivid.

4.5 Conclusion

The result of this study indicates that the weak (though relevant) antimicrobial activity was coupled by the high antioxidant potential of the extracts. The existence of the antibiotic fractions suggests that this herbal tea may be beneficial in treating infections of *S. aureus*, *S. pyogenes* and *C. albicans* infections.

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CHAPTER 5

5.1 General discussion

Rooibos and honeybush herbal teas have a long traditional use in South Africa in the treatment and prevention of a variety of ailments. Their compositional uniqueness to the traditional tea *Camellia sinensis* has significantly attributed to their popularity. Furthermore their different phenolic content to each other has led to increased scientific interest. Oxidative stress has been associated with a variety of life threatening conditions including cancer, cardiovascular diseases and diabetes. During this state, the production of reactive oxygen species is coupled by an incompetent antioxidant system. The oxygen species have the ability to attack the immune system cells, which may lead to an immune compromised status (Correa *et al*., 1999; Victor and De la Fuente, 2000). A weakened immune system allows the human body to be attacked by opportunistic pathogens that include *P. aeruginosa, S. aureus*, *S. pyogenes* and *C. albicans*. Associating the intake of rooibos and honeybush herbal teas with antioxidant and antimicrobial effects is very important, given the herbal teas easy accessibility, affordability and non-toxic nature. The results in this study are presented in two different chapters focusing on the antimicrobial and associated antioxidant effects of the various green and fermented extracts of rooibos and honeybush. The general discussion of this thesis will therefore be focused on the above parameters.

The zones of inhibition observed were dependent on the microorganism strain, extract type and concentration. Larger extract concentrations or volumes probably could have produced greater effects; however the restricted capacity of the discs utilized did not permit this. This study showed that the methanol extracts of rooibos and honeybush (green and fermented) proved to be the most potent antimicrobial agents against *S. aureus* and *C. albicans*, whilst chloroform extracts was most active against *S*. *pyogenes. S. pyogenes* was the most sensitive microbial strain studied, showing zones of inhibition in the presence of all the extracts except the ethyl acetate infusions. Though water is the most polar of all the solvents used in this study, methanol and chloroform extracts were shown to be most potent. Tian and collegues (2009) associated increased antibacteral activity with extracts of weaker polarity as they contain high molecular weight gallotannins. However the individual extracts had high MIC values against the sensitive microorganisms. Generally, the rooibos and honeybush extracts displayed inhibitory activity against the Gram-positive bacteria (*S. aureus* and *S. pyogenes*) and the fungus *C. albicans*,

whilst the Gram-negative *P. aeruginosa* exhibited overall resistance. The surrounding outer membrane possessed by Gram-negative bacteria tends to impede the inflow of hydrophobic compounds into the bacteria. The absence of this memabrane, as seen in Gram-positive bacteria, allows for easier diffusion of compounds that have detrimental effects on the microorganisms (McGaw *et al*., 2013). Similar results were reported by Hübsch *et al* (2014) and Coetzee *et al* (2008).

In this study, the MIC is defined as the lowest concentration of the extract that inhibits the growth of *S. aureus*, *S. pyogenes* and *C. albicans* after incubation (Das *et al*., 2010). The MIC values are high because a noteworthy antimicrobial agent has an MIC value of less than 1 mg/ml. With this fact, the whole plant extracts of both the green and fermented forms of rooibos and honeybush could not be considered noteworthy antimicrobial agents. However the antimicrobial activity displayed by the individual extracts against the studied microorganisms should be appreciated. The existence of other bioactive compounds within the extracts, which may have masked the inhibitory activity of the antimicrobial compounds present, also referred to as antagonism, could have led to such high MIC values. This phenomenon and its antonym synergism have been displayed and evaluated in antimicrobial and antioxidant studies of medicinal plants particularly the tea plant *Camellia sinensis* (Koech *et al*., 2013; Betts *et al*., 2013), but has never been reported for these two plants under study. This was made apparent by the clear zones of inhibition observed when the individual extracts were seperated using the TLC-bioautography assay. The compounds separated possessed different R_f values, showing their distinctiveness from one another. Three antibiotic compounds were separated from both rooibos and honeybush mainly due to the different extracting solvents utilized and the fermentation state of the whole plants. These TLC-bioautography results signify that these two plants may potentially yield purified compounds to be used as drugs that could improve the treatment approaches of infections caused by these microorganisms.

Many studies have shown that the green/unfermented forms of teas have stronger antioxidant properties than the fermented forms (Chan *et al*., 2011; Chan *et al*., 2010; von Gadow *et al*., 1997). In agreement with previous findings, the results of this study displayed the strong antioxidant properties of the green rooibos and honeybush, which were fairly higher than those exhibited by the fermented forms as shown graphically in Figures 3.2, 3.3, 3.4 for rooibos and Figures 4.2, 4.3, 4.4 for honeybush, respectively. ORAC, FRAP and TEAC were the assays

used to assess the antioxidant capacity of the whole plant extracts of rooibos and honeybush. The overall oxidation inhibition (ORAC) capacity of honeybush was greater than that of rooibos, whilst the ferric reducing ability (FRAP) of the extracts was higher for rooibos than honeybush. The free radical scavenging potential (TEAC) was generally higher in honeybush extracts than that observed in rooibos. These observations could have been as a result of the different principles of each assay used for this study allowing for the analysis of different antioxidant aspects of the two plants. Chan and colleagues (2011) associated the strong antioxidant activity of green and black teas (*Camellia sinensis teas*) to the phenolic composition of the teas. Flavonoids, which are subgroups of polyphenols, are known to significantly contribute towards the scavenging of oxygen species and ion reduction. Hence the strong antioxidant activity of the green and fermented rooibos extracts can be associated with the high levels of the flavonoids aspalathin, orientin and isoorientin, whilst that of green and fermented honeybush is due to high levels of mangiferin and hesperidin. Phytochemical analysis of the tea extracts indicated that the more polar solvents (methanol and water) allowed for the efficient extraction of flavonoids and polyphenol compounds from the green and fermented forms of rooibos and honeybush herbal tea. The high antioxidant capacity and polyphenolic content of rooibos and honeybush extracts observed in this study maybe an attributing factor to the health-promoting properties associated with these two herbal teas.

It has been demonstrated in the present study that the whole plant extracts of rooibos and honeybush may be classified as antioxidant-rich but not as notweworthy antimicrobial agents. This was observed as high antioxidant activity was coupled by weak, though relevant antimicrobial activity. This was in line with the results observed by Yildirim and collegues (2000), who reported a weak antimicrobial activity can be expected when a high antioxidant activity is recorded in medicinal plants. Regardless of this factor, the non toxic nature of both rooibos and honeybush herbal teas makes them ideal home therapies for infections and a variety of conditions. Marnewick *et al*. (2005) showed that a constant topical application of unprocessed/green rooibos and honeybush over 20 weeks did not induce any form of illness on the study mice. As confirmation, the consumption of 6 cups of fermented rooibos a day for a period of 6 weeks by human subjects did not cause any adverse effects, concluding that both the topical application and consumption of these herbal teas is more beneficial than harmful (Marnewick *et al*., 2011).

5.2 Conclusion and recommendations

The results of the current study provide novel evidence of the existence of a strong antioxidant activity and selective antimicrobial activity possessed by green and fermented rooibos (*A. linearis*) and honeybush (*C. intermedia*) extracts, to distinguishable degrees.

Due to the antioxidant and antimicrobial effects demonstrated by the various extracts of green and fermented rooibos and honeybush in this study, the constant intake of these teas could be considered a general source of natural antioxidants and antimicrobial therapeutics, leading to the enhancement of the general wellbeing of humans. In addition, these extracts can be developed into dietary supplements, henceforth contributing to improving the health of the general public. Furthermore these plants can be utilized in the food industry as natural preservatives as they can be used in preventing oxidation and also bacterial spoilage whilst maintaining the flavor quality of the food (Almajano *et al*., 2008). The acknowledgement of specific antimicrobial fractions within these extracts may provide the research community with new leads in the on-going pursuit for novel antimicrobial drugs.

While this study indicated that green and fermented forms of rooibos and honeybush extracts were strong antioxidant agents displaying selective antimicrobial activity, further studies should focus on the possible identity of the antimicrobially-active fractions of the various extracts. This can be achieved using an array of assays including the nuclear magnetic reasonance (NMR) and mass spectrometry (MS) (McGaw *et al*., 2013). Furthermore, the exploration of the antagonistic relations of the bioactive components present within the extracts could be performed so that the interactional mechanisms of these components may be well understood. Extensive research should also be conducted on the toxicity of the compounds in the rooibos and honeybush herbal plants considering their growing popularity.

Extract storage and other factors such as the harvest season for the rooibos and honeybush could have caused unclear results in some aspects of the study. When the extracts were stored at room temperature (during the pilot study), the antioxidant and antimicrobial activities of the

extracts were greatly weakened, a factor that was rectified by the storage of all extracts, after processing, at 4 ^{o}C. Therefore, studies with careful maintenance of the phytochemical nature of the extracts are recommended.

5.3. References

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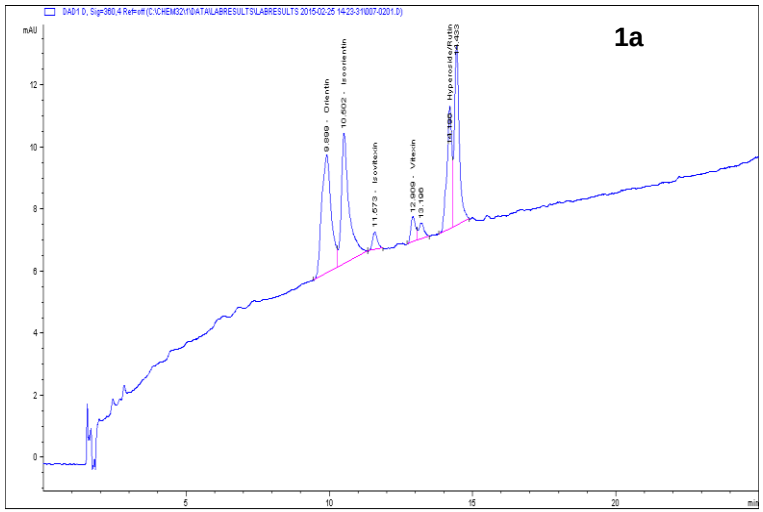
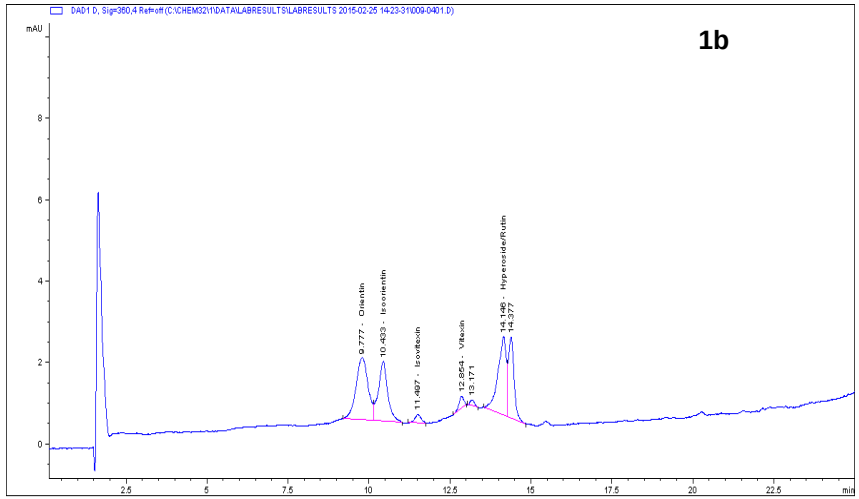
APPENDIX

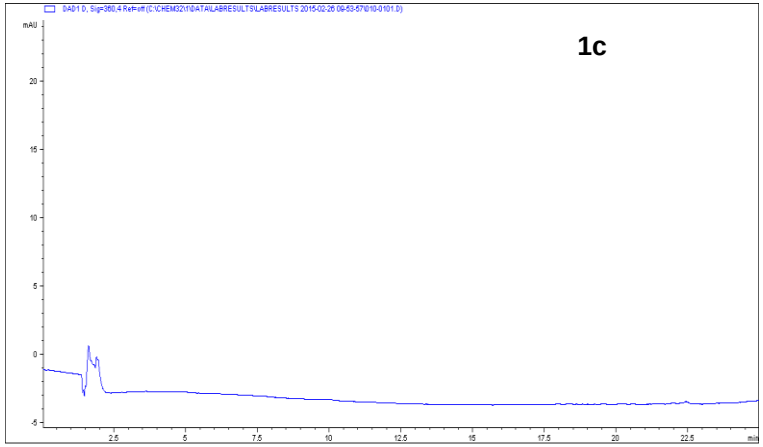
The HPLC quantification of the major flavonoids in green/unfermented and fermented rooibos and honeybush water, methanol, chloroform and ethyl acetate extracts.

Figures 1 and 2 depict the quantity of the flavonoids at 360nm and aspalathin at 287 nm respectively present in green/unfermented rooibos extracts of different polarity. Figures 3 and 4 display the quantity of the flavonoids at 360 nm and aspalathin at 287 nm respectively present in fermented water, methanol, chloroform and ethyl acetate rooibos extracts. Different wavelengths were used because these compounds are absorbed at distinct wavelengths.

Figures 5 and 6 depict the quantity of the flavonoids hesperidin and mangiferin at 280 nm present in green and fermented honeybush water, methanol, chloroform and ethyl acetate extract.







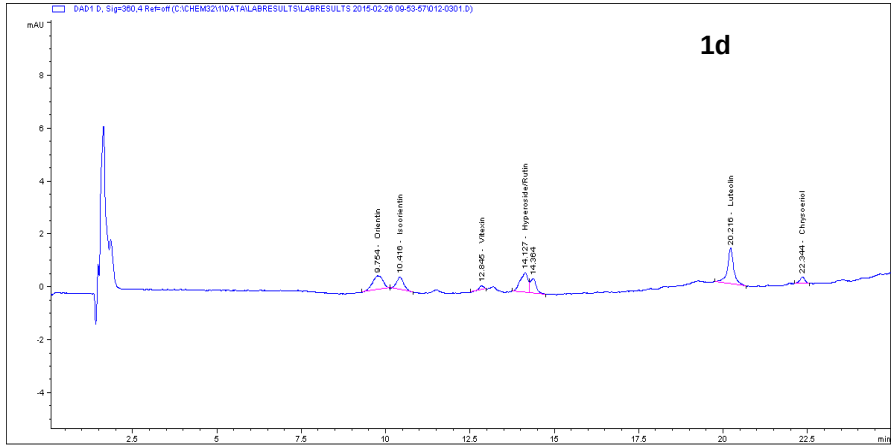


Figure 1: Quantification of the flavonoids orientin, isoorientin, isovetexin, vetexin, hyperroside, quercetin and luteolin in green/unfermented a) water b) methanol c) chloroform d) ethyl acetate rooibos extracts at 360 nm

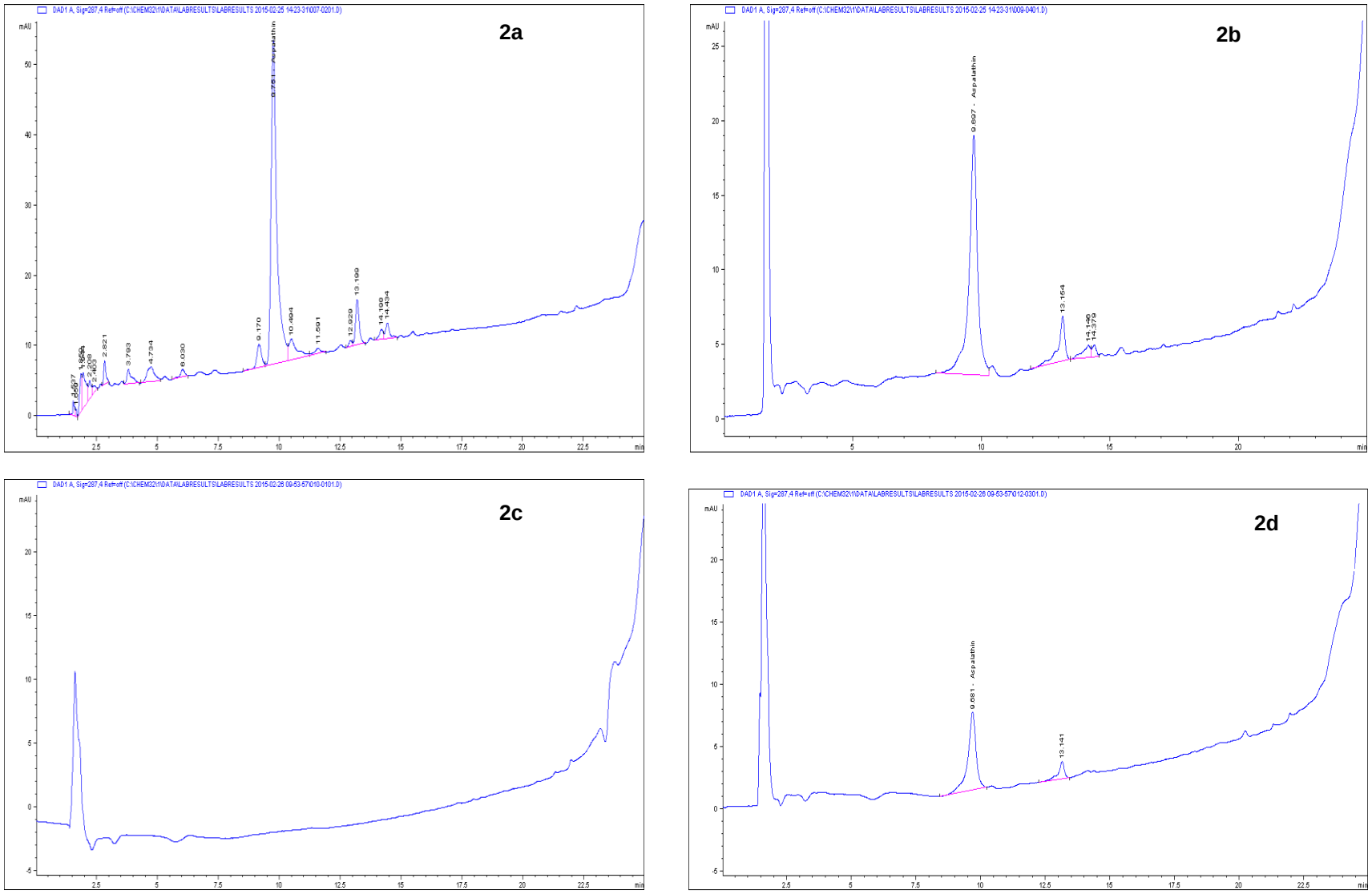


Figure 2: Quantification of the flavonoid aspalathin in green/unfermented a) water b) methanol c) chloroform d) ethyl acetate rooibos extracts at 287 nm

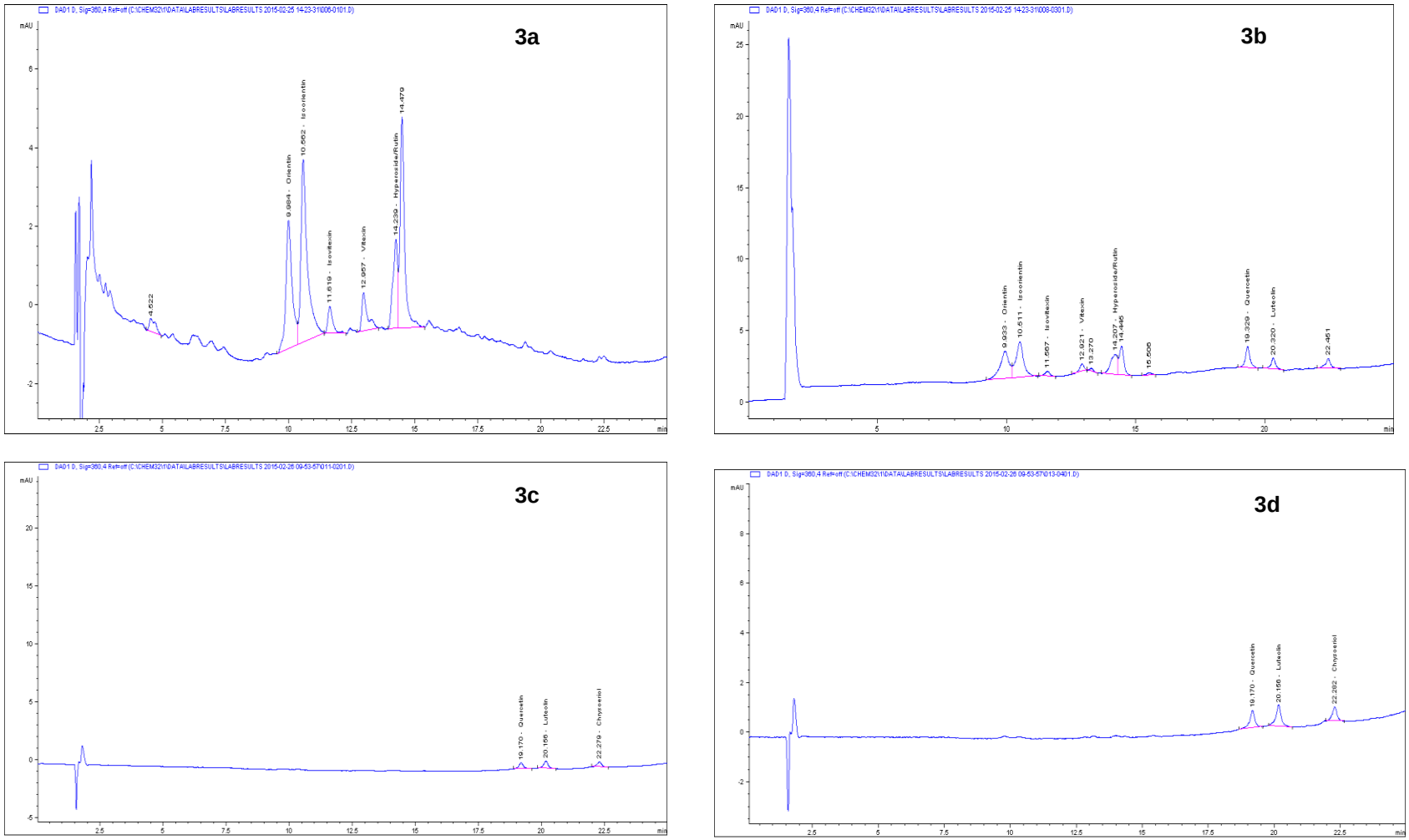


Figure 3: Quantification of the flavonoids orientin, isoorientin, isovetexin, vetexin, hyperroside, quercetin and luteolin in fermented a) water b) methanol c) chloroform d) ethyl acetate rooibos extracts at 360 nm

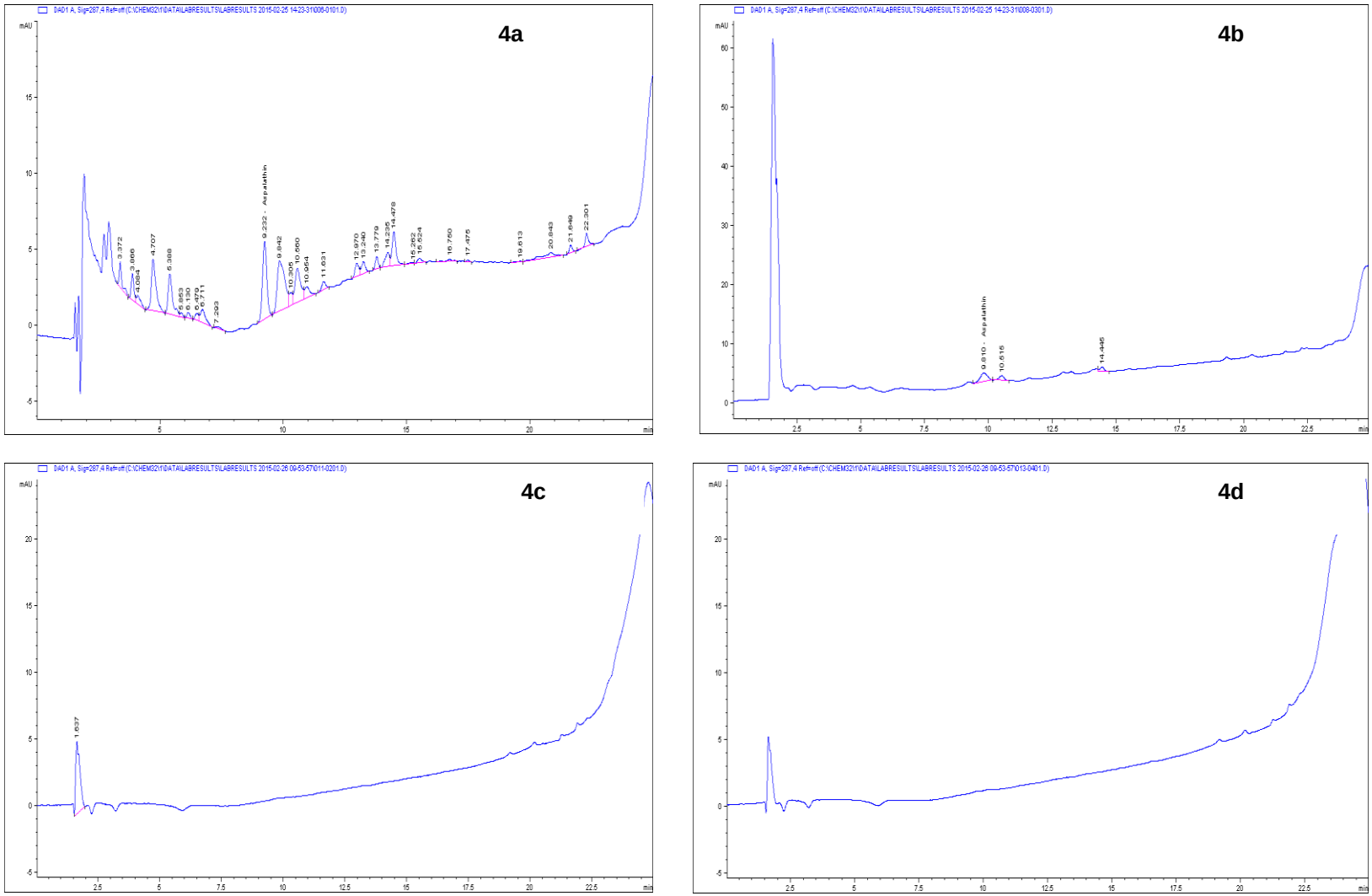


Figure 4: Quantification of the flavonoid aspalathin in fermented a) water b) methanol c) chloroform d) ethyl acetate rooibos extracts at 287 nm

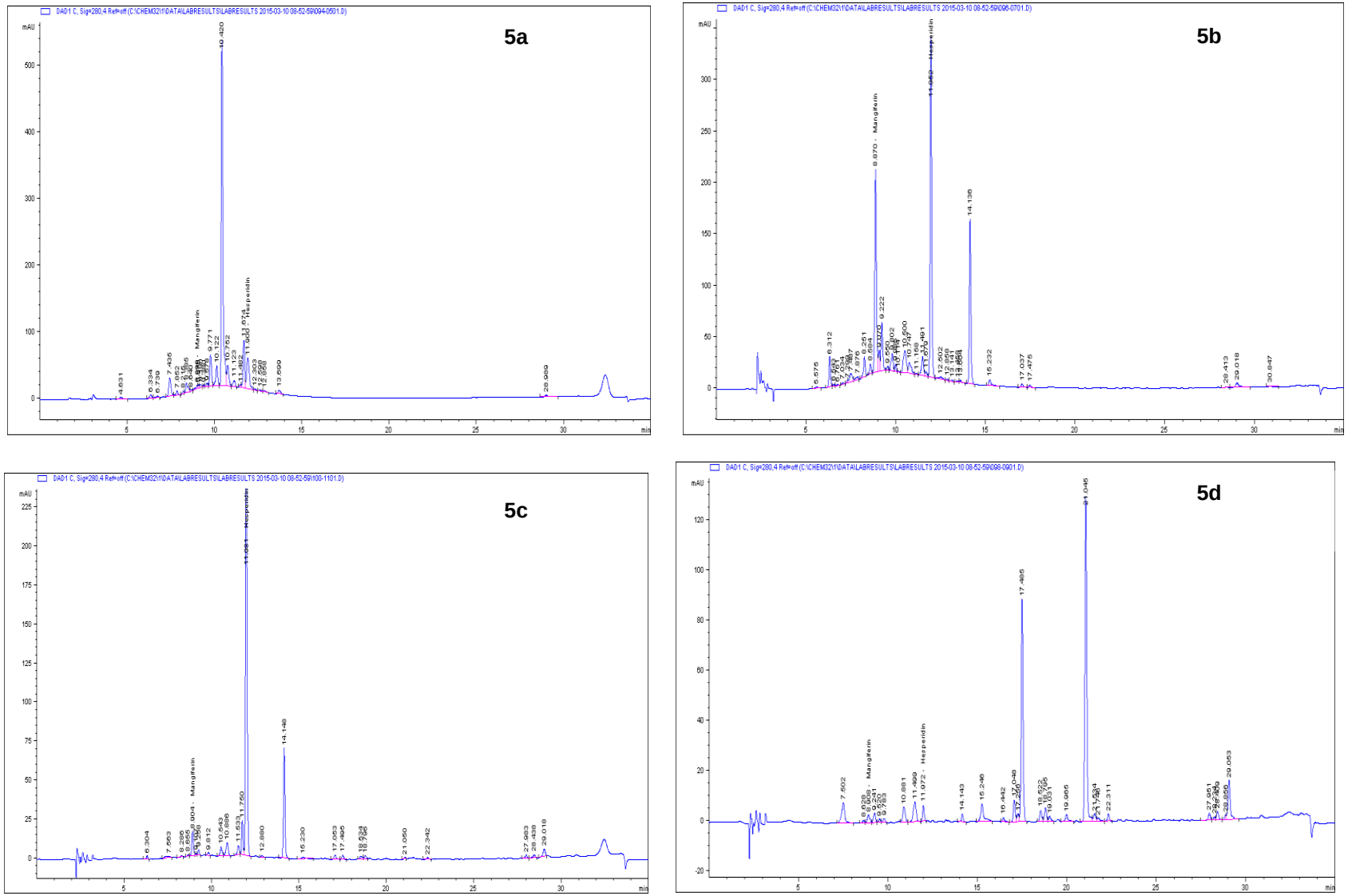


Figure 5: Quantification of the flavonoids hesperidin and mangiferin in green/unfermented a) water b) methanol c) chloroform d) ethyl acetate honeybush extracts at 280 nm

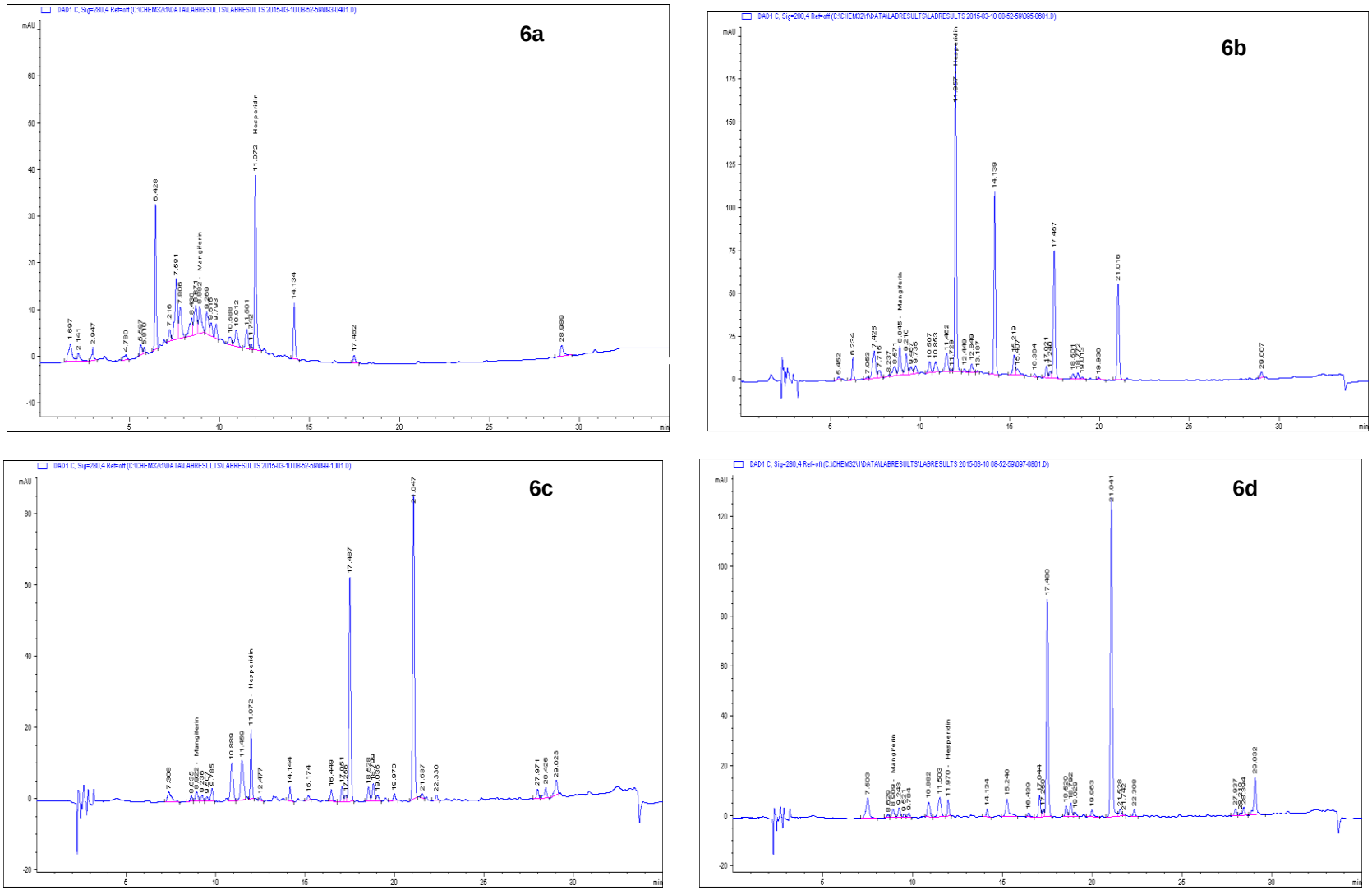


Figure 6: Quantification of the flavonoids hesperidin and mangiferin in fermented a) water b) methanol c) chloroform d) ethyl acetate honeybush extracts at 280 nm