ANTIOXIDANT ACTIVITY AND HEPATOPROTECTIVE POTENTIAL OF LEAF EXTRACTS FROM *MORELLA SERRATA* (LAM.) KILLICK (MYRICACEAE).

By

Mbhele, Nobuhle

A dissertation submitted in accordance with the requirements for the Magister Scientiae degree in the Faculty of Natural and Agricultural Sciences, Department of Plant Sciences at the University of the Free State, QwaQwa Campus.

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DECLARATION

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I, **Mbhele Nobuhle (Student number: 2009131158)**, do hereby declare that the dissertation hereby submitted for the qualification for the degree Master Scientiae in Botany at the University of the Free State represents my own original, independent work and that I have not previously submitted the same work for a qualification at another university.

I further cede copy right of the dissertation in favour of the University of the Free State.

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DEDICATION

To my late father Thamsanqa Jerom Mbhele.

"You left so soon dad, you know how you left us and it pains me deeply to think that you are not here anymore to share grateful times with me. I know you are looking back on us. You will always be loved and missed".

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ACKNOWLEDGEMENTS

Firstly, I would like to thank the Almighty God for leading, guiding and giving me unbelievable power when I needed it the most.

To my main supervisor Dr. Anofi Omatoyo Tom Ashafa, Acting Dean of Faculty of Natural and Agricultural Sciences, University of the Free State, Qwaqwa campus for his constant help, valuable guidance, pressure, constructive suggestions and most importantly father figure role you played in my life. Thank you for being a father to me. I will forever be grateful of meeting you in my life.

To my Co-supervisors Dr. Hafiz Abdelgadir and Dr. Ashwell Ndhlala from the Agricultural Research Council, thank you so much for such an opportunity you have given me. Thank you for your support and guidance throughout my studies.

My deep gratitude goes to two PhD students, Mr Fatai Balogun and Sabiu Saheed. Thank you for being a source of support and guidance. I thank God that I have met such wonderful souls during the period of my study and I will forever be grateful for that.

Miss Getrude Mahanke, thank you for your constant help sister. I will always remember the laughter we shared during the course of the study. Thank you so much and full of appreciation.

To PpRg members thank you all for the support, great efforts and sacrificing your time in the fulfilment of this work.

To Agro-processing members at the Agricultural Research Council- Vegetable and Ornamental Plants, I am very grateful that I have found sisters and brothers within you.

To my husband Bonga Njoko, thank you for your patience, love, support and encouragement everything else. Thank you my life partner.

To the lady who saw a potential in me, Miss Vezi. Thank you so much for your help and believing in me. I would not be where i am today if it wasn't for you. Thank you.

Finally, I would like to acknowledge the Agricultural Research Council and National Research Foundation for funding my studies.

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RESEARCH OUTPUTS

Conference proceedings

Mbhele, N., Abdelgaldir, H.A., Ndhlala, A.R., Ashafa, A.O.T., 2016. Phytochemical analysis and antioxidant activity of leaf extracts of *Morella serrata* (Lam.) Killick (Myricaceae). 42nd Annual Conference of the South African Association of Botanists (SAAB) and the 12th Southern African Society for Synthetic Biology (SASSB), 10 – 13 January 2016. University of the Free State, Bloemfontein.

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ABBREVIATIONS AND SYMBOLS

-: Negative

+: Positive

<: Less

>: Greater

±Plus or minus

ABTS: 2, 2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt

$AlCl_3$: Aluminium chloride

ALT: Alanine transaminase

ALP: Alkaline phosphate

ANOVA: One way analysis of variance

ARC: Agricultural Research Council

AST: Aspartate transaminase

BHA: Butylatedhydroxy anisole

BHT: Butylatedhydroxy toluene

b.w: Body weight

CAT: Catalase

CCl_4: Carbon tetra chloride

$CHCl_3$: Chloroform

DNA: Deoxyribonucleic acid

DPPH: 1, 1-diphenyl-2-picrylhydrazyl

EDTA: Ethylenediaminetetra acetic acid

et al.: and others

etc: et cetera

$FeCl_2$: Ferrous chloride

GAE: Gallic acid equivalents

H_2O_2: Hydrogen peroxide

H_2SO_4: Sulphuric acid

Hb: Haemoglobin

HCl: Hydrochloric acid

Hct: haematocrit

HIV/AIDS: Human Immunodeficiency Virus/ Acquired Immunodeficiency Syndrome

i.p: intraperitoneal

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IC_{50}: 50% of the amount of extract needed to inhibit free radicals at a specified time

In vitro: Laboratory experiment performed outside the specimen biological context

In vivo: Experiment performed in live specimen

MSLAEE: *Morella serrata* leaf aqueous-ethanol extract

N: Neutrophils

NaCl: Sodium chloride

NaCO3: Sodium carbonate

NRF: National Research Foundation

P: Platelets

PpRg: Phytomedicine and pharmacology research group

QE: Quercetin equivalents

RBC: Red blood cell

RNS: Reactive nitrogen species

RONS: Reactive oxygen and nitrogen species

ROS: Reactive Oxygen Species

SEM: Standard error of mean

TB: Tuberculosis

TBA: Thiobarbituric acid

TBARS: Thiobarbituric Acid Reactive Substances

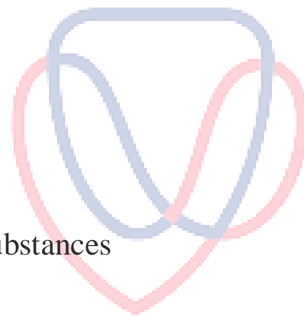
TCA: Trichloroacetic acid

UNAIDS: The Joint United Nations Programme on HIV and AIDS

WBC: White blood cell

WHO: World Health Organisation

α: Alfa



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LIST OF SI UNITS

% w/v: Percentage weight per volume

% w/w: Percentage weight per weight

%: Percentage

°C: Degrees centrigates

µl: Microlitre

µm: Micrometer

µM: Micromolar

g/dL: Gram per decilitre

g/L: Gram per litre

g: Gram

GAE/g: Gallic acid equivalents per gram

hr: Hour

L/L: Litre per litre

M: Molar

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mg/g: Milligram per gram

mg/kg: Milligram per kilogram

mg/ml: Milligram per millilitre

mg: Milligram

min: Minute

ml/kg: Milligram per kiligram

ml: Millilitre

mM: Millimolar

mol/L: Molar per litre

N: Normality

nm: nanometre

QE/g: Quercetin equivalents per gram

Rpm: Revolutions per minute

$x10^{12}$/L: Ten to the exponent of twelve per litre

$x10^{9}$/L: Ten to the exponent of nine per litre

ABSTRACT

Morella serrata L. Killick (Myricaceae) - is a South African plant finding therapeutic applications in oxidative stress related disorders including asthma, diabetes and male sexual dysfunction. The plant has not been scientifically investigated for its antioxidant and hepatoprotective activity. Thus the present study was aimed at determining the chemical constituents, antioxidant activity of *M. serrata* leaf extracts (ethanol, hydroalcohol and water) and hepatoprotective potential of aqueous-ethanol extract against carbon tetrachloride-induced liver injury in Wistar rats.

Phytochemical screening coupled with quantification of phenolic compounds was performed in extracts using standard methods. The preliminary screening of *M. serrata* leaf extracts revealed the presence of flavonoids, tannins, phenols, saponins, steroids, terpenoids and resins whilst alkaloids, phlabotannins as well as cardiac glycosides were not detected. The total phenolic, flavonoid and flavonol content of the extracts ranged from 0.06± 0.01 to 0.24±0.02 mg GAE/g; 1.25± 0.01 to 2.04± 0.03 mg QE/g; and 0.35± 0.01 to 0.50± 0.01 mg QE/g respectively.

The antioxidant activity of the extracts was assessed using DPPH, ABTS, nitric oxide, hydroxyl radical, reducing power, hydrogen peroxide and metal chelating assays using ascorbic acid as reference. Of all the tested extracts, the ethanol extract showed maximum free radical scavenging activity in the DPPH and nitric oxide scavenging activity assays while water extract showed maximum free radical scavenging activity in the ABTS, hydroxyl radical, hydrogen peroxide and metal chelating assay. Hydroalcohol extract showed maximum scavenging activity in the reducing power assay as compared to other extracts.

A 21-day daily double dose protective effect of the graded doses (100, 200, 400 mg/kg body weight) of *M. serrata* hydro-alcohol extract was tested against CCl_4-induced hepatotoxicity in Wistar rats using silymarin as a positive control. The effect of CCl_4 was investigated on liver and body weight, feed and water intake, haematological parameters, serum biochemical functions, liver marker enzymes and liver histology. Findings revealed a significant increase in liver weight in CCl_4-alone intoxicated rats compared to normal control. All groups intoxicated with CCl_4 displayed a loss in appetite after CCl_4 administration as compared to normal control. A decrease in body weight was observed in

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rats treated with CCl_4-alone which was reversed following treatment with extract and silymarin. CCl_4 intoxicated rats showed severe liver damage which was indicated by altered haematological parameters and elevated serum activity of ALP, ALT and AST. This was accompanied by a reduction in activity of marker enzyme CAT and a significant rise in TBARS concentration. This was however ameliorated in MSLAEE and silymarin treatments groups. Histopathological micrographs of hepatotoxic group revealed extensive liver damage characterised by severe necrosis, however, such damage was prevented in MSLAEE and silymarin pre-treated groups. The degree of damage in liver tissues was in the order CCl_4- alone treated rats > 200 mg/kg b.w MSLAEE treated rats > 400 mg/kg b.w treated rats > 100 mg/kg b.w treated rats > Silyamrin treated rats > Normal control.

Our findings from the research work provide support and evidence on the folkloric use *Morella serrata* as a potential natural antioxidant in treating oxidative stress induced ailments. The study also diverts from the perception that only the roots can be used to treat such ailments as the leaf extracts also showed effective antioxidant activity, thus contributing to the conservation of the plant. Data emanating from the further indicate that *M. serrata* was able to protect the liver against CCl_4-induced oxidative damage in rats which may be attributed to its antioxidant and free radical scavenging activities.

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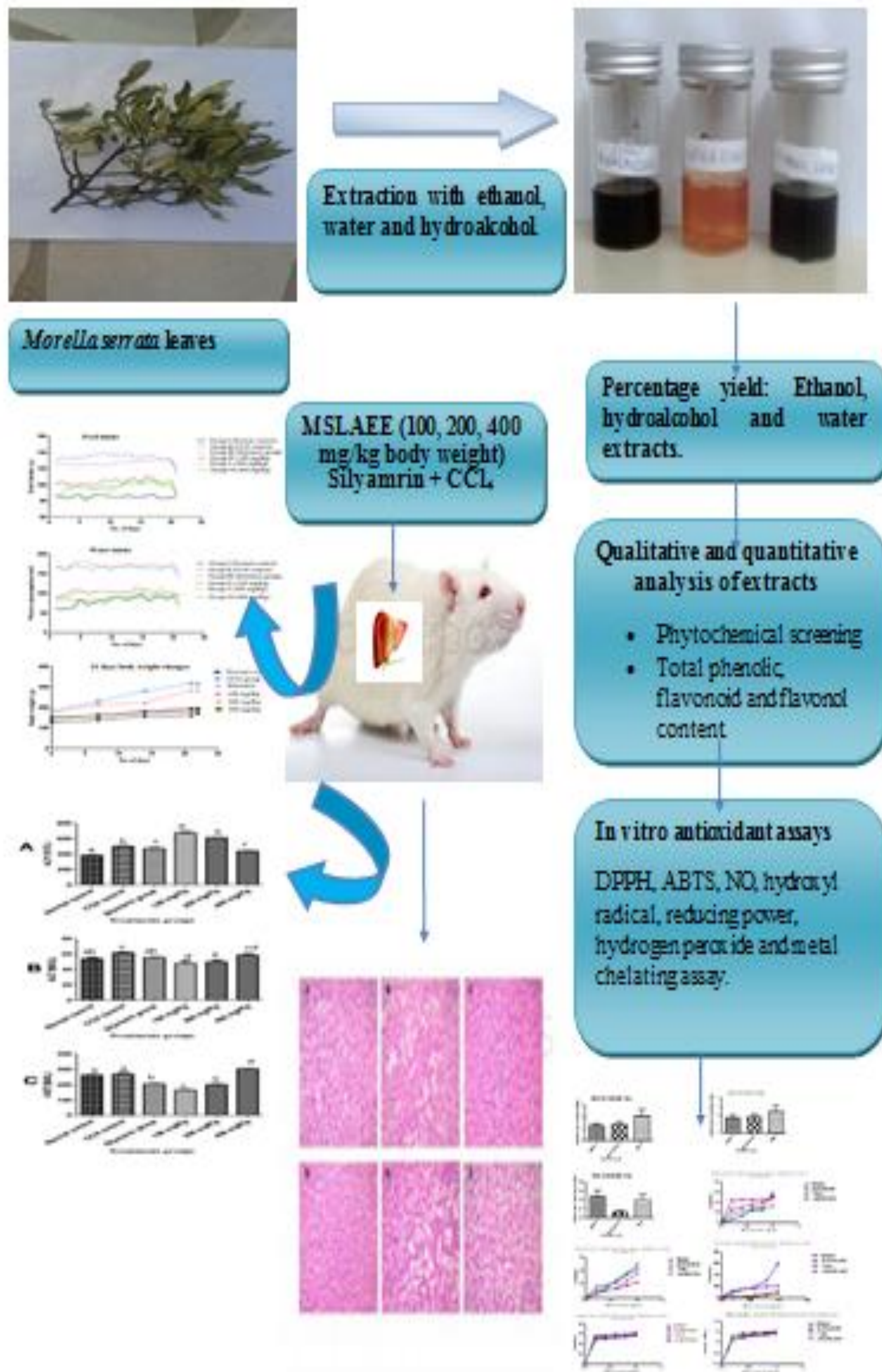
Keywords: Antioxidants, Carbon tetrachloride, Daily double dose, Free radicals, Hepatocytes, Liver injury, *Morella serrata*, Oxidative stress, Phenolic compounds.

GRAPHICAL ABSTRACT



CHAPTER 1

Introduction

1.1 Background of the study

Although free radicals form an essential part of a human life, they pose a major threat when they are over produced. Free radicals are of major concern worldwide as they are found to be associated with a number of deadly diseases. These include diabetes, skin lesions, immune depression, liver diseases, AIDS, infertility, pulmonary diseases, renal disorders, gastrointestinal diseases, tumour (Alessio and Blasi, 1997), premature infants diseases (O'donovan and Fernandes, 2004), neurodegenerative diseases, cancer, autoimmune diseases, ageing process, cardiovascular diseases (Pham-Huy *et al*., 2008) and eye disease (Kisic *et al*., 2014). Around the World, the damage caused by free radicals in human body is the leading cause of catastrophic diseases responsible for killing quite a number of people (Amić *et al*., 2003). According to Alwan (2011), 68% of deaths globally are due to noncommunicable diseases associated with free radicals.

There is an increasing interest and demand in research concerning liver diseases in recent years around the World. Liver disease is a global burden and it is among the leading cause of deaths and illness globally (Byass, 2014; Wang *et al*, 2014). Even with the advent of modern medicine, there are hardly any reliable drugs able to protect the liver from the damage or help regenerate damaged hepatocytes (Venukumar and Latha, 2002; Kissi, 2014). Over the counter available synthetic antioxidants used in the management of free radical associated diseases such butylatedhydroxy anisole (BHA) and butylatedhydroxy toluene (BHT) are dangerous to human health (Lobo *et al*. 2010) and to animals as they cause tissue toxicity, cell damage , inflammation, and atherosclerosis (Wintola and Afolayan, 2011). Since there are no alternatives, people still continue to use these products in managing free radical related diseases although they have been identified to be toxic. Plants have been used by humans for centuries in managing diseases and are considered safe and effective. Medicinal plants contain active principles which are used to cure various diseases or relief pain (Okigbo *et al*., 2008). Hence, research for new leads of natural

antioxidants as safe alternatives from medicinal plants is significant (Park *et al.*, 2004).

South African is exceptionally rich in plant biodiversity comprising about 30, 000 flowering plant species (Louw *et al.*, 2002), which accounts for 10% of the world's higher plants (Van Wyk and Gericke, 2000). Over 19, 500 of these higher plants are from about 350 families and indigenous to South Africa (Crouch *et al.*, 2008). South Africa is home to one of the six most recognised biodiversity hotspots due to its significant concentrations of plant biodiversity in the whole world, the Cape Floristic Region (Cowling and Richardson, 1995; Van Wyk and Smith, 2001). The opportunity for discovering and commercializing plant products from South African medicinal plants still remains underdeveloped (Street and Prinsloo, 2012). As diverse as South Africa is, only a small percentage of these plants have been investigated pharmacologically (Eloff, 1998). Hence, numerous plant species have the potential to be screened for active ingredients which can be used for pharmacological treatments.

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The highest deforestation rate has been recorded in the African continent (REUTERS, 2008; Gary and Thorpe, 2009). The continent loses about 1% of its land annually through deforestation (Iwu, 2014). Due to this, many medicinal plants may be lost even before they are discovered and documented. The trade of medicinal plants plays an important role in species disappearance in South Africa. Between 35, 000 to 70, 000 tons of plant materials in South Africa are used for herbal remedies (Mander, 1998). Medicinal plant knowledge is enormous and is passed verbally from one generation to another. However, if not rapidly researched and recorded, the knowledge will be lost with coming generations (Hostettmann *et al.*, 2000). Thus, the country will not only lose the precious plant diversity, but also the precious knowledge that goes with medicinal plants. Documenting and pharmacologically investigating the use of indigenous plant species will contribute to the knowledge of the people and to that of future generations. This will not only improve the knowledge of the people but will contribute to the conservation of indigenous plant species. By highlighting their importance in the communities and ensuring means of conservation.

1.2 Problem statement and justification of the study

As enormous as the botanical diversity of South Africa is, species like *Morella serrata* are among plant species that have not yet been investigated scientifically for their pharmacological use. *Morella serrata* L. Killick (Myricaceae) - is a South African plant finding therapeutic applications in oxidative stress related disorders such as asthma, diabetes and male dysfunction (Schimidt *et al*., 2002; Moffet, 2010). The present study is triggered by the fact that little is documented and investigated in terms of its phytochemicals and therapeutic application against oxidative stress associated conditions. The folkloric claims of this plant against oxidative stress associated conditions highlight its potential as a natural antioxidant. However, this has not been investigated and validated scientifically, thus it becomes of significant importance to conduct large-scale scientific research to verify its antioxidant potential.

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Plants are easily accessible to most South African communities, especially in poor rural areas. Plants have been used for hundreds of year and considered to be safe and effective. With the health care system not easily accessible in poor rural areas, individuals rely on plants to treat ailments and relive pain. Thus, it is of importance to investigate such plants for their safety and quality, additionally come up with sustainable interventions to conserve these plant species for sustainable use. The present study thus uses leaves to conduct the research for conservation purposes. This was triggered by the fact that in most cases, roots is the first point of attack when collecting plant material from the wild and is highly used in traditional system as compared to other parts of the plant. Taking into consideration that roots are the lifeline of plants, therefore it is a drawback to use non-renewable parts such as roots as a source of medicine as removing the roots leads to the death of the plant itself. It is also important to scientifically validate and divert from the perception that only the roots may be used to treat ailments as leaves, seeds, flowers and other parts of the plants have shown good activity (Mokgope, 2007; Otang *et al*, 2012; Arora *et al*., 2013).

The significance of finding active natural antioxidants in South Africa is of high importance as the country is faced with a combined contributing effect of HIV/AIDS

and tuberculosis (TB) syndemic (Kwan and Ernst, 2011). HIV/AIDS and TB treatments have been identified to result in liver toxicity to patients (Heil *et al*., 2010; Jong *et al*., 2013). South Africa occupies the highest cases of HIV/AIDS and tuberculosis (Evans, 2013) and a highest number of individuals on treatments for these diseases (UNAIDS, 2013). As a result, a high number of individuals are exposed to toxicity of these treatments. It is an undeniable fact that antiretroviral and antituberculosis drugs have significantly decreased morbidity and mortality cases (Heil *et al*., 2010). However, hepatotoxicity caused by antiretroviral (Montessori *et al*., 2004; Setzer *et al*., 2008, Kissi, 2014) and antituberculosis drugs (Chau, 2008; Hegde and Joshi, 2010; Jong *et al*., 2013) is highly recognised among HIV and TB-infected patients on treatment. Hence, it is of high importance to search for effective and safe alternatives or supplements of natural origin to protect the liver from the damage presented by these treatments.

Liver diseases caused by tobacco smoking and alcohol consumption accounts for more deaths (Hart *et al*., 2010, WHO, 2012) as an estimated 20, 000 deaths within a year are recorded worldwide (Bairwa *et al*., 2010). According to a report by Rehm *et al*. (2013), alcohol-attributed liver cirrhosis and alcohol-attributed liver cancer were respectively responsible for 493, 300 and 80, 600 deaths in the year 2010. Alcohol has been reported to triggers the over production of ROS in the liver (Galicia-Moreno and Gutiérrez-Reyes, 2014) and major factor posing risk and contributing to liver diseases worldwide (Rush, 1823). The consumption of alcohol in South Africa is unimaginable and findings revealed that South Africans consume an excess of 5 billion litres of alcohol annually (WHO; 2011). Similarly, Seggie (2012) describes South Africa as a heavy drinking country consuming about 5 billion litres of alcohol per annum and still rising giving the country a score of 4 out of 5 on risk of drinking patterns. For South Africa, this scale means there is greater alcohol-attributed disease burden (Seggie, 2012). According to the South African Community Epidemiology Network on Drug Use (2010), South African communities have highest rate of substance abuse of different kinds. Looking at the rate of alcohol and substance abuse in the country, there is high prevalence of liver diseases. This explains the significance of research on active antioxidants in South Africa.

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1.3 Aim and objectives of the study

1.3.1 Aim

Morella serrata is employed traditionally in treating oxidative stress implicated disorders such as asthma, diabetes and male dysfunction. Therefore, the aim of the study was to investigate the *in vitro* antioxidant potential of *M. serrata* leaf extracts and to further validate its potential in treating oxidative stress *in vivo* by evaluating the potential hepatoprotective effect of aqueous-ethanol extracts against carbon tetrachloride (CCl_4) induced liver damage in Wistar rats.

1.3.2 Objectives

The objectives of the study are to:

- Investigate the effect of extraction solvents on the percentage yield of the extract.

- Detect the presence of different phytochemicals in different leaf extracts (ethanol, hydroalcohol and water) from *M. serrata*, using different recognised standard methods.

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- Investigate the effect of extraction solvents on the total phenolic, flavonoid and flavonol content of the extracts.

- Evaluate the free radical scavenging and antioxidant potential of *M. serrata* leaf extracts (ethanol, hydroalcohol and water) using different *in vitro* approaches, and compare the results with those of a standard ascorbic acid.

- Investigate the contributing effect of detected phytochemicals on antioxidant activity of the extracts.

- Investigate the effect of single intraperitoneal dose of carbon tetrachloride (CCl_4) on feed and water intake, body weight, liver and liver-body weight in rats pre-treated with *M. serrata* leaf aqueous-ethanol extract at doses 100, 200 and 400 mg/kg body weight (b.w).

- Investigate the hepatoprotective effect of *M. serrata* aqueous-ethanol extract at doses 100, 200 and 400 mg/kg b.w and silymarin on CCl_4 -induce hepatotoxicity in Wistar rats by evaluating haematological parameters such as (Red blood cell, white blood cell, haemoglobin, haematocrit, Neutrophils and platelets.), liver function indices such as (aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphate (ALP), and liver marker enzymes such as catalase (CAT), thiobarbituric acid reactive species (TBARS) and compare the findings with that of a standard hepatoprotective drug, silymarin.

- Investigate the effect of different treatments (CCl_4, silymarin and MSLAEE) on the histopathology of the liver.

1.4 Hypothesis

- Leaves form part of a plant and are generally known to contain phytochemicals. The leaves of *M. serrata* may contain some phytochemicals that are found in the root of the plant as reported by research done by Ashafa (2013). Thus leaves can be used to outplace the roots when treating ailments for conservation and sustainable utilization of the plant.

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- The type of solvent used for extraction has an effect on the extract yield, extraction of phytochemicals, total phenolic compounds and antioxidant activity.

- Since the plant is used locally to treat free radical implicated diseases such as asthma, it may have good antioxidant activity.

- Rat treated with aqueous-ethanol extract or silymarin for 21 consecutive days could significantly decrease liver weight arising from treatment with CCl_4. Thus, the plant may have hepatoprotective activity and may be used to treat liver disease.

- The oral administration of aqueous-ethanol leaf extracts of *Morella serrata* has protective effect against CCl_4-induced liver toxicity in Wistar rats.

1.5 Significance of the study

The study will benefit the South African communities using the plant because it will scientifically investigate and validate the claims on the plant as a good antioxidant used in the management of free radical associated diseases like asthma, diabetes and sexual dysfunction. The study will recommend which solvent is suitable for extracting phytochemicals present in the leaves of *M. serrata* responsible for biological activities and investigate the possibility of the plant in treating liver disease. Furthermore, the study will contribute to the conservation means of sustainable usage of the plant.

1.6 Outline of the dissertation

The following outline gives a brief skeletal description of the content of this dissertation. The dissertation contains six chapters and the focal point of each chapter is as follows:

Chapter 1: Introduction

The chapter provides an overview and background of the study. The chapter also outlines problem statement, aims, objectives, and essence of the study.

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Chapter 2: Literature review: An overview on free radicals, oxidative stress, antioxidant and carbon tetrachloride-induced liver damage

The chapter outlines a comprehensive literature review relating to the study undertaken. It concentrated mainly on free radicals and oxidative stress implicated diseases of major threat around the world. The chapter also focuses on the mechanism of liver damage by CCl_4, additionally gives essential information on the choice of plant for the present study.

Chapter 3: Qualitative and quantitative analysis of leaf extracts from *Morella serrata*

The chapter outlines the first experimental methodology undertaken in the study. This includes the qualitative screening (preliminary screening of phytochemicals) and quantitative analysis (the total content determination of phenolic compounds) of leaf extracts from *M. serrata*.

Chapter 4: *In vitro* antioxidant activity and free radical scavenging activity of *Morella serrata* leaf extracts

The chapter outlines the second experimental methodology undertaken in the study. This includes the antioxidant and free radical scavenging activity of leaf extracts from *M.*

serrata using standard methods and comparing the results with that of a known standard (ascorbic acid).

Chapter 5: Hepatoprotective activity of *Morella serrata* ethanolic extract against carbon tetrachloride (CCl_4)-induced liver injury in Wistar rats

The chapter outlines the third part of experimental methodology undertaken in the study. In this chapter, different doses (100, 200 and 400 mg/kg body weight) of aqueous-ethanol leaf extracts of *M. serrata* were used to assess protective effect against CCl_4-injuced liver injury in Wistar rats. The findings were compared to that of standard silymarin which served as a positive control.

Chapter 6: Summary, Conclusion and Recommendations

This chapter summarises the findings, conclusion and recommendation on the study that may be undertaken to improve the present study in the future.

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CHAPTER 2

Literature review: An overview on free radicals, oxidative stress, antioxidant and Carbon tetrachloride-induced liver damage

2.1 Free radicals

Molecular oxygen (O_2) is an essential molecule needed by humans for survival. Oxygen inhaled is consumed by body cells to generate energy required for survival via the mitochondrial respiratory chain (Kabel, 2014). The mitochondrial respiratory chain is a major site of reactive oxygen species (ROS) production which utilizes about 80 to 90% of consumed molecular oxygen (Wu and Cederbaum, 2003). However it is stated that only about 2 to 3% of the consumed molecular oxygen is involved in ROS production (Chance *et al*., 1979). Free radicals form part of our daily lives as they are continuously produced during the normal body use of molecular oxygen. Free radical is a collective name for ROS and reactive nitrogen species (RNS) (Pham-Huy *et al*., 2008). Free radicals can be defined as molecules or molecular fragments containing one or more unpaired electrons in their atomic or molecular orbits (Halliwell and Gutteridge, 1999; Valko *et al*., 2007). An unpaired electron is an electron that occupies an atomic or molecular orbit singly, rather than as an electron pair. The presence of an unpaired electron(s) makes the molecule(s) unstable and highly reactive towards other molecules (Lobo *et al*., 2010) where they can behave as reductants or oxidants to other molecules (Cheeseman and Slater, 1993).

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In a normal human body, each body cell is attacked by an estimated 10, 000 to 20, 000 free radicals per day (Valko *et al*., 2006) and neutralization of these species is a major prerequisite of an aerobic life (Sies, 1986). For a free radical to neutralise itself, it seeks out and steal electrons from other molecules to gain stability (Wu and Cederbaum, 2003), allowing this molecule to become a free radical itself (Krishnamurthy and Wadhwani, 2012) that is less harmful (Lü *et al*., 2010). This reaction continues on and on resulting in a chain of reactions where in each step there is a formation of a newly generated free radical (Halliwell and Gutteridge, 2007). The chain reaction is thus maintained and continues which can be a thousand of events long (Valko *et al*., 2006). Chain reaction is thus defined as a series of reactions capable of initiating a new cycle of reactions in the process (Sen *et al*., 2010). Free radical chain reactions involve three distinct steps

namely: Initiation, propagation and termination (Manavalan and Ramasamy, 2001; Sarma *et al.*, 2010). Initiation involves formation of free radicals. During propagation, free radicals are generated repeatedly as a result of chain reaction while termination involves radical destruction.

2.1.1 Reactive oxygen species (ROS) and Reactive nitrogen species (RNS)

Reactive oxygen and nitrogen species (RONS) is a combined term for two classes of chemically reactive molecular fragments respectively containing ROS and RNS (Weidinger and Kozlov, 2015). ROS are derived from oxygen metabolisms while RNS are derived from nitric oxide and superoxide (Devasagayam *et al.*, 2004). The difference between the two is that RNS have a longer life compared to ROS thus making them more demanding (Balazy and Nigam, 2003). These species are both generated naturally in small amounts (Wu and Cederbaum, 2003) continuously during normal biochemical reaction in a human body, exposure to toxic environment and dietary xenobiotics (Bagchi and Puri, 1998). Both species react with molecules of cellular importance causing them to lose their structure and function (Krishnamurthy and Wadhwani, 2012).

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2.1.2 Sources of free radicals

There are three sources of free radicals namely internal, external and physiological sources (Kumar *et al.*, 2011). Internally generated sources of free radicals according to Ebadi (2001) include Inflammation, Xanthine oxide, Phagocytosis, arachidonate pathways and Ischemia/ reperfusions. Externally generated sources of free radicals includes cigarette smoke, environmental pollutants and radiation, certain drugs, pesticides, industrial solvents, ozone, or medication (Valko *et al.*, 2007; Lobo *et al.*, 2010), whilst physiological sources include disease conditions, mental conditions such as stress, emotions (Kumar *et al.*, 2011).

2.1.3 Beneficial and deleterious effects of RONS

Depending on the environment, ROS and RNS can be both beneficial and deleterious on the biological systems (Glade, 2003; Valko *et al*., 2004). ROS and RNS are essential in energy supply to the body, detoxification, chemical signalling and in the functioning of the immune system (Dimitrios, 2006; Gutowski and Kowalczyk, 2013). However, concentration in the system determines their effect. At low or moderate concentrations they are believed to exert beneficial effects on cellular response and immune function. However, in higher concentrations are toxic and exert deleterious effect due to oxidative stress resulting in several diseases (Valko *et al*., 2004; Halliwell and Gutteridge, 2007). At low concentration, RONS are involved in the maturation process of cellular structures and act as a weapon for the host defence system (Pham-Huy *et al*., 2008). At high concentration, they present oxidative stress characterised by alteration of cell structure, its content and other structures (Sies, 1986; Poli *et al*., 2004; Halliwell, 2007). However, Sarma *et al*. (2010) reported that some free radicals such as nitric oxide and superoxide when produced in very high amount become poisonous to foreign particles thus protecting against viruses and bacteria. This indicates that it is not only the low or moderate concentrations of RONS that is beneficial to the body, high concentrations of certain RONS are also beneficial to the system. Free radicals are essential for life thus complete elimination would also pose harmful effect (Bagchi and Puri, 1998). Thus, keeping them at normal concentrations as produced by the normal body biological activities is essential for human health without the involvement of external or physiological sources in the system.

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2.1.4 Oxidative stress and nitrosative stress

ROS and RNS are responsible for causing stress in different pathophysiological conditions (Kim and Byzova, 2014; Nimse and Pal, 2015). Oxidative stress and nitrosative stress are described as the harmful effect caused by free radicals of oxygen and nitrogen origin respectively resulting in potential biological damage (Ridnour *et al*., 2005). Oxidative or nitrosative stress represents an imbalance between the production and elimination of RONS in the body and also a decrease in antioxidant production (Salim, 2014; Li *et al*., 2015). Normally, there is a natural balance between the quantity of free radicals produced and antioxidants produced by the body (Masoko and Eloff, 2008). The balance prevents stress thus dominating the recurrence of oxidative or nitrosative stress implicated diseases. During stress, the production of free radicals exceeds the ability of

antioxidant to scavenge them. The excess free radicals will stabilize themselves by pairing with biological molecules (**Figure 2.1**) of significant importance such as lipids, proteins and DNA (Sen, 2003; Masoko and Eloff, 2008) leading to mutagenic changes, tissue damage and cell death (Valko *et al*., 2007; Sen and Chakraborty, 2011). It is not entirely that oxidative stress has negative effects as some positive effects have been recorded. According to Yoshikawa and Naito (2002), during delivery of the new born, oxidative stress induces apoptosis to prepare the mother's birth canal for delivery. The research further reports that oxidative stress strengthens biological defence mechanisms in our bodies during physical exercise and ischemia.

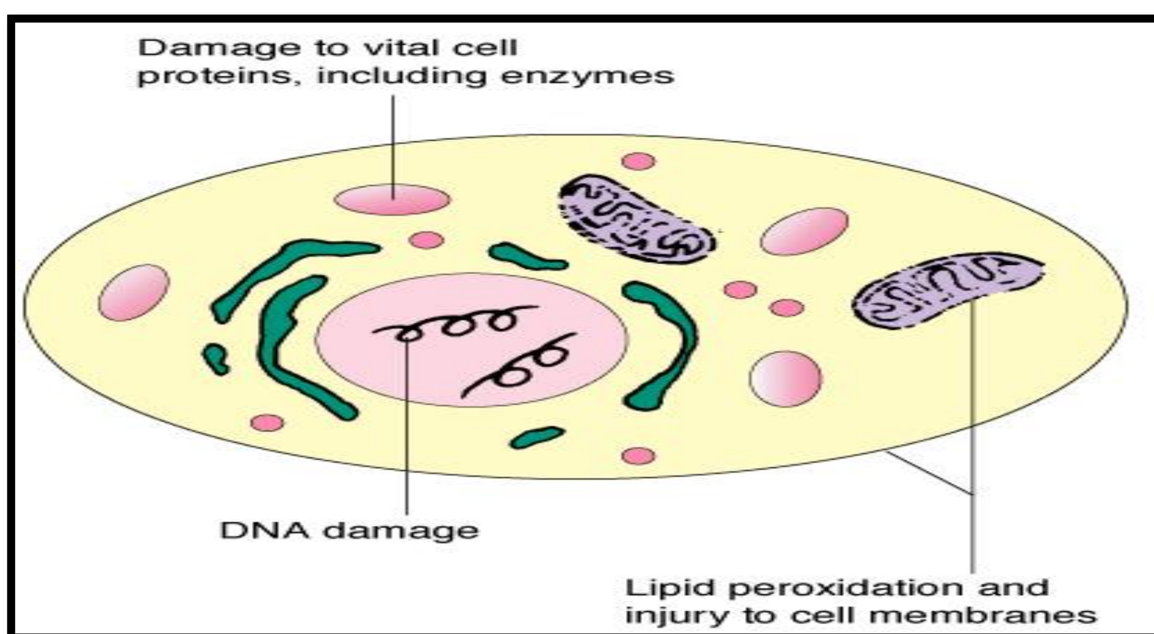

Figure 2.1 Three major cellular components attacked by free radicals (Sen, 2003)

2.1.5 Prevalence of pathological conditions associated with free radicals and oxidative stress Worldwide and in South Africa.

Abundant literature evidence points out oxidative stress as the primary factor in development of numerous pathological diseases. At least 50 diseases are associated with free radicals (Halliwell, 1994). Many body organs are attacked by excess free radicals leading to different pathological conditions in humans as shown in **Figure 2.2** (Pham-Huy *et al*., 2008). Pala and Gürkan (2008) classify these pathological conditions into two groups namely Mitochondrial and inflammatory oxidative stress conditions. Gunalan *et al*. (2012) reported that about 95% of pathological conditions observed in individuals who

are older than 35 years old of age are associated with production and accumulation of excess free radicals in the body.

A report by Puoane *et al* (2008) indicates that between the years 1998 to 2000 in South Africa, a large increase of prevalence of diseases associated with free radicals such as hypertension, stroke, diabetes, asthma and cancer was observed. Similarly Norman *et al* (2007) reported a number of free radical related diseases contributing to mortality in South Africa such as heart diseases, stroke, diabetes mellitus, asthma and cancer. A total of 65 000 lives are claimed by these diseases annually (Bradshaw *et al.*, 2003)

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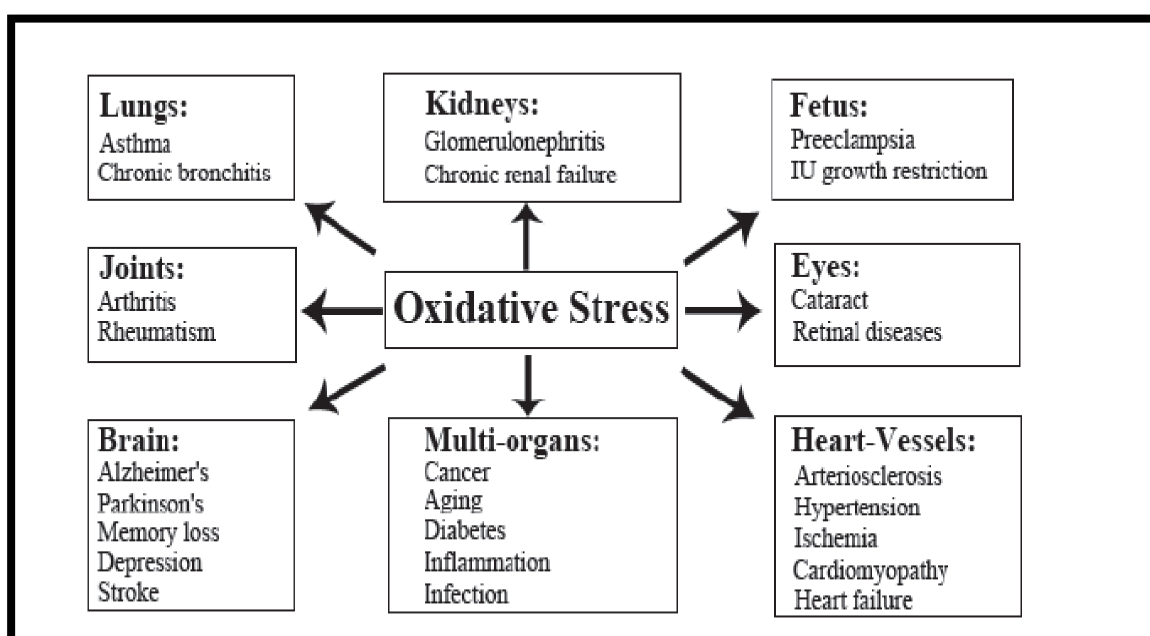

Figure 2.2 Oxidative stress-induced diseases in humans (Pham-Huy *et al.*, 2008)

2.2 Antioxidants

Antioxidants are substances capable of delaying or inhibiting the oxidation of a substrate when present at a lower concentration than that of an oxidized substrate (Gutteridge, 1994; Antolovich *et al.*, 2002; Gupta and Sharma 2006). Antioxidants are a first line of defence against free radical damage capable of stabilizing or deactivating free radicals before they attack cells (Rakesh *et al.*, 2010). In simple terms, Young and Woodside (2001) defined antioxidants as molecules that inhibit or quench free radical reaction and delay or inhibit cellular damage. Antioxidants are naturally produced by the body's

normal metabolism, however in the case of overproduction of free radicals in the system, antioxidants actions are disturbed and are said to decrease with age (Sies, 1997). According to Prakash *et al*. (2001), the main feature of an antioxidant is its potential to trap free radicals, which defines its ability as an antioxidant. The antioxidant activity of a substance is based on its ability to donate or accept hydrogen atom from the free radical. When free radicals are trapped, this terminates the chain reaction where by the reaction with reactive oxygen species is prevented thus maintaining the free radical in its redox state which leads to its inability to reduce molecular oxygen (Flora, 2009). The antioxidant hypothesis states that if antioxidants can prevent oxidative damages, thus increased intake from the diet will also reduce the risks of chronic diseases (Stanner *et al*., 2004).

When antioxidants react with free radicals, they may directly destroy free radicals or render them less reactive, free-lived and less dangerous newly born free radicals (Lü *et al*., 2010). In this case, they further react with other antioxidants to terminate their radical status. According to Nimse and Pal (2015), antioxidants can be categorised based on their activity, solubility in water or lipids and size. Whereas Jadhav *et al*. (1996) classified antioxidants into two based on their functions as primary (chain breaking antioxidants) and secondary (preventing antioxidants). Due to the difference in their mechanism of action when scavenging free radicals, they are thus classified in two major groups' namely enzymatic and non-enzymatic antioxidants. Irshad and Chaudhuri (2002) classified enzymatic antioxidant as first line of defence and non-enzymatic antioxidants as second line of defence. Enzymatic antioxidants include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidise (GPx), glutathione reductase (GR), etc. and non-enzymatic antioxidants include Glutathione (GSH), vitamin C (ascorbic acid), Vitamin E (α-tocopherol), etc (Goodman *et al*., 2011).

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2.2.1 Natural antioxidants

Natural antioxidants are antioxidants derived from plant sources (Pandey and Rizvi, 2009) and are present as chemical compounds in all plant parts (Asif, 2015) such as fruits, vegetables, nuts, seeds, leaves, root and root bark (Pratt and Hudson, 1990). Thus the basic sources of natural antioxidants for humans are plant derived products. **Figure 2.3** gives a classification of natural antioxidants (Brar *et al*., 2014). Natural antioxidants include phenolics and polyphenolic compounds, chelators, vitamins and enzymes, as well

as carotenoids and carnosine (Shahidi, 1997). Plants natural products such as flavonoids, anthocyanins, carotenoids, dietary glutathionine, vitamins, and endogenous metabolites are rich in antioxidant activities, thus they are commonly known as free radical scavenging molecules (Kivits *et al*., 1997). Recent findings from different researchers have indicated that consumption of plant foods and natural antioxidant supplements significantly protects the body against oxidative stress implicated conditions (Vinson *et al*., 1995; Dhalla *et al*., 2000; Prior and Cao, 2000; Sabu and Kuttan, 2002; Sen *et al*., 2010). Natural antioxidants help the endogenous antioxidant system to reverse oxidative damage or protect against conditions where oxidative stress is implicated (Elmastas *et al*., 2007; Sen and Chakraborty, 2011).

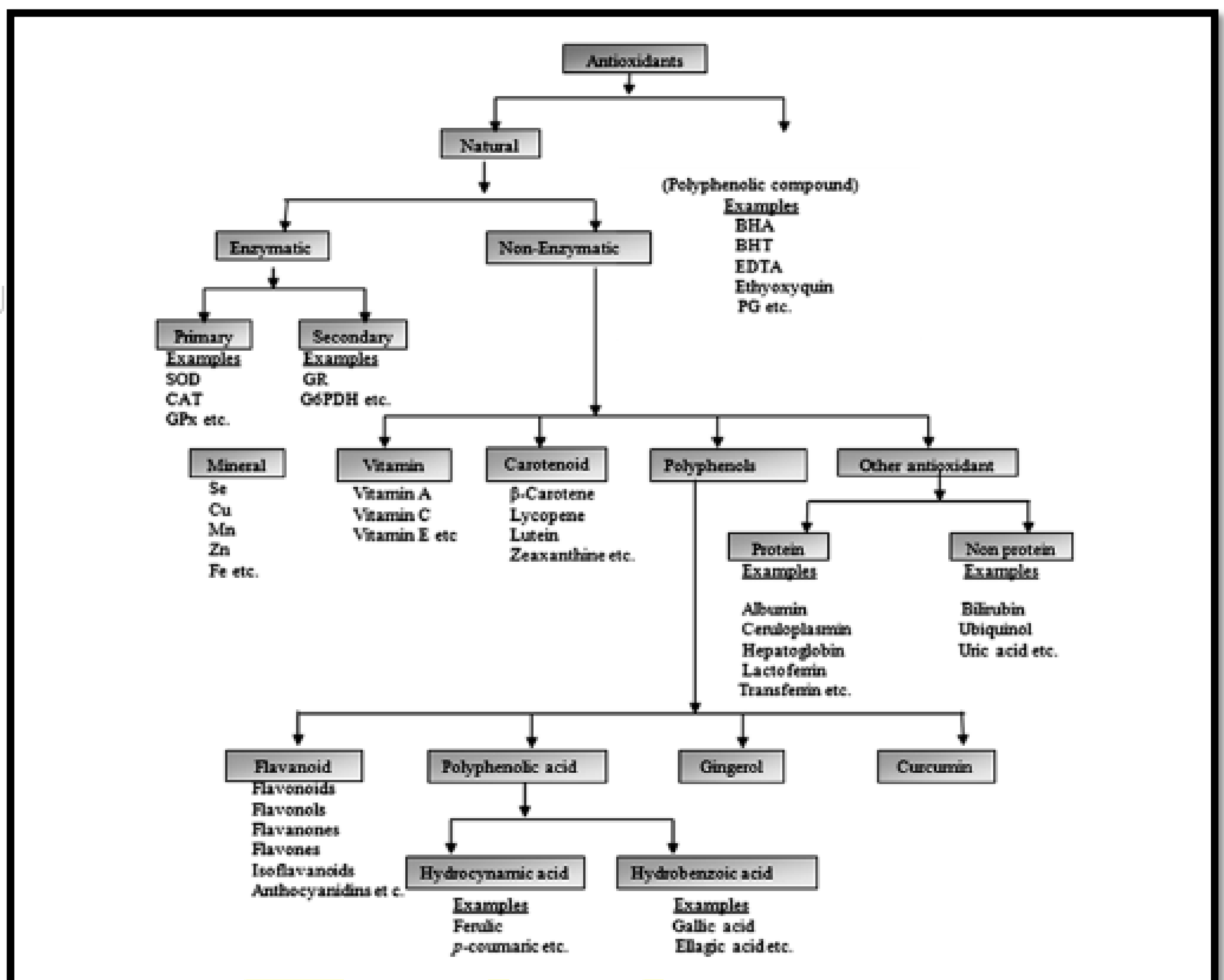

Figure 2.3 Schematic classifications of antioxidants (Brar *et al*., 2014)

2.2.2 Natural antioxidants versus synthetic antioxidants

Synthetic antioxidants are antioxidants of chemical origin. They include butylatedhydroxy toluene (BHT), butylatedhydroxy anisole (BHA), tert-butyl hydroquinone (TBHQ) and gallates and are widely used in the food industry, thus are supplemented in the human diet (Leclercq *et al.* 2000) whilst natural antioxidants are antioxidants of plant origin (Potterat, 1997) Both synthetic and natural antioxidants have a protective effect on body cells against the damage of free radicals and oxidative stress. Back then, synthetic antioxidants gained popularity because of their affordability, accessibility, consistent quality and greater antioxidant activity (Pokorný, 1991) and were frequently considered better than natural antioxidants (Ningappa *et al.*, 2008). However nowadays, tables have turned as the commonly used synthetic antioxidants have been identified as toxic to human health (Lobo *et al.*, 2010) and their antioxidant potential, safety and toxicity has often been a point of contention and concern as they are implicated in causing liver damage and carcinogenesis (Grice, 1986; Brewer, 2011). This has resulted in their reduced usage (Ito *et al.*, 1985). Due to these findings, the food industry is motivated to seek effective and non-toxic natural alternatives with effective antioxidant activity (Gupta and Sharma, 2006).

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The antioxidant activity of a compound highly depends on the number of -OH group present in it. Synthetic antioxidants such as BHA, BHT and propyl gallate have only one –OH group whilst natural antioxidants such as flavonoids and anthocyanins contain more than one –OH groups thus making these natural antioxidants more effective than synthetic antioxidants (Brewer, 2011). Another possible reason why natural antioxidants are better than synthetic ones is that they are composed of a variety of antioxidant compounds each exhibiting its antioxidant activity which may work together or independently to scavenge free radicals (Shahidi *et al.*, 1994; Podsędek, 2007). In simple terms, it is the activity of phenolic compounds within the natural antioxidants that exhibits the antioxidant activity. According to Munir *et al.* (2013), numerous antioxidants compounds with potential therapeutic effects to chronic diseases are present in high concentrations in plants. Natural antioxidants are thus considered easily accessible, reliable additionally safer than synthetic antioxidants (Zhang and Gao, 2014).

2.3 Secondary metabolites

Plants produce chemicals called Secondary metabolites to enhance survival and overcome different stresses presented by its environment (Kennedy and Wightman, 2011). These chemicals are normally referred to as phytochemicals. Due to the fact that plants are naturally sessile, they have developed defence mechanism by means of releasing chemicals to protect themselves from any stress that may be directed to them by herbivores, pathogen or either insects. These chemicals are also involved when plants attack each other under certain environmental stresses (Demain and Fang, 2000). In simple terms, secondary metabolites function as a weapon which plays a protective or a defensive role against different stresses and plants use these chemicals to counteract and adapt to different stresses pose to them by their environment. These include oxidative stress, excess UV radiation, pathogen attack, chemical oxidants and other kinds of stresses (Matkowski and Wołniak, 2005). Examples of secondary metabolites include terpenes, phenolics, and nitrogen containing compounds (Agostini-Costa *et al*., 2012). Due to the precious nature of these chemicals and their activities, some of them are used by humans for medicinal, flavouring and recreational purpose. Research on these phytochemicals is now of interest as they are viewed as future sources of drugs of natural origin, antibiotics, and insecticides (Dewick, 2002). In several studies, phytochemicals from plant extracts have displayed biological activities beneficial to human health. Such activities include antimicrobial (Maddox *et al*., 2010, Pinho *et al*., 2014), anti-atherosclerotic effects (Krishnaiah *et al*., 2011), anticancer (Chusri *et al*., 2015) and anti-inflammatory (Cuong *et al*., 2015) to name a few. Such activities bring evidence of the potential of phenolic compounds in medicine. Thus the presence of phenolic compounds in plants allows them to be used as potential chemopreventives.

2.4 Liver

Liver is the largest glandular and chief metabolic organ in a human body. The liver has more functions than any other organ in a human body (Brynie, 2002). This organ lies below the diaphragm in the thoracic region of the abdomen and is dark reddish-brown in colour due to the large quantity of blood flowing through it. This gland has two important lobes namely: right and left lobe each made up of thousands of lobules which are connected to small ducts leading to a larger hepatic duct. The liver has a wide range of physiological functions ranging from metabolism, secretion, storage and detoxification of

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toxic substances. Major functions of the liver include metabolism of carbohydrates, proteins and fat, secretion of bile, detoxification and storage of vitamins, iron and glucose or glycogen (Refaey *et al*., 2015). Due to the huge role it plays in metabolizing substances taken by the body, no wonder Govind (2011) calls it “the engine-room of the body”.

All foods, drugs and water taken to the system are metabolized and detoxified by this organ (Govind, 2011). During metabolism and detoxification of these substances, a number of potentially dangerous by- products are generated (Eidi *et al*., 2012). As such, it is the main site vulnerable to free radical attack (Kumar *et al*., 2008; Sánchez-Valle *et al*., 2012). Due to this, the liver has a mechanism of maintaining an equilibrium state of free radicals produced and their elimination to keep it functioning normally (Li *et al*., 2015) as shown in **Figure 2.4**. Hepatocytes (liver cells) are the main component responsible for regulating various metabolic activities taking place in the liver (Yahya *et al*., 2013). These cells are very active during the process of metabolism (Timbrell, 2001). Hence, this could be another reason why the liver is vulnerable to free radical attack. Hepatocytes have an antioxidant defence system which it utilizes for protection against oxidative stress. This antioxidant system compromises of antioxidant compounds such as GSH, ascorbic acid, and vitamin E and antioxidant enzymes such as SOD, catalase, and GPx (Kaplowitz and Tsukamoto, 1996).

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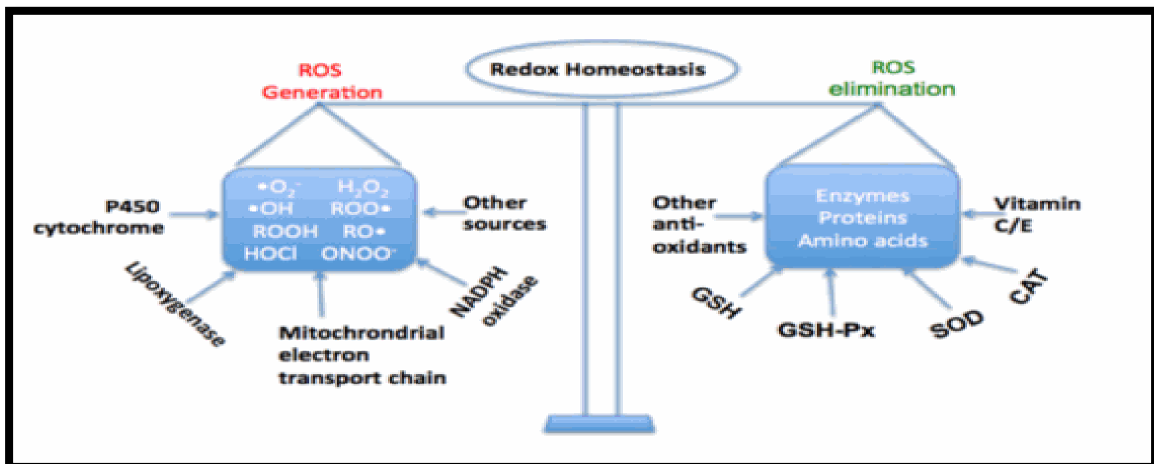

Figure 2.4 Mechanism of liver redox homeostasis by means of balancing and eliminating ROS (Li *et al*., 2015)

2.4.1 Liver diseases

The term liver disease describes any condition or disorder that interferes with the normal functioning of the liver which may eventually lead it to dysfunction or total loss of function. Liver diseases may be classified as acute or chronic hepatitis, hepatosis and cirrhosis (Govind, 2011). Rajaratnam *et al*. (2014) identifies alcohol-induced hepatitis, drug-induced hepatitis, Non-alcoholic steahepatitis (NASH) and viral hepatitis as major liver diseases. According to Nadeem *et al*. (1997) drugs are the most contributing factor towards liver diseases. Li *et al*. 2015 highlighted various other factors playing a role in oxidative stress in **Figure 2.5**. To name a few, toxic chemicals, excess consumption of alcohol, infections, autoimmune disorders (Rao *et al*., 2014) and obesity (Marchesini *et al*., 2008, Li *et al*., 2015) are among causative agents of liver diseases. Due to this reason, so many drugs have been withdrawn from the market (Butura, 2008). These agents pose oxidative stress in the liver by means of generating excess toxic free radicals. A study by Muriel (2009) demonstrated that increased levels of RONS and decreased levels of antioxidant compounds is associated with a kind of liver disease known as Hepatitis C. Thus, it is always important to be careful on what to consume as it will have either a positive or a negative effect to the liver.

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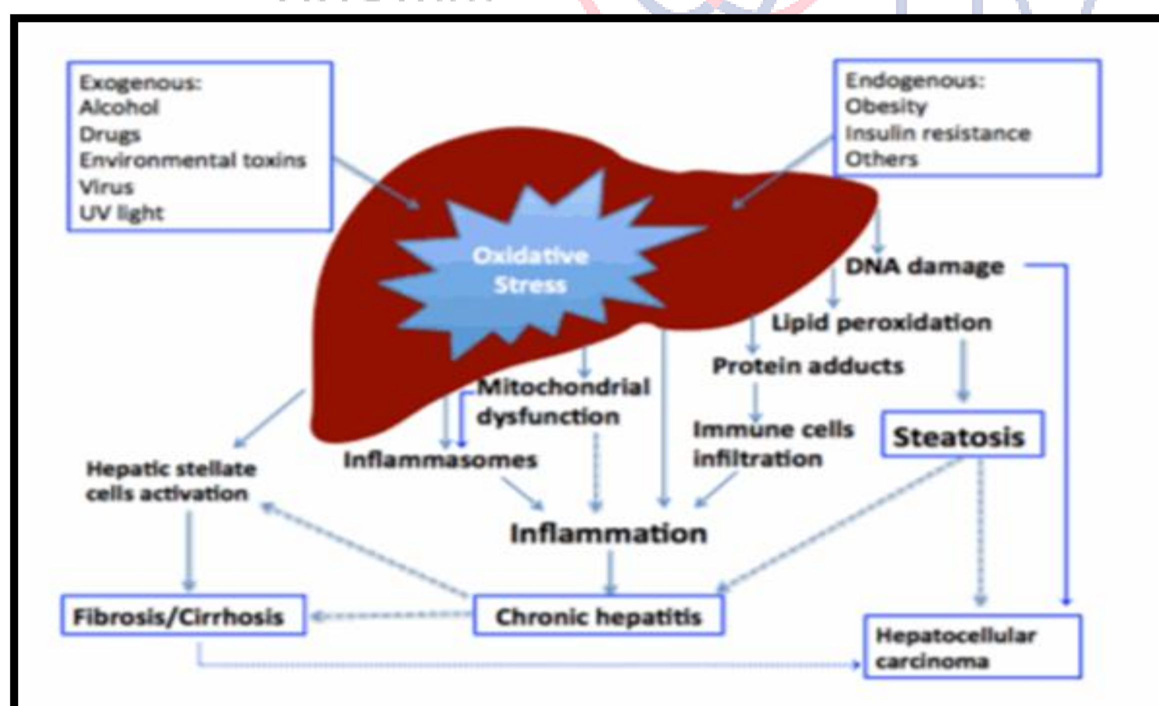

Figure 2.5 Mechanism of oxidative stress induced by various factors on liver disease (Li *et al*., 2015)

2.4.1.1 Prevalence of liver diseases

Lozano *et al.* (2013) and Murray *et al.* (2012) argue that liver diseases have been found to contribute significantly on the global mortality and morbidity burden. A report on global burden of liver diseases by Rehm *et al.* (2013) reported that in 2010, alcohol-attributed liver cirrhosis and liver cancer were responsible for an estimated 493,300 and 80,600 deaths, respectively and 14,544,000 and 2,142,000 Disability adjusted life years (DALYs), respectively. Another report by WHO (2011), reveals that about 3.3 million deaths and 139 million DALYs which occurred in 2012 globally were attributed to alcohol consumption mostly in developing countries. In South Africa, Hepatitis B is the endemic liver disease (Spearman, 2008).

2.4.1.2 Limitations of treatments of liver diseases

The liver is the major organ responsible for the process of metabolism in our body. Substances taken up by the body firstly pass through the liver to be metabolised into different compound that may be used by the body. When a toxic substance such as alcohol is metabolized, toxic by–products are generated in the process. A number of liver treatments are available to protect or reverse any damage caused by these substances in the liver (Muriel and Rivera-Espinoza, 2008). However, according to Govind (2011), until recently it was accepted that no effective satisfactory treatments of liver diseases exists. Despite the statistics in the mortality and morbidity rate due to liver diseases around the world, liver disease treatments of synthetic origin are considered inadequate and most importantly found to have serious side effects than the healing effect (Prakash *et al.*, 2008). Botterweck *et al.* (2000) has reported on the carcinogenic and anticarcinogenic properties of BHT and BHA which highlights the danger of these synthetic antioxidants. Thus, there is a great demand for the development of alternatives which will be adequate, safe to use with less or no side effects.

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2.4.1.3 Medicinal plants as a cure for liver diseases

The role of medicinal plants in the health system worldwide is enormous. The use of medicinal plant preparations in the management of liver diseases has long been in the picture. Hundreds of plants have been investigated to be taken for a wide spectrum of liver diseases up to date (Asadi-Samani *et al.*, 2014). However, their substantial usage in the management of liver diseases still lacks legal regulations and pharmacological vigilance (Rajaratman *et al.*, 2014). A number of studies have demonstrated that

medicinal plants extracts have antioxidant compounds which have the ability to protect the liver against hepatotoxicity caused by CCl_4 (Pandey, 1990; Kumar *et al*, 2011; Refaey *et al*., 2015). There are a number of herbal formulations used in the management and treatment of various liver disorders or hepatotoxicity (Pandey, 1990; Govind, 2010). The hepatoprotective activity displayed by these plant formulations is due to their antioxidant properties (Madrigal-Santillán *et al*., 2014). With this being said, natural remedies of plant origin are considered to be effective and safe alternative for the treatment of hepatic injury (Ranawat *et al*., 2010; Huo *et al*., 2011).

2.5 Hepatotoxicity

Navarro and Senior (2006) define hepatotoxicity as a liver injury caused by exposure to a drug or non-infectious agent leading to weakening or alteration of the normal functioning abilities of the liver. When the liver is damaged or weakened, over production of RONS may result. **Figure 2.6** shows pathways in which RONS are generated in the liver (Singal *et al*., 2011).

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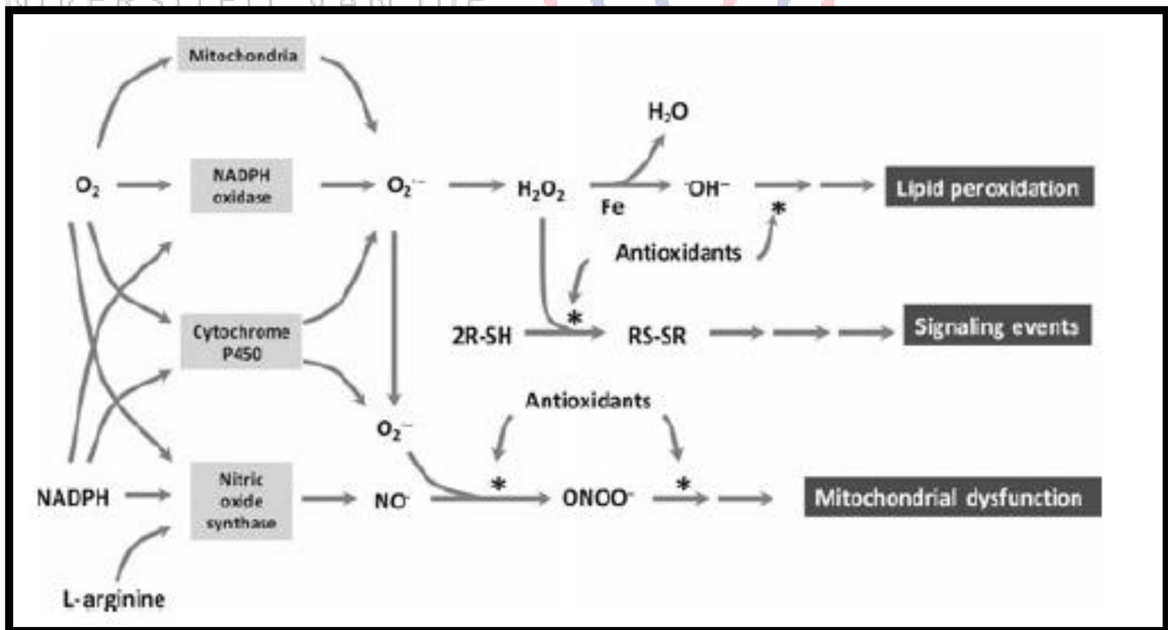

Figure 2.6 Pathways generating RONS in the liver during hepatotoxicity (Singal *et al*., 2011)

2.5. 1 Hepatotoxin: Carbon tetrachloride (CCl_4)

2.5.1.1 Mechanism of Hepatotoxicity caused by carbon tetra-chloride in the liver

Carbon tetrachloride is widely used as an industrial solvent, degreasing and cleaning agent (Elagib *et al*., 2014). It is a synthetic hepatotoxin widely used in the animal model to study hepatotoxicity (Recknagel *et al*., 1989; Lee *et al*., 2003; Huo *et al*., 2011), cellular mechanism behind oxidative damage (Basu, 2003) and hepatoprotective effect of drugs and plant extracts (Suja *et al*., 2002; Refaey *et al*., 2015). **Figure 2.7** shows the mechanism of action of CCl_4 hepatotoxicity (Timbrell, 2009). CCl_4-induced cell damage occurs in the smooth endoplasmic reticulum (SER) and follows the electron transfer-dependent homolytic cleavage of the carbon-chloride bond (Dianzani, 1987). The reduction of CCl_4 takes place in hepatocytes by means of cytochrome P_{450} (Fang *et al*., 2008; Refaey *et al*., 2015). The mechanism of action of CCl_4 initially includes the reduction of carbon tetrachloride (CCl_4) molecule to highly reactive trichloromethyl (Onyesom *et al*., 2008) and trichloromethylperoxyl radicals (Huo *et al*., 2011). A free radical trichloromethyl ($CCl^{\bullet}_3$) and a chorine ion (Cl^-) are produced as by-products of this cleavage (Timbrell, 2009). The $CCl^{\bullet}_3$ radical formed is responsible for initiating the precipitation of the membrane lipid peroxidation of the cytoplasmic membrane phospholipids leading to liver damage (Reinke *et al*., 1988). Trichloromethylperoxyl radical is formed when trichloromethyl free radical reacts with oxygen (Refaey *et al*., 2015) and may attack lipids on the membrane of endoplasmic reticulum to elicit lipid peroxidation (Recknagel *et al*., 1989; Singhal and Gupta, 2012), ultimately resulting in fatty liver, cirrhosis and necrosis as pathological characteristics (Recknagel *et al*., 1989). Toxic levels of CCl_4 in the system results in fatty accumulation in the liver due to blockage in the synthesis of lipoproteins responsible for transporting triglycerides away (Lima *et al*., 2010), thus leading to deformation of normal metabolic functions of the liver (Bhuvaneswari *et al*., 2014). This is a result of reduced antioxidant defence function and susceptibility of the organ during exposure to the xenobiotic (Lee *et al*., 2003) and distortion of its metabolic functions (Wolf, 1999).

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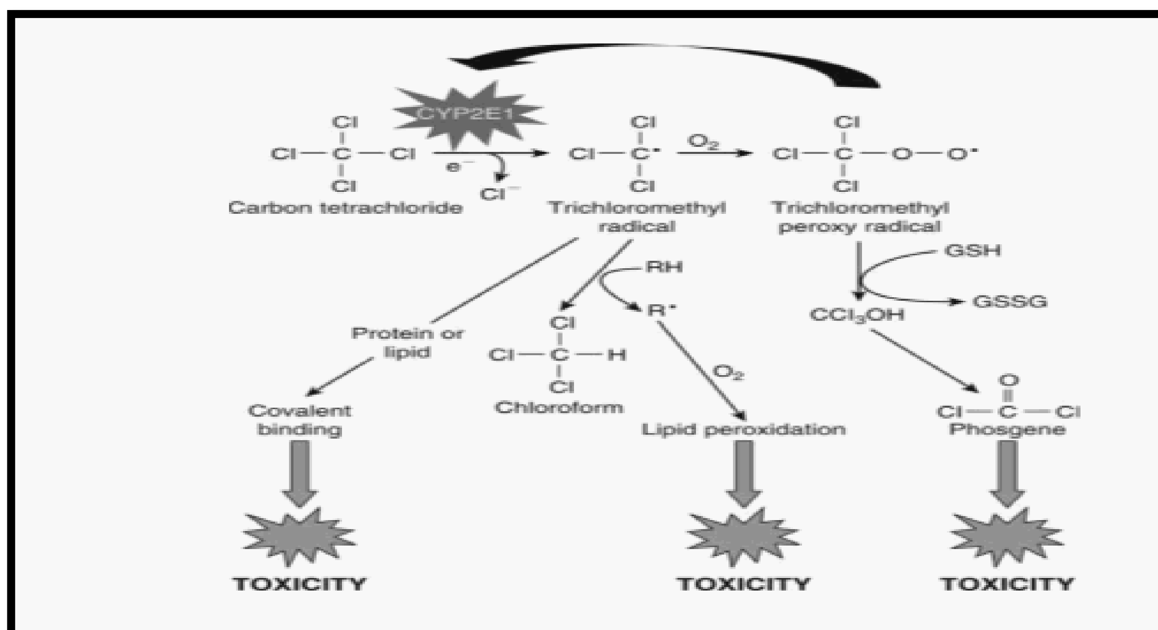

Figure 2.7 Mechanism of action of CCl_4 hepatotoxicity (Timbrell, 2009)

2.6 Antihepatotoxin: Silymarin

Silymarin is a natural antioxidant standardised plant extract made from a mixture of flavonol–lignin extracted from milk thistle (*Silybum marianum*) which protects liver and kidney cells from toxic effects of drugs (Post-White *et al*., 2007). The extract has been used for almost 2000 years in traditional medicine as a liver stimulant (Surai, 2015). The extract has good safety profile, easily accessible and cost effective with no known side effects (Dixit *et al*., 2007). The extract contains a mixture of four flavonolignan isomers, namely sylibin, isosylibin, silydianin and silychristin (Mahli *et al*., 2015). Among these components, silibinin (sylibin) is the primary active ingredient (Surai, 2015) with hepatoprotective properties (Fraschini *et al*., 2002; Hackett *et al*., 2013). This herbal hepatoprotective agent has been widely studied clinically and chemically in major liver diseases (Rajaratman *et al*., 2014). Other than hepatoprotectivity, silymarin has several recognised effects such as antioxidant, anti-lipid-peroxidant, antifibrotic, anti-inflammatory, immunomodulating (Luper, 1998) and anti-fibrosis effect (Abenavoli *et al*., 2010). Due to its strong antioxidant and tissue regeneration properties, the extract is further studied for neuro-, nephro- activity and cardio-protective activity (Milić *et al.,* 2013; Farhana Mohd Fozi *et al*., 2013; Zholobenko and Modriansky, 2014). Silymarin as a natural antioxidant contributes or help the existing normal body mechanism of natural antioxidants present in cells to fight against oxidative stress. It does this in different ways including scavenging free radicals directly, inhibiting free radical producing enzymes,

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activating an array of enzymatic and non-enzymatic antioxidants responsible for scavenging free radicals, additionally activating vitagenes responsible for synthesis of protective molecules (Surai, 2015).

2.7 Medicinal plants

2.7.1 The use, threats and conservation means of medicinal plants in South Africa

The use of medicinal plants in South Africa plays a major role in on both health and livelihood of many people who depend on them for medication. This is not only driven by the fact that Southern Africa is home to three world's biodiversity hotspots namely the Cape Floristic Region, Succulent Karoo and Albany-Maputuland Corridor, but by the diversity of the cultures (Makunga, 2009) and unaffordability of Western medicine (Taylor *et al*., 2001). South Africa is home to about 30, 000 flowering plant species (Louw *et al*., 2002), 4, 000 of these plant species are ethnobiotanically significant, with some 3, 000 being medicinally useful (De Wet *et al*., 2013; Makunga, 2009). Due to the increased urbanisation, poverty and unemployment, there is an observed increased in the medicinal plants demand (Cunningham, 1993; Mander, 1998) resulting in some species in the wild becoming rare, vulnerable, threatened or purely extinct (Cunningham, 1993; Coetzee *et al*., 1999; Afolayan and Adebola, 2004). In addition to this, wild plants are under pressure of increased demand for local and international markets (Street and Prinsloo, 2012) resulting in severe harvesting (Wiersum *et al*., 2006). Mander (1998) estimates that there are between 35, 000 and 70, 000 tons of medicinal plants exported annually. Therefore, documenting of medicinal plants and sustainable use interventions are first line of defence in saving the precious species and the knowledge that accompany them. One strategy involves World Health Organisation, Medical Research Council, and Council for Scientific and Industrial Research where by an institute for African traditional medicine has been established with the aim to develop new remedies for chronic diseases and secure the indigenous knowledge (Crisp and Ntuli, 1999). This extends to government departments, academics and research institutions all over the country.

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2.8 Choice of plant of study: *Morella serrata* (Lam.) Killick (Myricaceae)

Figure 2.8 ***Morella serrata*** **tree and leaves**

2.8.1 Classification of *Morella serrata* (Hassler, 2016)

Kingdom: Plantae (Plants)

Phylum: Tracheophyta (Vascular plants)

Division: Magnoliophyta (Flowering plants)

Class : Magnoliopsida (Dicotyledons)

Order : Fagales

Family: Myricaceae

Genus : *Morella*

Species: *M. serrata*

2.8.2 *M. serrata* common names

In the Southern Africa, the plant is known as *Lance-leaved waxberry* (English), *Smalblaarrwillbebessie* (Afrikaans), *Umqhetayo* (Xhosa), *Umlulama*, *uMakhuthula* (IsiZulu), *Uleti* (Swaziland), *Monnamotsho* (Sesotho) (Schmidt *et al*., 2002; Loots, 2005; Raimondo *et al*. 2009) and *Maleleka* (Lesotho) (Kose *et al*., 2015).

2.8.3 Botany of *M. serrata*

Myrica serrata now reclassified as *Morella serrata* (MacDonald *et al*., 1989) is a shrub or small trees used by South African communities in traditional medicine (**Figure 2.8**). The plant is formerly called *Myrica serrata* Lam. *M. serrata* belongs to the shrub and small tree family (Myricaceae). *M. serrata* usually have several erected stems (Zukulu *et al*., 2012; Ashafa, 2013) and grows 2 to 6 m tall, along the drainage lines and streams along the escarpment in grasslands and open woodlands (Schmidt *et al*., 2002). Leaves are alternate, narrowly elliptic, apex and base narrowly tapering; margins entire to broadly serrata, hairy and glabrous (Schmidt *et al*., 2002; Zukulu *et al*., 2012). The plant flowers during August and September (Schmidt *et al*., 2002; Curtis and Mannheimer, 2005). The plant is actinorhizal having a symbiotic relationship with actinobacteria genus *Frankia* (Gtari and Dawson, 2011).

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2.8.4 Geographical distribution of *M. serrata*

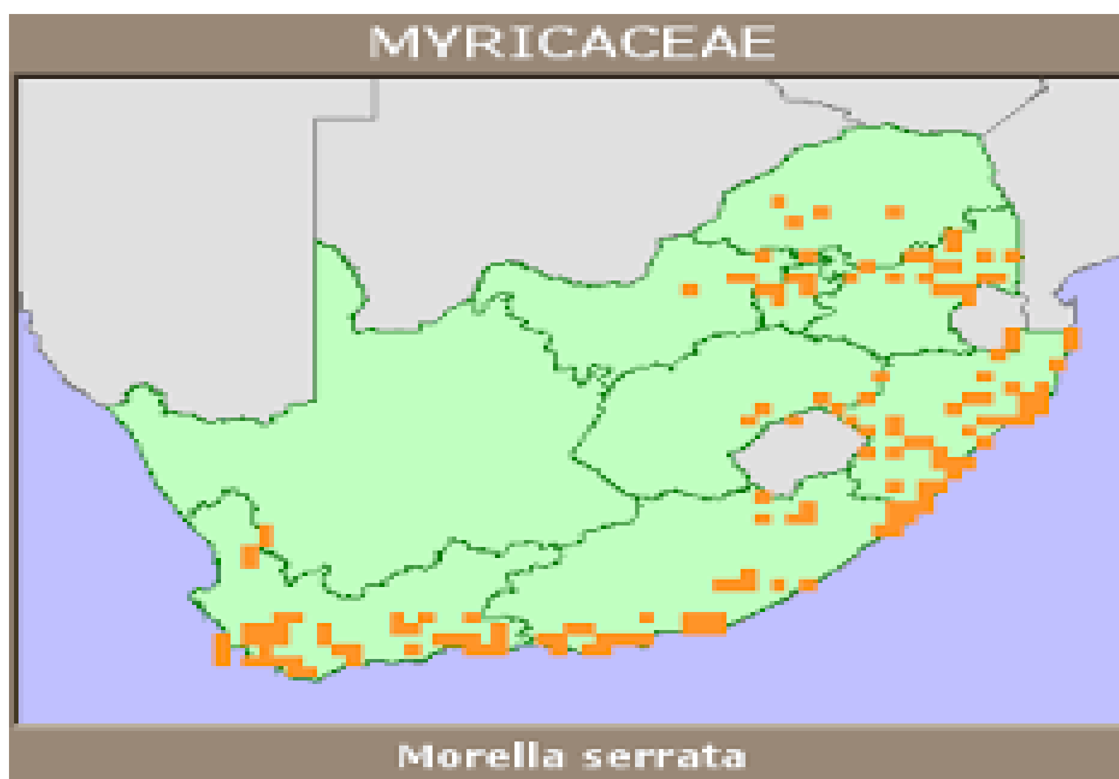

Figure 2.9 Geographical distribution of *Morella serrata*

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The plant is not endemic to South Africa, however, indigenous (**Figure 2.9**). It is distributed in South Africa and Southern African countries extending into tropical Africa (Silva *et al*., 2015).

2.8.5 Threat to *M. serrata*

The plant is identified as Least Concern in the Red list (Loots, 2005; Raimondo *et al*., 2009; Kose *et al*., 2015) and collection is a major factor which threatens it (Golding, 2002).

2.8.6 Traditional uses of *M. serrata*

2.8.6.1 Medicinal uses

Traditional uses of *M. serrata* include treating asthma, coughing, shortness of breath (Schimidt *et al*., 2002). Among the *Xhosa* tribe, the decoction of the root when taken tastes like ginger and causes irritation of the throat, thus called *Umqhetwayo* meaning "the itchy one" and the plant is mix with alum granules and used as a purgative against bad luck (Zukulu *et al*., 2012). The decoction of the root is also used to treat

dysmenorrhoea (Hutchings and Van Staden, 1994), cold and coughs (Moffet, 2010), TB, mental illness (Kose *et al*., 2015) and diabetes mellitus (Balogun *et al*., 2016). Its various organs have been used to manage sugar related disorders, headache, as laxative and to enhance male sexual performance hence the name '*Monnamontsho'* (the black man) among the *Basothos* of South Africa (Ashafa, 2013). In the Maseru district of Lesotho, the plant is mixed with cows, dogs, or pigs dung or milk to chase away insects introduced by witchcraft (Kose *et al*., 2015). After death in the Zulu culture, during mourning and period of wearing the black attire symbolizing death, it is believed that the cows in the yard can feel the mourning within the family thus they shed their weight. In order to protect them from grieving and becoming leanness, a mixture of their dung and plant is prepared and sprinkled around the yard and their kraal.

2.8.6.2 Non-medicinal uses

In Zimbabwe and Swaziland, the plant is cut for fuel, while its branches are burnt to enhance or promote crops growth (Hutchings *et al*. 1996). The fruits are boiled and the wax obtained is used to make candles (Schmidt *et al*, 2002). Palgrave (2002) reported that the fruit can be eaten raw.

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2.8.7 Phytochemical and pharmacological review of *M. serrata*

Very little research has been done and documented on the chemistry and pharmacology of this plant species. A study done by Gafner *et al*. (1996) study reported the dichloromethane leaf extract of *Morella serrata* to inhibit the growth of *Bacillus subtilis* and *Escherichia coli* strains which are respectively implicated with Cholangitis and diarrhoea. Another study by Ashafa (2013) on the phytochemical screening of root extracts of *M. serrata* revealed the presence of alkaloids, saponins, tannins, phlabotannins, terpenoids, and steroids. The study also displayed the potential of *M. serrata* as an antimicrobial and antitumor agent. Recent work by Balogun *et al*. (2016) displayed the potential of the plant in treating diabetes mellitus. The plant is widely used among the Southern African communities to treat free radical and oxidative stress implicated conditions such as asthma, diabetes and sexual dysfunction. However, there are no scientific reports available verifying and supporting such claims. In this view the present study was undertaken to investigate the plants' antioxidant and hepatoprotective potential, in addition to screening and quantifying the phytochemicals responsible for such activity.

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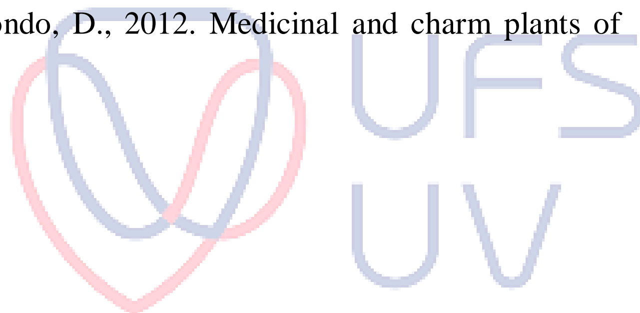

CHAPTER 3

Qualitative and quantitative analysis of leaf extracts from *Morella serrata*

3.1 Introduction

Medicinal plants usage in treating ailments dates back to time immemorial. The curing and healing properties in medicinal plants are due to the presence of phytochemicals (Nostro *et al*., 2000). Medicinal plants are used by communities for health, relieving symptoms and treating different ailments. However, there is lack of scientific validation of these claims (Tabassum and Agrawal, 2003). These medicinal plants contain different phytochemicals which are naturally produced by plants as mechanism of protection against any danger. Literature surveys have revealed the presence of different phytochemicals in medicinal plants with various pharmacological properties ranging from antimicrobial (Srinivasan *et al*., 2001; Ashafa, 2013), antioxidant (Kähkönen *et al*., 1999; Abdennacer *et al*., 2015), anticancer (Bomser *et al*., 1996; Bagattoli *et al*., 2016), anti-inflammatory (Falcão *et al*., 2005; Zhen *et al*., 2015), antimalarial (Carvalho *et al*., 1990; Bankole *et al*., 2016), anti-tuberculosis (Arya, 2011; Kumar *et al*., 2014), anti-helminthic (Ibrahim, 1992; Panda *et al*., 2015) to name a few. These activities in a range of plant extract support the effectiveness and genuity of medicinal plants. Previous research findings on plant extracts have resulted in elevation of medicinal plants research as better solutions in treating different ailments which are burden not only to human beings, but to animals as well for a better health. The present study thus investigates the phytochemicals and quantifies the antioxidant compounds in different leaf extracts of *Morella serrata.*

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3.2 Material and methods

3.2.1 Collection, identification and authentication of plant material

Fresh leaves of *Morella serrata* were collected in February 2014 around Phuthaditjhaba area, Witsishoek (28°39’31.3”S and 28°52’47.4”E; altitude 2047 m), Free State Province, South Africa. The plant was identified, authenticated (Voucher specimen No: MbhMed/02/2014/QHB) and deposited at the University of the Free State Herbarium, Qwaqwa Campus. The leaves were washed under running tap water to remove all contaminants, shade-dried to constant weight and pulverized to fine powder using Warring Commercial Laboratory electric blender. The powdered leaves were then stored in airtight glass containers at 5°C in a refrigerator until further use.

3.2.2 Chemicals and reagents

Quercetin, gallic acid, Folin-Ciocalteu and ascorbic acid were products of Sigma-Aldrich. All other chemicals were of analytical grade. Distilled water was prepared in the Department of Plant Sciences, University of the Free State, Qwaqwa Campus.

3.2.3 Preparation of crude extracts

In three different beakers, 30 g each of the powdered sample was separately extracted with 1L ethanol, hydroalcohol (50% ethanol and 50 % distilled water), and distilled water ending up with three different extracts namely, ethanol, hydroalcohol and water extracts. The set ups were placed in a Labcon shaker (Laboratory Consumables, PTY, Durban, South Africa) for 24 h with agitation. The resulting infusions were filtered using Whatsman No. 1 filter paper. The ethanol crude extract was concentrated in a rotary evaporator (Cole Parmer SB 1100, Shangai, China) under reduced pressure while aqueous (hydroalcohol and water) extracts were concentrated in a water bath at 40°C. The dry crude extracts were weighed and kept in air-tight containers prior to use.

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3.2.4 Qualitative analysis of leaf extracts from *M. serrata*

3.1.4.1 Preliminary phytochemical screening of *M. serrata*

Phytochemical composition of the leaf extracts (Ethanol, hydroalcohol and water) was determined using various standard methods (Harbone, 1973; Sofowara 1993).

Detection of alkaloids

In a test tube, 3mL (20 mg/mL) of extract was deposited and 1 mL of 1% hydrochloric acid was added. This was treated with few drops of Meyer's reagent. A creamy white precipitate served as an indication of the presence of alkaloids.

Detection of tannins

A volume of 1 mL (20 mg/mL) extract was added to 1 mL of 3% ferrous chloride in a test tube. A green black precipitate signified the presence of tannins.

Detection of phlabotannins

A volume of 1 mL (20 mg/mL) extract was boiled with 2 mL of 1% hydrochloric acid in a test tube. A red precipitates indicated the presence of phlabotannins in the extract.

Detection of saponins

In a test tube, 5 mL (20 mg/mL) extract was shaken vigorously to obtain a stable persistent froth. The froth was then homogenised with 3 drops of olive oil and allowed to settle down. The formation of emulsion served as an indication to presence of saponins.

Detection of flavonoids

A volume of 1 mL (20 mg/mL) extract was added to 1 mL of 10% lead acetate in a test tube. The mixture was then gently shaken. Formation of a muddy brownish precipitate served as an indication of the presence of flavonoids.

Detection of steroids

A volume of 2 mL of acetic anhydride was added to 0.5 g of dried extract with 2 mL concentrated sulphuric acid in a test tube. Colour change from violet to blue or green was an indication of steroids being detected.

Detection of terpenoids

A volume of 5 mL (20 mg/mL) extract was mixed with 2 mL chloroform in a test tube. Three mL of concentrated sulphuric acid was carefully added into the mixture. A reddish brown coloration between upper and lower layer signified the presence of terpenoids.

Detection of cardiac glycosides

In a test tube, a volume of 5 mL (20 mg/mL) of the extract was treated with 2 mL of glacial acetic acid containing one drop of ferric chloride solution. This was mixed with 1 mL concentrated sulphuric acid. A brown ring of the interface formation was an indication of the presence of cardiac glycosides.

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Detection of resins

In a test tube, a volume of 5 mL of ethyl alcohol concentration (95%) was added to 0.5 g of dried extract after leaving in a water bath to a boil for two minutes. The mixture was then cooled and filtered. To the filtrate, 10 mL of distilled water acidified hydrochloric acid concentrate, where inferred the existence of resin materials emergence of turbidity.

Detection of phenols

In a test tube, a volume of 2 mL (20 mg/mL) of extract was taken into water and warmed at 45-50ºC. Then 2 mL of 3% ferrous chloride was added. Formation of green or blue colour was an indication of presence of phenols.

3.2.5 Quantitative determination of leaf extracts of *M. serrata*

3.2.5.1 Preparation of standard solution and test concentrations

Known weight (10 mg) of Gallic acid, quercetin and *M. serrata* leaf extracts aliquot (ethanol, hydroalcohol and water) were accurately taken into different 10 mL volumetric flask, and each dissolved in 10mL distilled water to make a stock solution of 1 mg/ml. Standards stock solutions were further diluted to five different final concentrations (0.2,

0.4, 0.6, 0.8, 1.0 mg/mL) in separate centrifuge tubes (**Table 3.1**). Test extracts were only tested at a concentration of 1 mg/mL while standards (gallic acid and quercetin) were tested at all five concentrations. The total phenolic, flavonoid and flavonol content of the extracts were interpolated from standards.

Table 3.1 Preparation of test concentrations of extract/ standard

Concentration (mg/mL)	Extract / standard (µl)	Distilled water (µl)
0.2	200	800
0.4	400	600
0.6	600	400
0.8	800	200
1.0	1000	0

3.2.5.2 Total phenolic content, total flavonoid content and total flavonol content determination

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Total phenolic

The total phenolic content in the leaf extracts of *M. serrata* was estimated spectrophotometrically using the Folin-Ciocalteu reagent method defined by Wolfe *et al.* (2003). Precisely, 0.5 mL, 1mg/mL of extract aliquot was mixed with 2.5 mL of 10% Folin-Ciocalteu reagent and 2 ml of sodium carbonate. The resulting mixture was then vortexed for 15 s and incubated at 40ºC for 30 min for colour development. The sample absorbance was read at 765 nm. The same procedure was followed for all the concentrations (0.2, 0.4, 0.6, 0.8, 1.0 mg/mL) of standard gallic acid.

Total flavonoids

The total flavonoid content was estimated using the method defined by Ordonez *et al.* (2006) based on the formation of a complex flavonoid-aluminium. Precisely, 5 mL of 2% Aluminium chloride ethanol solution was added to 0.5 mL of extract solution. The mixture was incubated at room temperature for 1 hr and the absorbance was read at 420 nm. The same procedure was followed for all the concentrations (0.2, 0.4, 0.6, 0.8, 1.0 mg/mL) of standard quercetin.

Total flavonols

The total flavonols content was estimated using the procedure described by Kumaran and Karunakaran (2007). Extract (2 mL); 2 ml $AlCl_3$ prepared in ethanol and 3 mL of sodium acetate solution (50 g/L) were mixed together. The mixture was then incubated at 20 ℃ for 2.5 h and read at absorbance of 440 nm. The same procedure was followed for all the concentrations (0.2, 0.4, 0.6, 0.8, 1.0 mg/mL) of standard quercetin.

3.2.6 Statistical analysis

Liner equation and the coefficient of determination (R^2) were estimated using the linear calibration curves of standards. $R^2 > 0.9$ was considered significant. Total contents of extracts were interpolated from respective equations of standards. Statistical analysis was performed using a Graph Pad Prism version 5.00 statistical package (Graph Pad software®, San Diego, CA, USA). Results were expressed as mean of three determinations ± standard error of mean (SEM) and subjected to one way analysis of variance (ANOVA) followed by Bonferroni comparison test to determine the significant difference in all parameters. P value < 0.05 was considered statistically significant.

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3.3 Results

3.3.1 Percentage yield of *M. serrata* leaf ethanol, hydroalcohol and water extracts

Three different extractants (ethanol, hydroalcohol and water) were employed during the extraction process. The extracts were prepared from equal amount (30 g) of powdered plant material. However, the results showed varying percentage yield. The extract yield varied with extraction solvent as shown in **Table 3.2**. Maximum percentage yield was observed in the hydroalcohol extract 20.28% followed by ethanol (15.44%) and water (13.24%) extracts.

$$\% \text{ Yield: } \frac{weight\ of\ dry\ crude\ extract\ (g)}{weight\ of\ powdred\ plant\ material\ used\ for\ extraction\ (g)} X100 \ldots\ldots\ldots\ldots\ldots\ (1)$$

Table 3.2 Percentage yield of different leaf extracts of *M. serrata*

Solvent	Initial weight (g)	Final weight (g)	% Yield
Ethanol	30	4.63	15.44
Hydroalcohol	30	6.08	20.28
Water	30	3.97	13.24

3.3.2 Phytochemical analysis of *M. serrata* leaf ethanol, hydroalcohol and water extracts

The presence of different bioactive constituents in ethanol, hydroalcohol and water extracts of *M. serrata* leaves were investigated as part of the preliminary study (**Table 3.3**). The screening revealed the presence of tannins, flavonoids and phenols in all the extracts. Saponins and steroids were respectively only detected in water and ethanol extracts, while resins were only detected in the ethanol and hydro-alcohol extracts. Terpenoids were detected in both hydroalcohol and water extracts while resins were detected in both ethanol and hydroalcohol extract. Nonetheless, alkaloids, phlabotannins, and cardiac glycosides were not detected in all the extracts.

Table 3.3 Phytochemical composition of different leaf extract from *M. serrata*

Phytochemicals	**Extracts inference**		
	Ethanol	**Hydroalcohol**	**Water**
Alkaloids	-	-	-
Tannins	+	+	+
Saponins	-	-	+
Phlabotannins	-	-	-
Flavonoids	+	+	+
Steroids	+	-	-
Terpenoids	-	+	+
Cardiac glycosides	-	-	-
Phenols	+	+	+
Resins	+	+	-

+: Detected -: Not detected

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3.3.3 Quantitative analysis of *M. serrata* leaf ethanol, hydroalcohol and water extracts

3.3.3.1 The effect of extractants on total content of phenolics, flavonoids and flavonols.

Total phenolic content

The total phenolic content was expressed as mg/g gallic acid equivalent using the standard curve (**Figure 3.1**):

$$y = 0.3915x + 0.2085$$

$$R^2 = 0.9420,$$

Where y is the absorbance and x is the total phenolic content in the extract.

The total phenolic content of different *M. serrata* leaf extracts is presented in **Figure 3.2.** Among the tested extracts, there was no significant ($p>0.05$) difference in phenolic content observed in ethanol and water extracts of *M. serrata*, whilst was the phenolic content of hydroalcohol extract was significantly ($p<0.05$) lower when compared with other two extracts (Ethanol and water). Maximum phenolic content was observed in the ethanol extract exhibiting 0.24 mg GAE/g, followed by water extract exhibiting 0.20 mg GAE/g with hydroalcohol extract showing least phenolic content of 0.06 mg GAE/g.

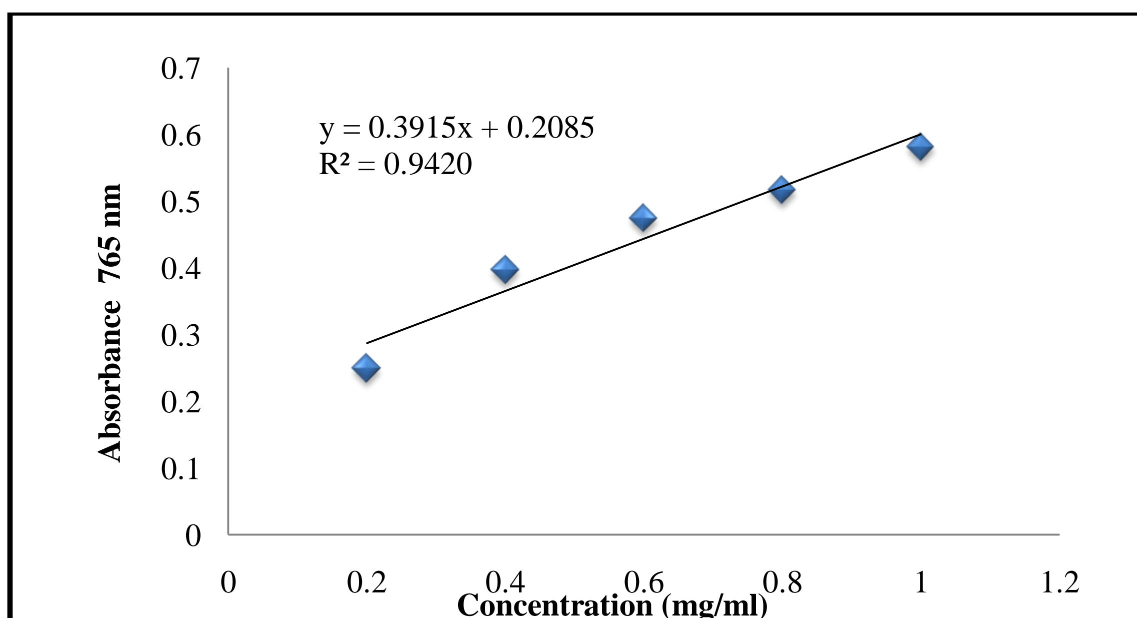

Figure 3.1 Linear calibration curve of standard gallic acid

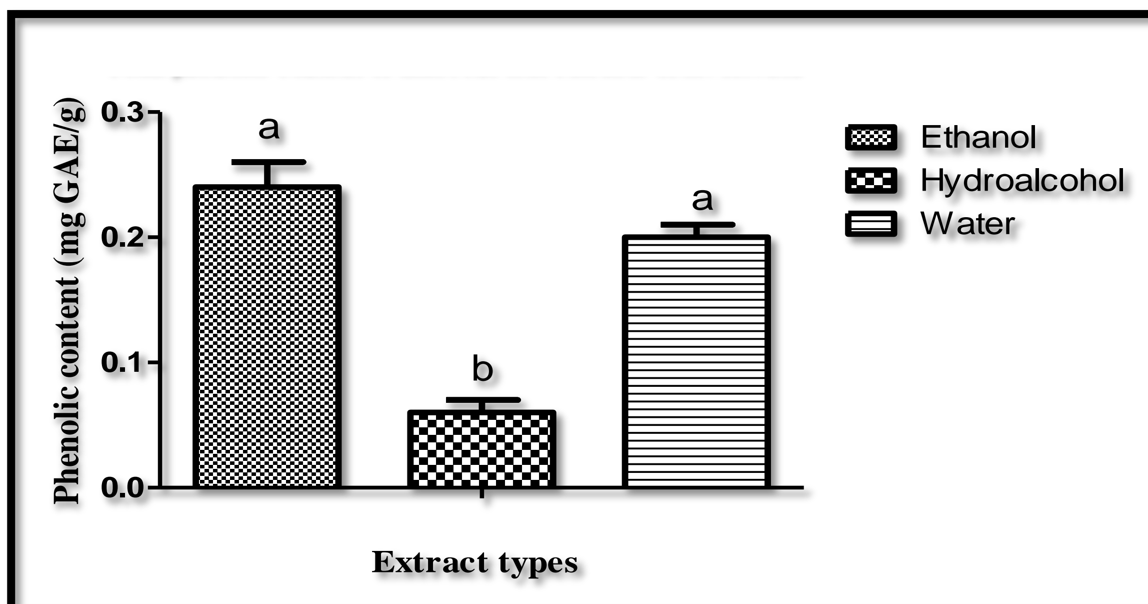

Figure 3.2 Total phenolic content of different leaf extracts of *M. serrata.* Values are presented as mean ± SEM (*n=3*). Means sharing the same letter are not significantly different (p > 0.05)

Total flavonoid content

The total flavonol content were expressed as mg/g quercetin equivalent using the standard curve (**Figure 3.3**):

$$y = 0.8185x + 2.3679$$

$$R^2 = 0.9219,$$

Where y is the absorbance and x is the total flavonoid content in the extract.

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The total flavonoid contents of both ethanol and hydroalcohol extracts were not significantly different (p>0.05) from each other but significantly reduced (p<0.05) in comparison with water extract (**Figure 3.4**). The total flavonoid content was in order water> hydroalcohol> ethanol. The total flavonoid content of each extract was 2.04 mg QE/g for water extract 1.37 mg QE/g for hydroalcohol extract and 1.35 mg QE/g for ethanol extract.

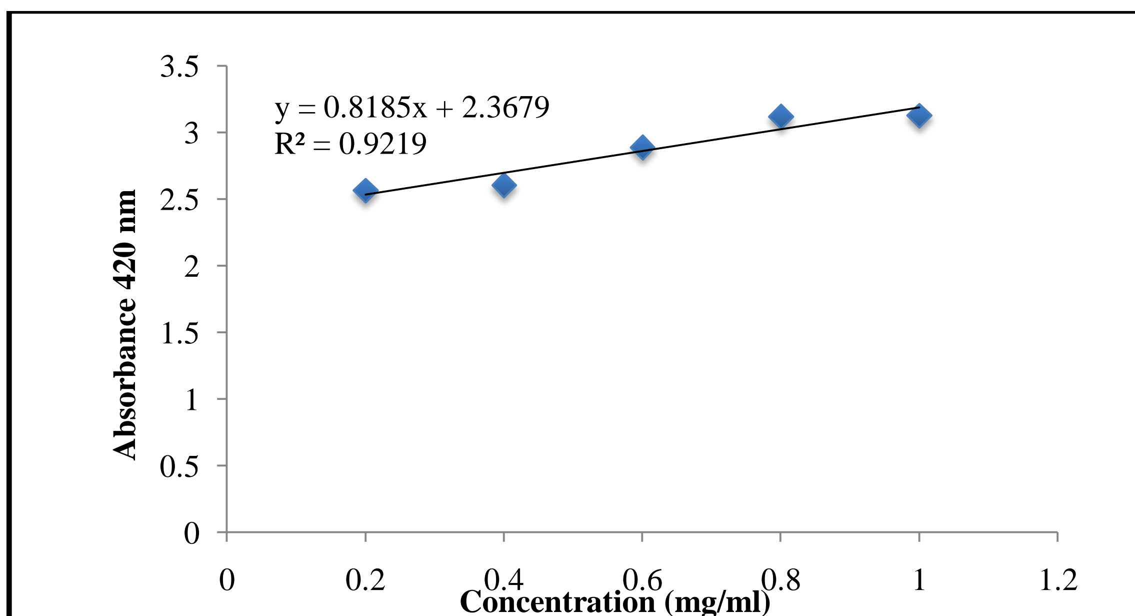

Figure 3.3 Linear calibration curve of standard quercetin

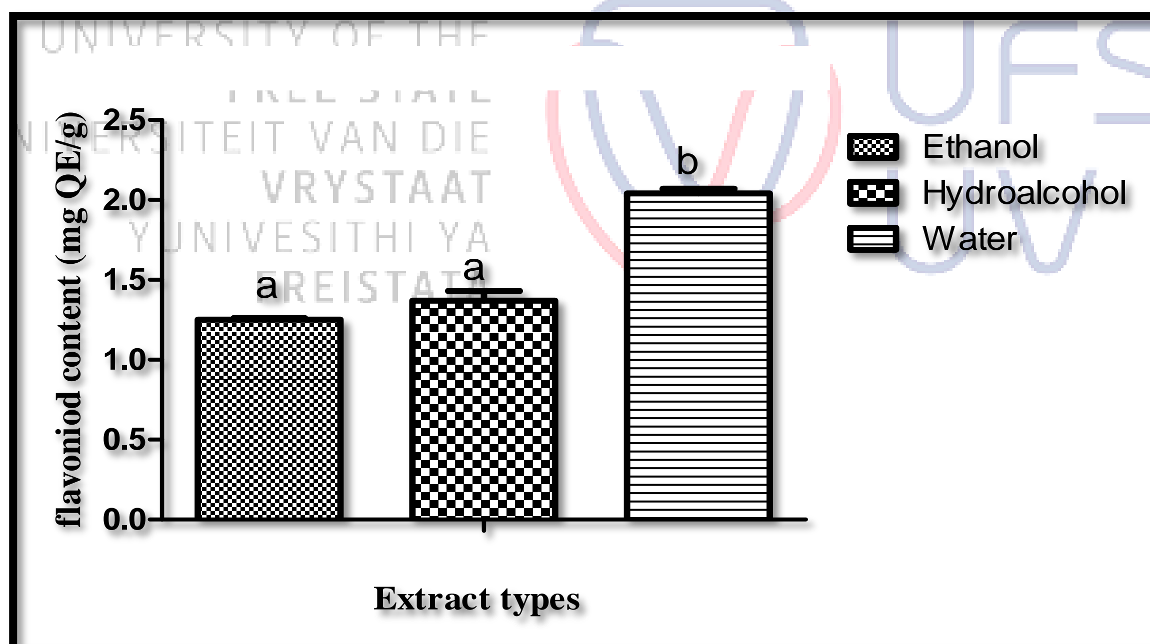

Figure 3.4 Total flavonoid content of different leaf extracts of *M. serrata.* Values are presented as mean ± SEM (*n=3*). Means sharing the same letter are not significantly different ($p > 0.05$)

Total flavonol content

The total flavonol content were expressed as mg/g quercetin equivalent using the standard curve (**Figure 3.5**):

$$y = 2.0175x + 1.5855$$

$$R^2 = 0.9041,$$

Where y is the absorbance and x is the total flavonol content in the extract.

All the tested three extracts of *M. serrata* showed no significant difference ($p > 0.05$) in total flavonol content (**Figure 3.6**). Maximum flavonol content was observed in the water extract, followed by hydroalcohol extract with ethanol extract showing least flavonol content each exhibiting a total flavonol content of 0.50, 0.37 and 0.35 mg QE/g respectively.

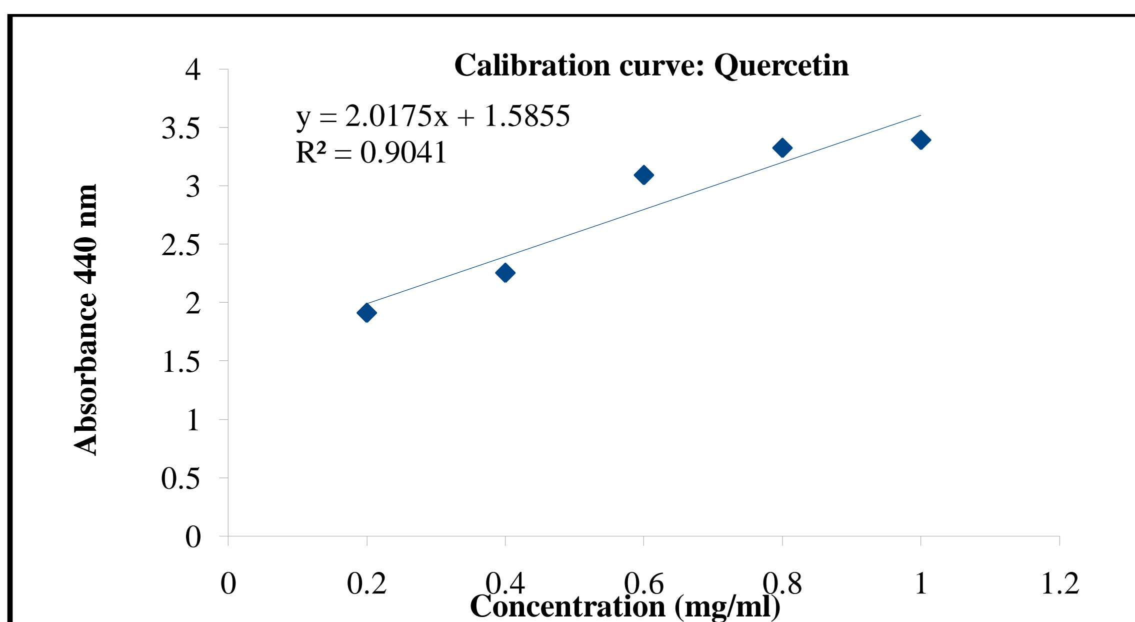

Figure 3.5 Linear calibration curve of standard quercetin

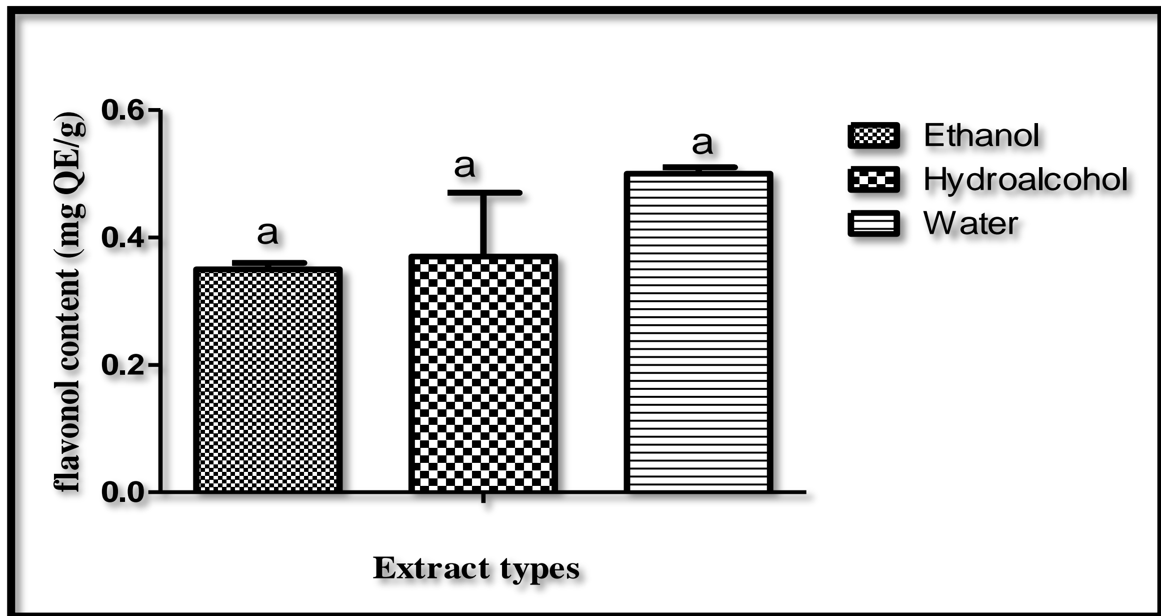

Figure 3.6 Total flavonol content of different leaf extracts of *M. serrata*. Values are presented as mean ± SEM (*n=3*). Means sharing the same letter are not significantly different (p > 0.05)

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3.4 Discussion

In this study, equal portions of powdered *M. serrat*a leaves (30 g) were extracted with three extractants of different polarities (ethanol, hydroalcohol and water) to determine their extraction yield capacity on plant material. The three crude extracts were further investigated for the capacity of extracting phenolic compounds in leaves of *M. serrata* by measuring the content of phenolics, flavonoids and flavonols extracted by each solvent. Ethanol, water and their mixtures are commonly used for extracting phenolic compounds from plant materials due to their high polarity (Alothman *et al*., 2009; Durling *et al*., 2007). The mechanism of extraction involves the separation of active ingredients from the tissues of plant material by means of a solvent. During the process of extraction, the extractant in question diffuses in the plant material and solubilise compounds with the same polarity as the extractant. In the present study, the extractants displayed different capacities of extracting the *M. serrata* leaf material resulting in variation in the calculated percentage yields. According to Złotek *et al.* (2015), different extractants have unique polarities, just as phenolic compounds have unique structural composition which determines their rate of solubility in the extractant in question. Solubility of phenolic compounds is better seen in extractants of high polarity (Naczk and Shahidi, 2006). Generally, water is polar than ethanol. In the present study however, the hydroalcohol solvent displayed a better potential on extracting *M. serrata* leaf plant material as

compared to water and ethanol. Addition of water to ethanol significantly improves the extraction rate (Spigno *et al*., 2007). This may be attributed to the fact that the combined solvents (water and alcohol) have the capacity to extract compounds that are both soluble in water and/ or organic solvents (ethanol). Thus, the combined effect has double the amount of extraction leading to a high percentage yield in the hydroalcohol extract compared to other solvents. Similar findings were observed by Do *et al*. (2014) and Sulaiman (2015) who assessed the extraction capacities of different extractants of different polarities. Both studies reported that hydroalcohol extract produced a better yield as compared to water and alcoholic solvents.

Plants contain a number of phytochemicals which in recent years have drawn attention to the research due to their role in health. These bioactive principles in plants have been reported to play a significant role in prevention of numerous diseases and to improve health (Hosseinzadeh *et al*., 2015). Preliminary phytochemical screening of ethanol, hydroalcohol and water *M. serrata* leaf extracts revealed the presence of some secondary metabolites such as tannins, saponins, flavonoids, steroids, terpenoids, and resins. Earlier, a study by Ashafa (2013) on roots of *M. serrata* revealed the presence of phytochemicals such as alkaloids, saponins, tannins, terpenoids, and steroids. The phytochemical compounds detected in *M. serrata* leaf extracts are well known for their pharmacological and medicinal importance. For instance, Tannins are reported contain antioxidant, cardioprotective, antitumor, antibacterial, antiviral anti-inflammatory and immune-modulatory activities (Bagchi *et al*., 1999; Liu *et al*., 2010). They were also found to exhibit a positive effect on diabetes management. A study done by Rao and Sung (1995), reported on anti-carcinogenic and antifungal activity expressed by saponins. Flavonoids have demonstrated anti-inflammatory, antioxidant (Houghton *et al*., 2003), antimicrobial, cytotoxicity, antitumor (Saxena *et al*., 2013), antimalarial (Dua *et al*., 2013), antispasmodic and pharmacological effects (Thite *et al*., 2013). Flavonoids have been associated with enzyme inhibition, oestrogenic activity, anti-allergic activity, and vascular activity (Tapas *et al*., 2008). Yang *et al*. (2001) reported a positive corelation between increased flavonoids consumption and reduced risk of cardiovascular and cancer diseases. Terpenoids are correlated with lowering the blood glucose level and prevent complications related to diabetes (Abulude *et al*., 2010). On the other side phenols are claimed to inhibit different stages of cancer processes (Wattenberg, 1992) and protect against cardiovascular diseases (Sholihah et al., 2012).

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Water, ethanol, methanol, acetone, and their aqueous mixtures are among the most common solvents used to extract phenolic compounds in plants (López *et al*., 2011; Do *et al*., 2014). Phenolic and flavonoid compounds are recognised as material base of the antioxidant activity of plant extract (Kähkönen *et al*., 1999; Pietta, 2000). Hence, explains their quantification in the present study. Different phenolic compounds respond differently on the Folin-Ciocalteu reagent (Mohammedi and Atik, 2011), thus resulting in the observed phenolic content variation in the present study. Among the tested extracts of *M. serrata* leaves, highest phenolic content was observed in the ethanol extract. Similar findings were observed by Do *et al.* (2014) and Ngo *et al*. (2017) where ethanol extract displayed a high content of phenolic compounds when compared to other solvents. Ethanol is a good solvent for polyphenols extraction (Do *et al*., 2014) due to the fact that it is more efficient at penetrating the cellular membranes of the plant material to extract intracellular ingredients (Valko *et al*., 2005). Thus, alcoholic solutions provide satisfactory results for the process of extraction (Mohammedi and Atik, 2011) and addition of water counteract the action as seen in the hydroalcohol extract which displayed lowest phenolic content. This is in agreement with findings of Do *et al*. (2014) where it was reported that hydroalcohol extract exhibits the lowest phenolic content as observed in the present study.

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There are over 6000 identified flavonoids (Ferrer *et al*., 2008). Flavonoids are water-soluble (Harbone, 1998). Flavonol are a class of flavonoids (Graf *et al*., 2005). From the results, the amount of total flavonoid and flavonol varied in different analysed extracts. Highest total flavonoid and flavonol content was confined to water extracts compared to the ethanol and hydroalcohol extracts. Similar findings were observed by Skowyra (2014). Zainol *et al.* (2009) reported that some flavonoids such as flavan-3-ol, cathechin and gallocatechin are more stable in water due to their intermolecular hydrogen bonding. According to Mierziak *et al*. (2014), flavonoids are the most abundant class of secondary metabolites in plants responsible for major functions especially when interacting with their environment. Deshpande and Kadam (2013) reported that geographical location of plants is also a determining factor of distribution in the phytochemicals within the plant. Furthermore, flavonoids increase when plant experience stress in its environment (Agati *et al*., 2012). Witsishoek, situated in Phuthaditjhaba Free State where the plant *M. serrata* was located, is along the mountains; thus the plant experienced stress from herbivores and

other plants; in addition to the winter freezing temperatures of the mountain. Thus, this possibly explains the presence of high flavonoid content in the leaves of the plant.

3.5 Conclusions

Results from the present study showed that hydroalcohol has high capability of extracting leaves of *M. serrata* as displayed by high percentage yield. Thus for conservation purposes, where high yield is of importance, the study encourages the use of hydroalcohol to obtain such yields in order to avoid unnecessary waste of raw material. The phytochemical screening of the three extracts of *M. serrata* leaves has also revealed that the plant is endowed with phytochemicals of pharmacological importance. Traditional healers or practitioners commonly use water as an extraction solvent in preparation of decoctions. The study has indicated that water extract has the capacity of extracting high content of phenolics, flavonoids and flavonol. Thus, this justifies the use of water in extracting active ingredients by the South African people. However, the study also encourages the use of hydroalcohol or alcoholic solvents in extraction of *M. serrata* leaf material in order to recover different kinds of active principles as they have also shown potential in extracting active ingredients.

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CHAPTER 4

In vitro antioxidant and free radical scavenging activity of *Morella serrata* leaf extracts

4.1 introduction

Antioxidants are important in maintaining good health. Our everyday life styles usually interfere with the normal body functions. The food and drinks consumed as well as daily activities somehow exhaust the amount of antioxidants in our bodies. Thus, supplementing our bodies with natural antioxidants from plants is essential. The potential of antioxidant activity in plants is due to the presence of wide range of compounds which have the ability to neutralise free radicals (Saeidnia and Abdollahi, 2013). From the findings of chapter 3, the extracts from *M. serrata* leaves contained phytochemical compounds with known pharmacological roles. Thus, the present study made use of these extracts (ethanol, hydroalcohol and water) to investigate the in *vitro* antioxidant activity of *Morella serrata* leaves and also validate the folkloric use of the plant in treating free radical implicated diseases such as asthma, diabetes and sexual dysfunction by communities in South Africa.

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4.2 Material and methods

4.2.1 Collection, identification and authentication of plant material

Fresh leaves of *Morella serrata* were collected in February 2014 around Phuthaditjhaba area, Witsishoek (28°39’31.3”S and 28°52’47.4”E; altitude 2047 m), Free State Province, South Africa. The plant was identified, authenticated (Voucher specimen No: MbhMed/02/2014/QHB) and deposited at the University of the Free State Herbarium, Qwaqwa Campus. The leaves were washed under running tap water to remove all contaminants, shade-dried to constant weight and pulverized to fine powder using Waring Commercial Laboratory electric blender. The powdered leaves were then stored in airtight glass containers until further use.

4.2.2 Chemicals and reagents

DPPH (1, 1–Diphenyl–picrylhydrazine), ABTS (2, 2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt), ascorbic acid, Trichloroacetic acid (TCA) and Thiobarbituric acid (TBA) were products of Sigma–Aldrich. All other chemicals were of analytical grade. Distilled water was prepared in the Department of Plant Sciences, University of the Free State, Qwaqwa Campus.

4.2.3 Preparation of crude extracts

In three different beakers, 30 g of the powdered sample was separately extracted with 1L of ethanol, hydroalcohol (50% ethanol and 50 % distilled water), and distilled water, thus ending up with three different extract namely, ethanol, hydroalcohol and water extracts. The extracts were placed in a Labcon shaker (Laboratory Consumables, PTY, Durban, South Africa) for 24 h with agitation. The resulting infusions were filtered using Whatsman No. 1 filter paper. The ethanol crude extract was concentrated in a rotary evaporator (Cole Parmer SB 1100, Shanghai, China) under reduced pressure while aqueous (hydroalcohol and water) extracts were concentrated in a water bath at 40° C. The dry crude extracts were then kept in air-tight containers in a refrigerator prior to use.

4.2.4 Preparation standard solutions and test concentrations

Precisely, 10 mg of standard ascorbic acid and extracts aliquot (ethanol, hydroalcohol and water) were accurately weighed into separate 10 ml volumetric flasks, and each dissolved in 10 mL defined solvent with respect to the antioxidant assay to make a stock solution of 1mg/ml. The stock solutions were further diluted to obtain five different concentrations (0.2, 0.4, 0.6, 0.8, 1.0 mg/ml) in separate centrifuge tubes (**Table 4.1**).

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Table 4.1 Preparation of test concentrations of extracts/ ascorbic acid

Concentration (mg/ml)	Extract / Ascorbic acid (µl)	Distilled water (µl)
0.2	200	800
0.4	400	600
0.6	600	400
0.8	800	200
1.0	1000	-

Figure 4.1 Performing antioxidant activities at the PpRg lab, University of the Free State, QwaQwa campus

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4.2.5 *In vitro* antioxidant assays

The free radical scavenging and antioxidant activity of *M. serrata* was evaluated using standards assays including DPPH, ABTS, nitric oxide, hydroxyl radical scavenging, reducing power, hydrogen peroxide and metal chelating activity with ascorbic acid (Vitamin C) as reference.

4.2.5.1 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity assay

The antioxidant activity of ethanol, hydro-alcohol and distilled water extract were studied using the DPPH method.

Principle of the method

The method was developed by Blois in 1958 based on determination of antioxidant activity using the stable free DPPH radical. This method is rapid, simple and inexpensive involving the use of DPPH to measure the antioxidant capacity (Prakash, 2001). The antioxidant activity of an extract is measured by its potential to donate electrons or protons (H^+) to the stable and nitrogen-centred violet coloured DPPH free radical (Govindan and Muthukrishnan, 2013), thus, measuring the antioxidant scavenging capacity of scavenging substance towards it (Kedare and Singh, 2011). The DPPH in methanol solution is deep purple or violet in colour and absorb at an absorbance of 517 nm (Stanković, 2011). This deep purple or violet colour is due to delocalization of the electron on the DPPH (Alam *et al*., 2013). Towards most compounds, DPPH is said to

have low deterioration and reactivity, thus only good hydrogen atom donors are likely to react with this stable radical (Schwarz *et al*., 2001).When a substrate that acts as an antioxidant (hydrogen atom donor) is mixed with a solution of DPPH, it gives rise to a reduced form of DPPH characterized with the loss of a violet colour and a deceased in absorbance at 517 nm band (Amarowicz *et al*., 2003). Reduction of $DPPH^+$ (Free radical) to DPPH-H (nonradical) by an antioxidant (Hydrogen donor) is shown in **Figure 4.2** (Molyneux, 2004).

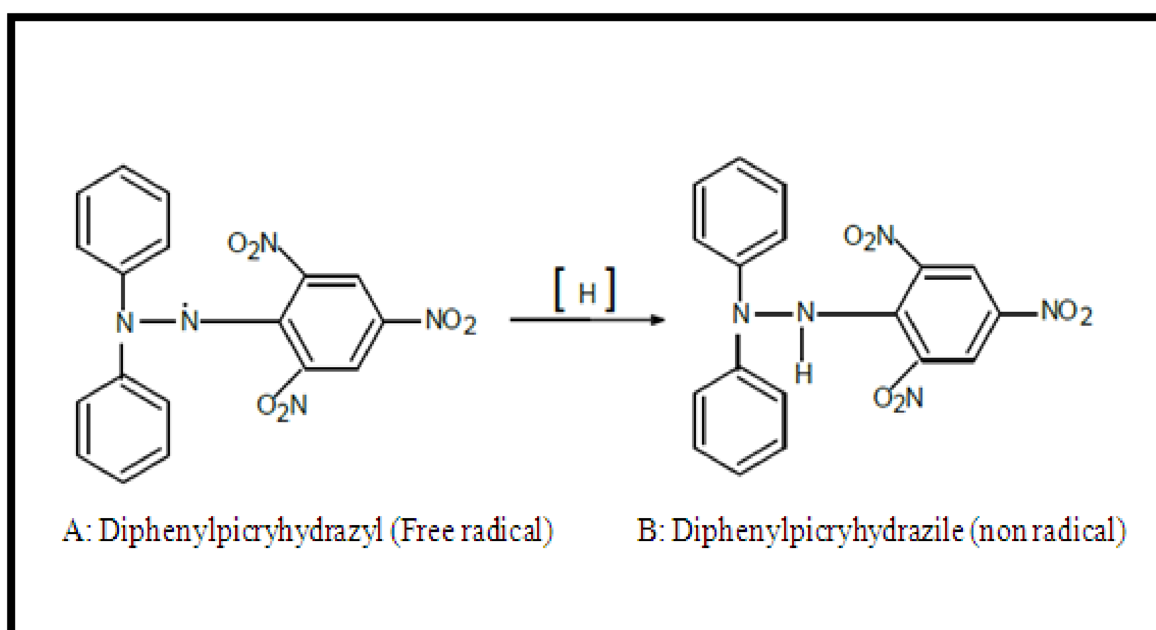

Figure 4.2 Reduction of $DPPH^+$ (Free radical) to DPPH-H (nonradical) by an antioxidant (Hydrogen donor) (Molyneux, 2004)

Protocol

The method of Liyana-Pathirana and Shahidi (2005) was used to determine the effect of crude extracts and standard ascorbic acid on the DPPH radical. A solution of 0.135 mM DPPH in methanol was prepared and 1.0 mL of this solution was mix with 1.0 mL of varying concentrations (0.2, 0.4, 0.6, 0.8 and 1.0 mg/mL) of extracts prepared in methanol. The reaction mixture was vortexed thoroughly and left in the dark at room temperature for 30 min. The absorbance of the mixture was measured at 517 nm using a microplate reader. The same procedure was performed for ascorbic as a standard. The ability to scavenge DPPH radical was calculated by the following equation:

DPPH radical scavenging activity (%) = $[(A_0-A_1)/A_0] \times 100$ where A_0 is the absorbance of the control (DPPH radical + methanol), and A_1 is the absorbance of DPPH radical + sample extract / standard.

4.2.4.2 2, 2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) free radical scavenging activity assay

Principle of the method

This assay was first reported by Miller *et al* (1993) and improved by Rice-Evans *et al.* (1996) and Re *et al*. (1999). The method is also known as Trolox equivalent anti-oxidant capacity (TEAC) assay. The assay assesses the total radical capacity of plant extracts through their ability to scavenge the long-lived specific ABTS cation chromophore. The assay is based on the principle that when ABTS is incubated with a peroxidase such as metmyoglobin and H_2O_2, it results in a formation of a relatively stable radical cation ABTS+ (Badarinath *et al*., 2010). The generation of the ABTS+ blue/green chromophore occurs through the oxidation of the ABTS diammonium salt in the presence of potassium persulfate, with the absorption maxima occurring at wavelengths 645, 734 and 815 nm. If the plant extract has potential antioxidants activity, it will reduce the preformed radical cation to ABTS thus bringing about decolourisation of $ABTS^+$ to a colourless product. Formation of ABTS radical after addition of potassium persulphate is shown in **Figure 4.3** (Pannala *et al*., 2001). The extent of this decolourisation is a measure of the $ABTS^+$ radical that has been scavenged, after a fixed time period. The drawback of this method is that it only indicates the presence of antioxidant compounds in the solution, but does not infer structure-activity relationship (Nenadis *et al*. 2004).

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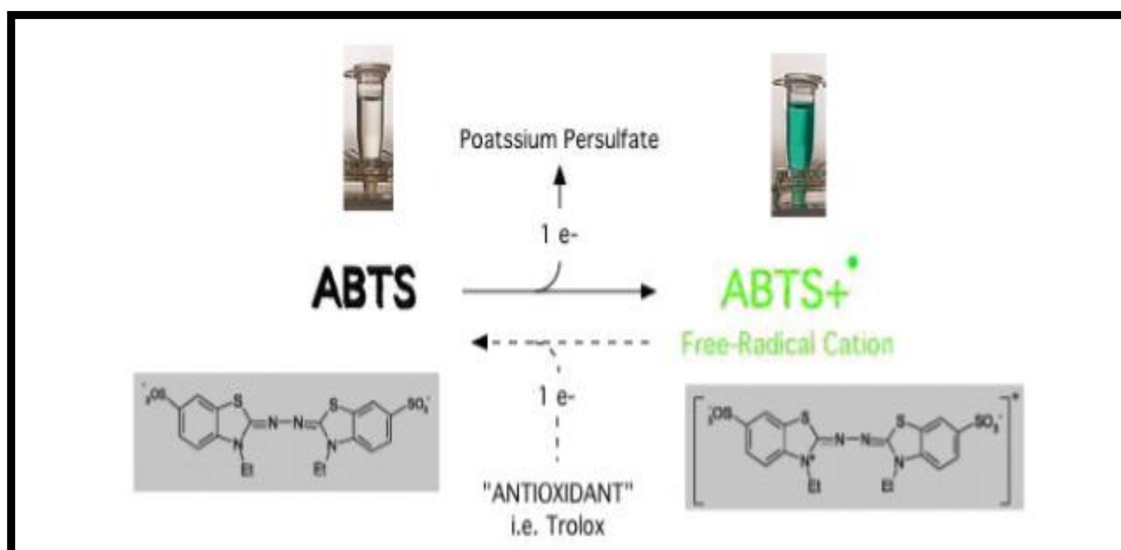

Figure 4.3 Formation of ABTS radical after addition of potassium persulphate and regeneration of ABTS after an antioxidant intervention (Pannala *et al*., 2001)

Protocol

A method by Proestos *et al*. (2013) was adopted for this assay. Solutions of 7 mM ABTS and 2.45 mM Potassium persulfate were separately prepared in deionised water. The two solutions were mixed in a ratio of 1:1 and kept in the dark for 30 h prior to use. A dilution ratio of 1:25 ABTS solution in aqueous methanol was prepared from ABTS radical solution. Varying concentrations (0.2, 0.4, 0.6, 0.8 and 1.0 mg/ml) of the extract were prepared in aqueous methanol extracts at a ratio of (1:10) after which 20 µl of each concentration was added to 2 mL of ABTS radical solution and kept at 30º C standard temperature. The absorbance was measured at 734 nm using a microplate reader (Bio-Rad Model 680, Japan) in 0.5, and 10 min after initial mixing. The same procedure was performed for ascorbic as a standard. The percentage inhibition of $ABTS^{\cdot +}$ was calculated using the following formula:

$$ABTS^{\cdot +} \text{ Percentage inhibition} = [(A_0 - A_1)/A_0] \times 100$$

Where A_0 is the absorbance of the control (ABTS radical + aqueous methanol), and A_1 is the absorbance of the ABTS radical + extract / standard.

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4.2.5.3 Nitric oxide scavenging activity assay

Principle of the method

Nitric oxide (NO) is a reactive nitrogen produced species functioning as a bio regulatory molecule responsible for controlling blood pressure, neural signal transduction, platelet function, antimicrobial and antitumor activity (Jagetia, 2004). The mechanism of the nitric oxide scavenging assay is based on sodium nitroprusside in aqueous solution at physiological pH spontaneously generating nitric oxide, which interact with oxygen to produce nitrite ions and can be determined by the use of the Griess reagent. Scavengers of nitric oxide compete with oxygen, leading to reduced production of nitrite ions (Ebrahimzadeh *et al*., 2010). The nitrite ions produced diazotizes with sulphanilamide acid and couple with naphthyl ethylenediamine give a pink colour chromophore which has a maximum absorption at 546 nm (Balakrishnan *et al*., 2009).

Protocol

The nitric oxide scavenging activity of the extracts was determined using previously reported method (Ebrahimzadeh *et al*., 2008). A volume of 2 mL sodium nitroprusside (10 mM) prepared in 0.5 mM phosphate buffered saline (pH 7.4) was mixed with 0.5 mL of varying concentrations (0.2, 0.4, 0.6, 0.8 and 1.0 mg/mL) of extract. The mixture was then incubated at 25ºC for 150 min. Aliquot of each extract (0.5 mL) was added to 0.5 mL Griess reagent (1.0 mL Sulfanic acid reagent (0.33%) prepared in 20% glacial acetic acid for 5 min with 1 mL naphthy ethylenediamine chloride (0.1% w/v). The mixture was then incubated at room temperature for 30 min. The chromophore formed was read at 546 nm using the microplate reader (Bio-Rad Model 680, Japan). The same procedure was performed for ascorbic as a standard. The NO radical was calculated using the following formula:

$$\text{NO radical scavenging activity} = [(A_0 - A_1)/A_0] \times 100$$

Where A_0 is the absorbance of the control (NO radical + methanol) and A_1 is the absorbance of NO radical + extract / standard

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4.2.5.4 Hydroxyl radical scavenging assay

Principle of the method

The hydroxyl radical scavenging assay is used to measure the scavenging activity of free hydroxyl radical such as hydrogen peroxide which damages the body in the presence of different concentration of plant sample. Hydrogen peroxide reacts with ferrous to form hydroxyl radicals which attack deoxyribose. The reaction is based on the quantification of the 2-deoxyribose degradation product, malonaldehyde, by its condensation with TBA to give a yellow colour which absorbs at 532 nm (Adjimani and Asare, 2015). The role of plant extract is to scavenge the hydroxyl radicals and prevent the degradation of 2-deoxyribose (Halliwell and Gutteridge, 1981).

Protocol

The hydroxyl radical scavenging activity of the extracts was determined following the method of Mathew and Abraham (2006). A reaction mixture containing 100 µl of extract aliquot at varying concentrations (0.2, 0.4, 0.6, 0.8 and 1.0 mg/ml), 120 µl Deoxyribose (20 mM), 400 µl phosphate buffer (0.1M), 40 µl H_2O_2 (20 mM), 40 µl ferrous sulphate (500 µM) and 100 µl H_2O was prepared and incubated at 37 ºC for 1 h. The reaction was stopped with 0.5 ml TCA (28%) and 400 µl TBA (0.6%). The set up was incubated in hot water for 20 min and read at 532 nm using microplate reader (Bio-Rad Model 680, Japan). The same procedure was performed for ascorbic as a standard. The hydroxyl radical scavenging activity was calculated using the following formula:

Hydroxyl radical scavenging activity: $[(A_0-A_1)/A_0] \times 100$

Where A_0 is the absorbance of the control (HO radical + methanol) and A_1 is the absorbance of HO radical + extract / standard.

4.2.5.5 Reducing power capacity assay

Principle of the method

Reducing power is a measure of the extracts ability to reduce Fe^{3+} to Fe^{2+}. The antioxidant ability of the extract is shown when the extract react with potassium ferricyanide (Fe^{3+}) to form potassium ferrocyanide (Fe^{2+}) and then reacts with ferric chloride to form ferric-ferrous blue complex with maximum absorbance at 700 nm. The increase in the absorbance of the mixture as the concentration increases explains the

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increase in the antioxidant activity of the extract under investigation. (Jayanthi and Lalitha, 2011)

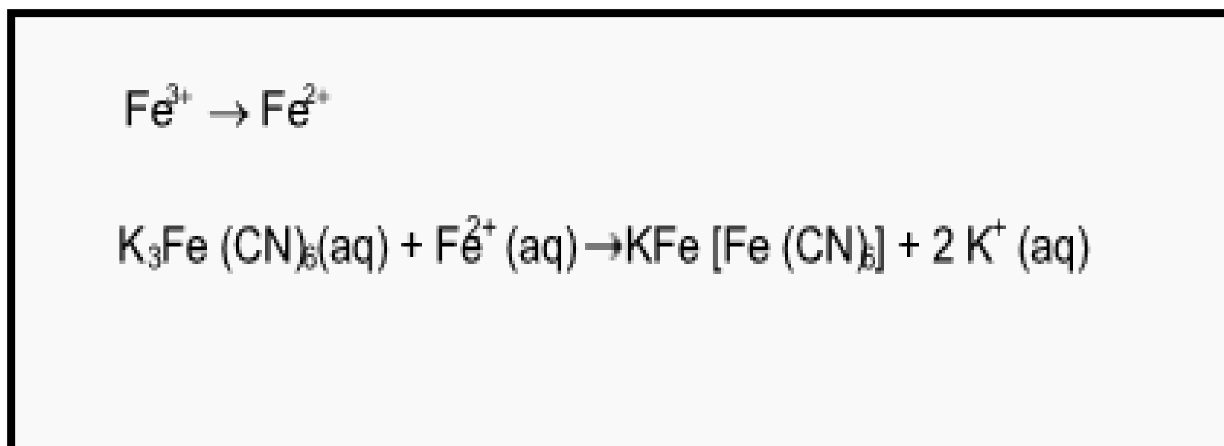

Figure 4.4 Reduction of Fe^{3+} to Fe^{2+} by a possible antioxidant

Protocol

The reducing power of the extracts was evaluated according to the method described by Oyaizu (1986). A volume of 1 mL of extract at varying concentrations (0.2, 0.4, 0.6, 0.8 and 1.0 mg/mL) was prepared in distilled water and mixed thoroughly with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of $K_3Fe(CN)_6$ (1% w/v). The resulting mixture was incubated at 50 ºC for 20 min. This was followed by addition of 2.5 ml of TCA (10% w/v) and centrifuging at 3000 rpm for 10 min. The upper layer of the solution was collected and mixed with 2.5 ml of distilled water, followed by 0.5 mL of ferrous chloride (0.1% w/v). The absorbance was read at 700 nm using microplate reader against blank sample. The same procedure was performed for ascorbic as a standard. Increased absorbance of the reaction mixture indicated higher reducing power of the plant extract.

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4.2.5.6 Hydrogen peroxide activity assay

Principle of the method

Hydrogen peroxide is a weak oxidizing agent which has the ability to directly inactivate few enzymes, usually by oxidation of essential thiol group (-SH). Hydrogen peroxide also has the ability to cross the cell membrane, where it reacts with Fe^{2+}, and possibly Cu^{2+} ions to form hydroxyl radical and this may be the origin of many of its toxic effects. It is therefore biologically advantageous for cells to control the amount of hydrogen peroxide that is allowed to accumulate. Hydrogen peroxide free radical has the ability to accept

protons (H^+) or electrons from other substances. When it accepts electrons or protons, it is reduced to water. In the assay, hydrogen peroxide is reduced to H_2O by iron chelators via donation of an electron or proton to it (Adjimani and Asare, 2015). Upon the acceptance of an electron, a reduction in the absorbance of H_2O_2 is observed (Malik *et al.*, 2011).

Protocol

The hydrogen peroxide scavenging activity of the extracts was determined according to the procedure described by Ruch *et al.* (1989). A volume of 0.6 mL of 40 mM hydrogen peroxide solution prepared in phosphate buffer (pH 7.4) was mixed with varying concentrations (0.2, 0.4, 0.6, 0.8 and 1.0 mg/mL) of extracts. The mixture was incubated for 10 min at room temperature and absorbance was read at 230 nm using spectrophotometer. The percentage of hydrogen peroxide scavenging activity was calculated according to the following formula:

$$\text{Hydrogen peroxide scavenged (\%)} = [(A_0 - A_1)/A_0] \times 100$$

Where A_0 is the absorbance of the control solution (containing all reagents except extract) and A_1 is the absorbance of all reagents + extract / standard.

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4.2.5.7 Metal chelating ability assay

Principle of the method

Ferrozine can quantitatively chelate Fe^{2+} and form a red coloured complex. This reaction is limited in the presence of other chelating agents and results in a decrease of the red colour of the ferrozine - Fe^{2+} complex. Measurement of the colour reduction estimates the chelating activity to compete with ferrozine for the ferrous ions (Soler-Rivas *et al.*, 2000). The antioxidants present in plant extract forms a coordinate complex with the metal ions (chelating activity) and inhibit the transfer of electrons. Thus, oxidation reaction is arrested and no free radicals are produced.

Protocol

The ferrous ions chelating activity of extracts was estimated by the method of Dinis *et al.* (1994). Briefly, different concentrations (0.2, 0.4, 0.6, 0.8 and 1.0 mg/mL) of extracts were added to 0.1 mL solution of 2 mM ferrous chloride ($FeCl_2$). A volume of 0.2 mL (5 mM) ferrozine was added to the mixture to initiate the reaction. The mixture was then shaken vigorously and kept at room temperature for 10 min after which the absorbance

was measured spectrophotometrically at 562 nm. The percentage of inhibition of ferrozine – Fe^{2+} complex was calculated using the formula:

Ferrous ions chelating ability (%) = $[(A_0-A_1)/A_0] \times 100$

Where A_0 is the absorbance of the control solution (containing all reagents except extract) and A_1 is the absorbance of all reagents + extract / standard.

4.2.6 Statistical analysis

IC_{50} values were calculated using linear regression. Mean and SEM of three replicates were calculated. Statistical analysis was performed using a Graph Pad Prism version 5.00 statistical package (Graph Pad software®, San Diego, CA, USA). Results were expressed as mean of three determinations ± standard error of mean (SEM) and subjected to one-way analysis of variance (ANOVA) followed by Bonferroni comparison test to determine the significant difference in all parameters. P value < 0.05 was considered statistically significant.

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4.3 Results

4.3.1 *In vitro* antioxidant activity and free radical scavenging activity of *M. serrata* leaf extracts and ascorbic acid.

4.3.1.1 DPPH-free radical scavenging activity

The DPPH radical scavenging activity of *M. serrata* leaf extracts is presented in **Table 4.2**. The DPPH scavenging activity of ethanol extract was significantly ($p<0.05$) higher than the other tested extracts (hydroalcohol and water) and to that of standard. This was followed by hydroalcohol extract, ascorbic acid with the least inhibition seen in the water extract. The DPPH radical scavenging activity of the extracts was dose-dependent as shown in **Figure 4.5**.

Table 4.2 DPPH IC_{50} values of different leaf extracts of *M. serrata*

Extract type	DPPH (IC_{50} value (mg/ml))
Ethanol	1.00 ± 0.26^{a}
Hydroalcohol	1.05 ± 0.21^{ac}
Water	1.94 ± 0.34^{bc}
Ascorbic acid	1.65 ± 0.14^{bc}

Values are means± SEM of triplicates. Means sharing the same letter superscript in the same column are not significantly different from each other (p > 0.05)

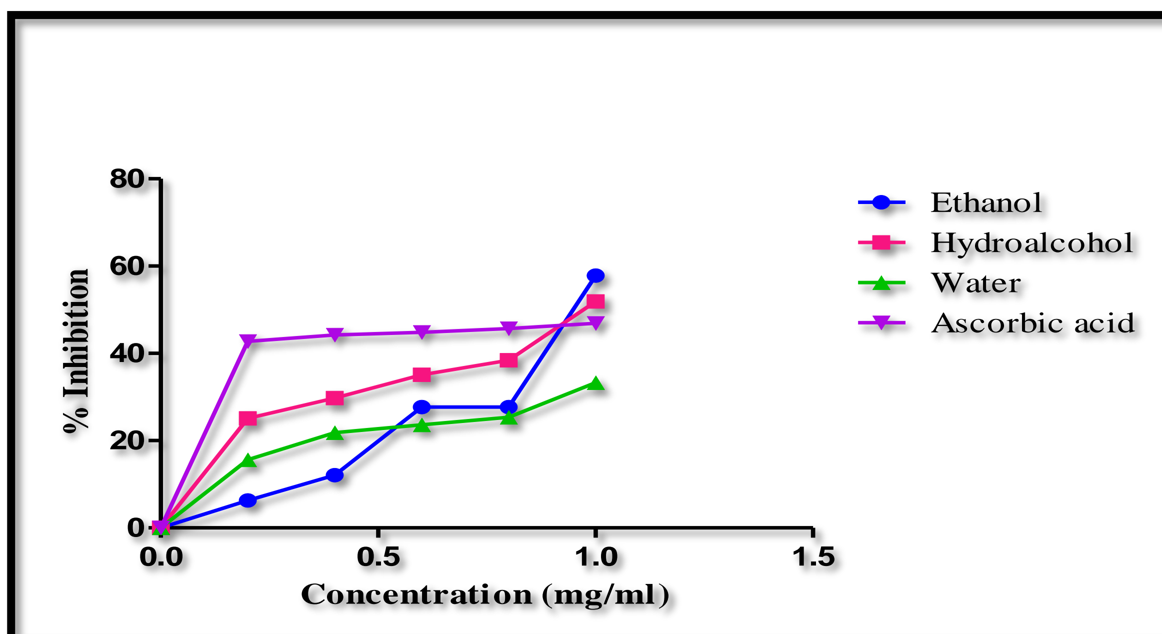

Figure 4.5 Dose dependent curves for the % inhibition of DPPH free radical scavenging activity of *M. serrata* extracts and ascorbic acid. Values are presented as mean ± SEM (*n=3*)

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4.3.1.2 ABTS free radical scavenging activity

The ABTS inhibition of ethanol and water extracts were significantly ($p<0.05$) higher than that of ascorbic acid whilst the hydroalcohol extract was significantly ($p<0.05$) different from ascorbic acid with hughest IC_{50} value of 2.65 mg/mL. ABTS inhibition was in order of water> ethanol> ascorbic acid. Hydroalcohol. as shown in **Table 4.3**. The percentage free radical scavenging activity was plotted against concentration of the extracts as shown in **Figure 4.6**. The antioxidant activity was observed to incease with an incease in the extracts concentration.

Table 4.3 ABTS IC_{50} values of different leaf extracts of *M. serrata*

Extract Type	ABTS (IC_{50} value (mg/ml))
Ethanol	1.26±0.22[a]
Hydroalcohol	2.65±0.28[b]
Water	1.04±0.11[a]
Ascorbic acid	1.42±0.21[a]

Values are means± SEM of triplicates. Means sharing the same letter superscript in the same column are not significantly different from each other ($p > 0.05$)

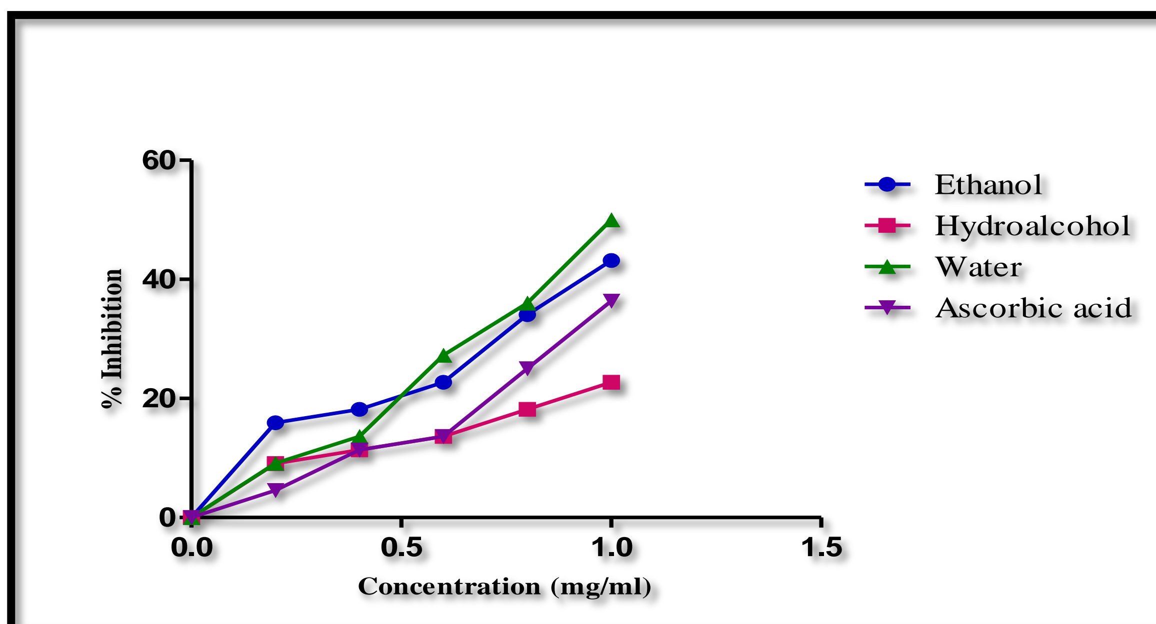

Figure 4.6 Dose dependent curves for the % inhibition of ABTS free radical scavenging activity of *M. serrata* leaf extracts and ascorbic acid. Values are presented as mean ± SEM (*n=3*)

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4.3.1.3 Nitric oxide scavenging activity

The maximum free radical scavenging activity and potency of the extracts were interpolated from **Figure 4.7** to give results of IC_{50} as shown in **Table 4.4**. Among the tested extracts, only the ethanol extract had significantly ($p<0.05$) better NO inhibition than the ascorbic acid. The ABTS inhibition of hydroalcohol and water extract was significantly ($p<0.05$) lower than that of ascorbic acid. The antioxidant activity was highly dependent on the concentration of the extract as shown in **Figure 4.7**.

Table 4.4 Nitric oxide IC_{50} values of different leaf extracts of *M. serrata*

Extract type	Nitric oxide (IC_{50} value (mg/ml))
Ethanol	1.01 ± 0.35^{a}
Hydroalcohol	4.44 ± 0.61^{b}
Water	5.60 ± 1.26^{b}
Ascorbic acid	3.40 ± 0.96^{ab}

Values are means± SEM of triplicates. Means sharing the same letter superscript in the same column are not significantly different from each other ($p > 0.05$)

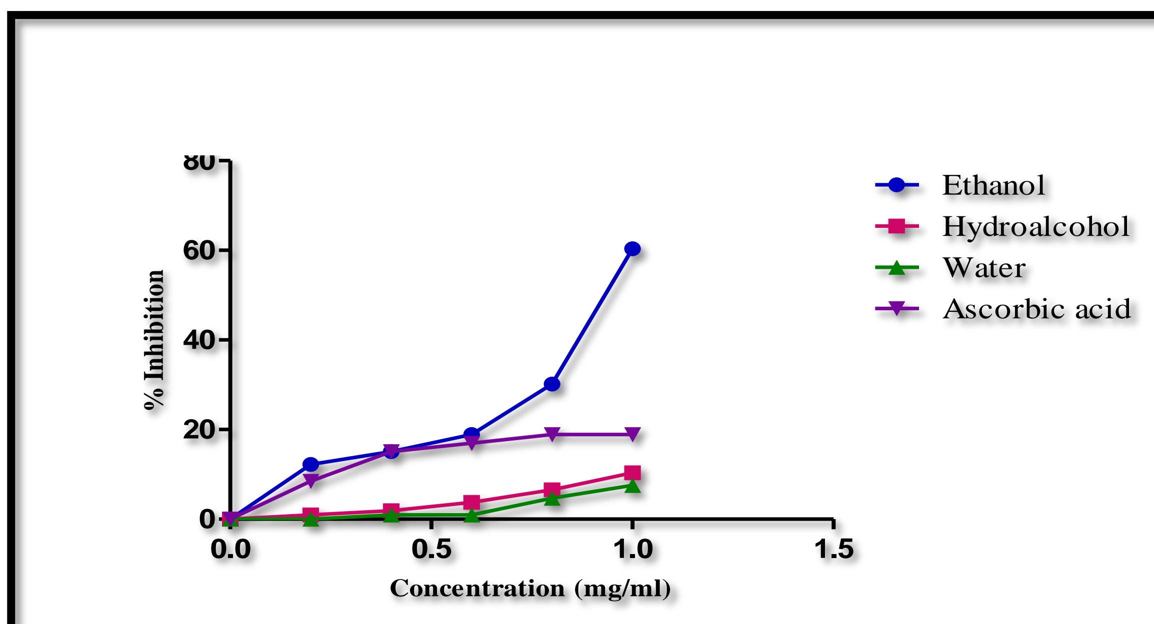

Figure 4.7 Dose dependent curves for the % inhibition of nitric oxide scavenging activity of *M. serrata* leaf extracts and ascorbic acid. Values are presented as mean ± SEM (*n=3*)

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4.3.1.4 Hydroxyl radical scavenging activity

All the tested extracts of *M. serrata* displayed no significant difference ($p<0.05$) when compared to ascorbic acid. The three extracts of *M. serrata* displayed better scavenging activity compared to ascorbic acid. Maximum scavenging activity of hydroxyl radical was observed in the water extract followed by hydroalcohol and ethanol extracts with ascorbic showing the least scavenging activity as shown in **Table 4.5.** Percentage inhibition of the extracts was dose dependent and increased with an increase in concentration as shown in **Figure 4.8**.

Table 4.5 Hydroxyl radical IC_{50} values of different leaf extracts of *M. serrata*

Extract type	**Hydroxyl radical (IC_{50} value (mg/ml))**
Ethanol	0.45±0.07[a]
Hydroalcohol	0.32±0.05[a]
Water	0.13±0.07[a]
Ascorbic acid	0.52±0.14[a]

Values are means± SEM of triplicates. Means sharing the same letter superscript in the same column are not significantly different from each other ($p > 0.05$)

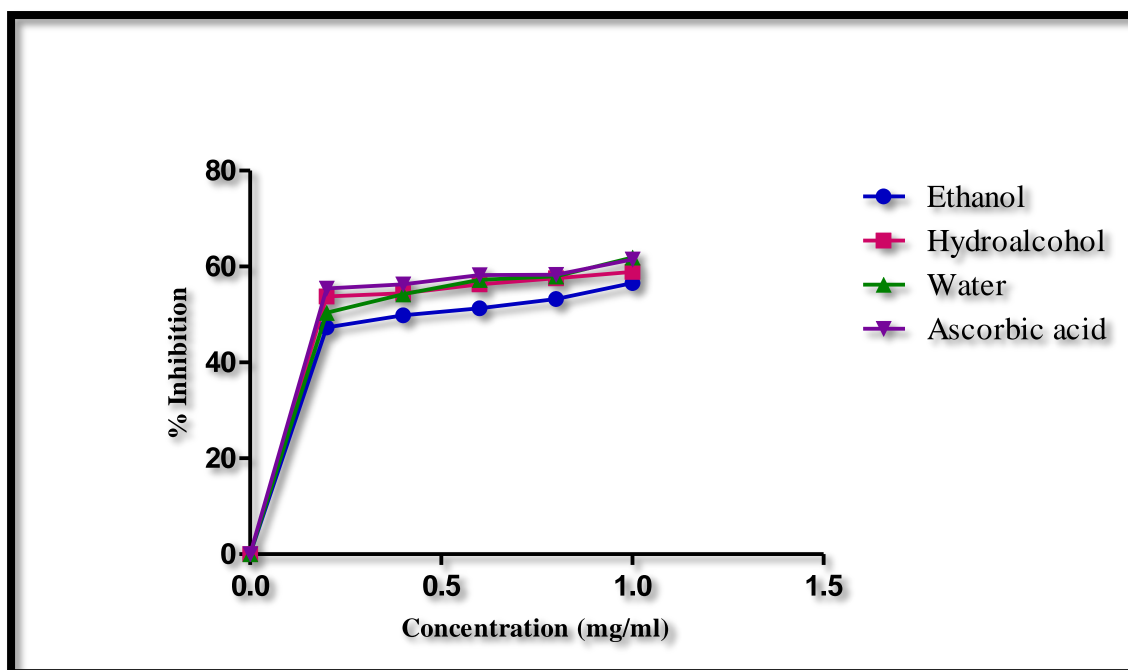

Figure 4.8 Dose dependent curves for the % inhibition of hydroxyl radical scavenging activity of *M. serrata* leaf extracts and ascorbic acid. Values are presented as mean ± SEM (*n=3*)

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4.3.1.5 Reducing power activity

The reducing power of the reaction mixture resulted in a dose dependent absorbance (**Figure 4.9**). The higher absorbance of the reaction mixture a, the higher would be its reducing power. The order of reducing power was found to be Hydroalcohol> Ethanol> Ascorbic acid> Water.

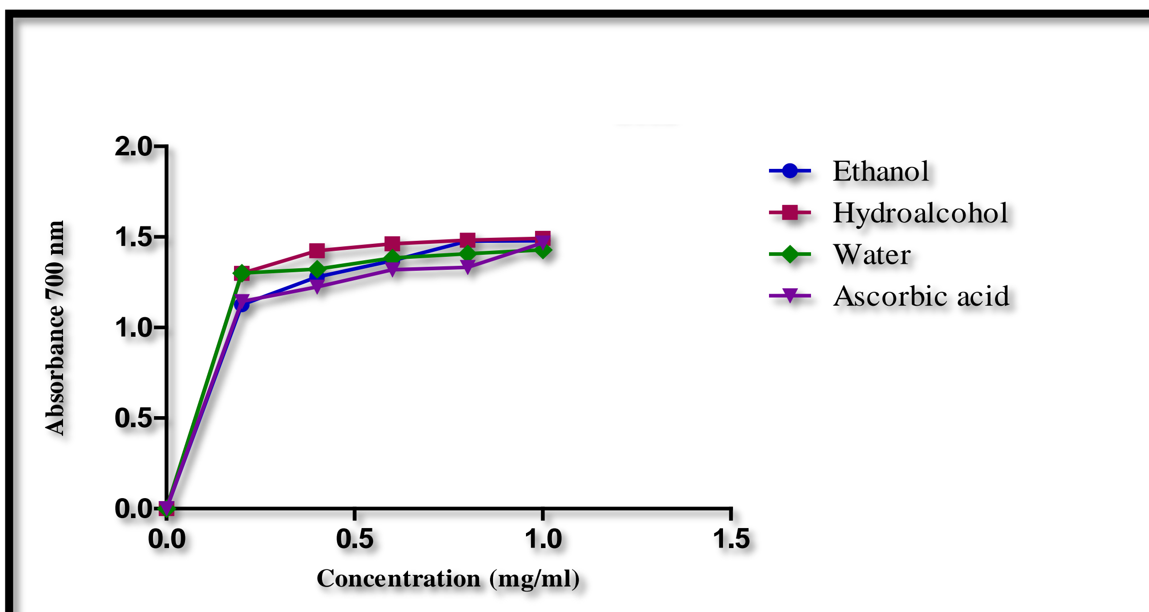

Figure 4.9 Dose dependent curves for the absorbances of *M. serrata* leaf extracts and ascorbic acid in the ferric reducing power activity assay. Values are presented as mean ± SEM (*n=3*)

4.3.1.6 Hydrogen peroxide antioxidant activity

No significant difference ($p< 0.05$) was observed among tested extracts in the hydrogen peroxide antioxidant assay as shown in **Table 4.6**. All the extracts of *M. serrata* showed activity that is comparable to that of standard ascorbic acid. However, greater inhibition was observed in the water extract, followed by ascorbic acid and ethanol with hydroalcohol exhibiting the lowest antioxidant activity. Percentage inhibition of the extracts was observed to fo,llow a dose dependent dependent manner as shown in **Figure 4.10**.

Table 4.6 Hydrogen peroxide IC_{50} values of different leaf extracts of *M. serrata*

Extract type	Hydrogen peroxide (IC_{50} value (mg/ml))
Ethanol	0.55±0.08[a]
Hydroalcohol	0.62±0.08[a]
Water	0.41±0.11[a]
Ascorbic acid	0.42±0.15[a]

Values are means± SEM of triplicates. Means sharing the same letter superscript in the same column are not significantly different from each other ($p > 0.05$)

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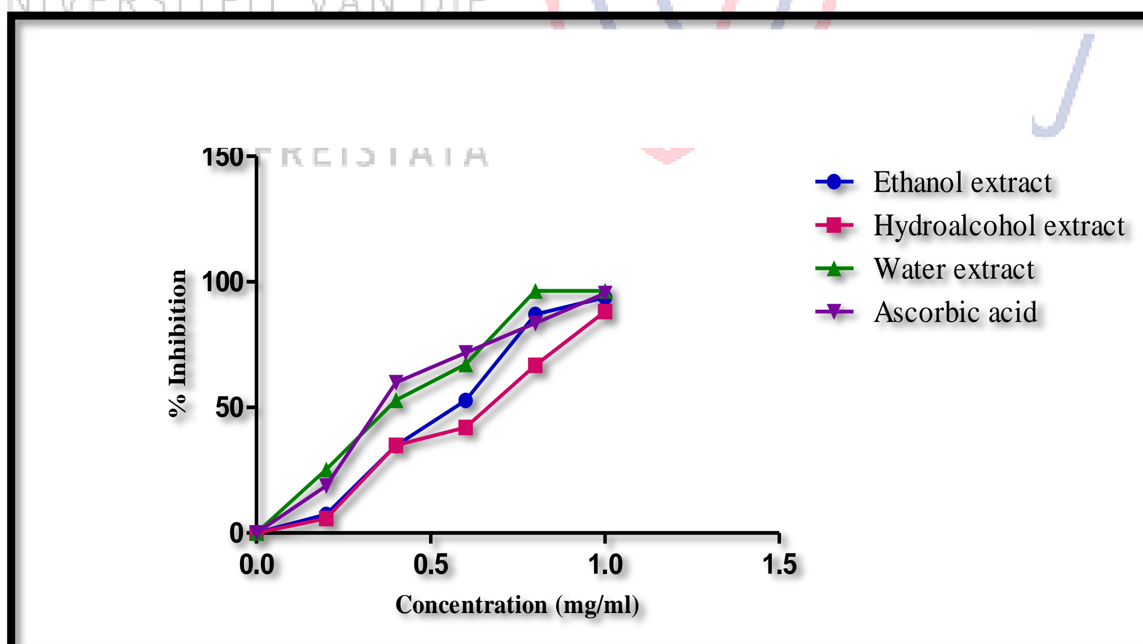

Figure 4.10 Dose dependent curves for the % inhibition of hydrogen peroxide antioxidant activity of *M. serrata* leaf extracts and ascorbic acid. Values are presented as mean ± SEM (*n=3*)

4.3.1.7 Metal chelating antioxidant activity

In the metal chelating antioxidant activity assay, all the tested extracts had no significant difference ($p>0.05$) from each other and were comparable to ascorbic acid.(**Table 4.7**). Extracts of *M. serrata* displayed stronger metal chelating acivity when compared to the standard ascorbic acid. Highest metal chelating activity was seen in the water extract, followed by ethanol and hydroalcohol extract with least metal chelating activity being observed in the ascorbic acid. Percentage inhibition of the extracts was observed to follow a dose dependent dependent manner as shown in **Figure 4.11.**

Table 4.7 Metal chelating assay IC_{50} values of different extracts of *M. serrata* leaves

Extract type	Metal chelating (IC_{50} value (mg/ml))
Ethanol	0.33±0.03[a]
Hydroalcohol	0.35±0.10[a]
Water	0.31±0.12[a]
Ascorbic acid	0.73±0.16[a]

Values are means± SEM of triplicates. Means sharing the same letter superscript in the same column are not significantly different from each other ($p > 0.05$)

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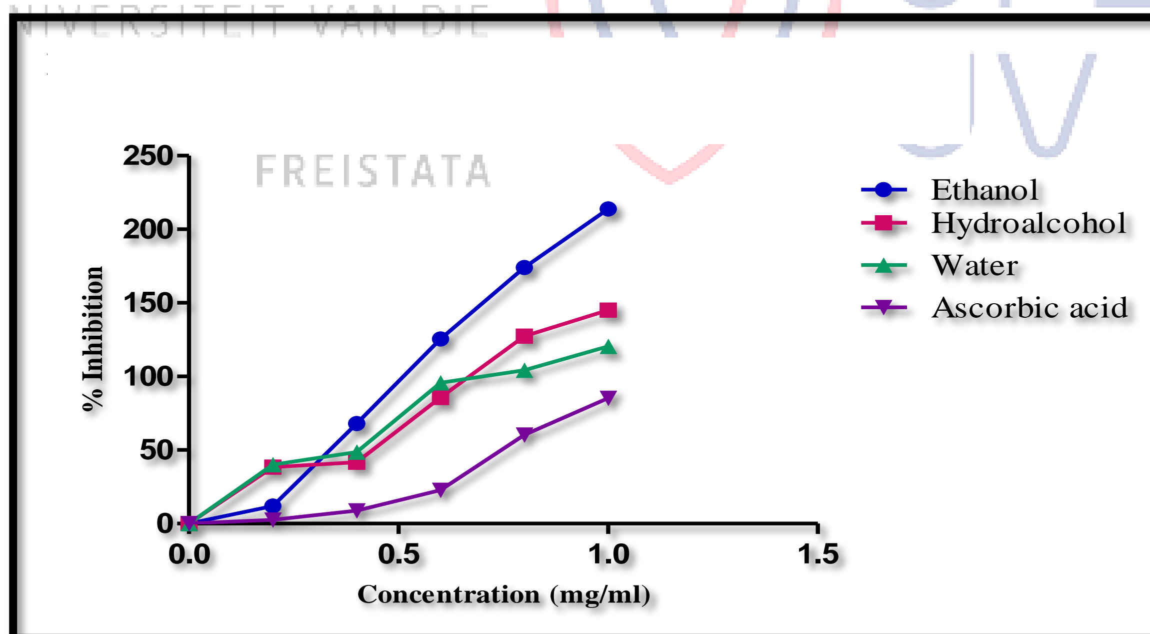

Figure 4.11 Dose dependent curves for the % inhibition of metal chelating activity of *M. serrata* leaf extracts and ascorbic acid. Values are presented as mean ± SEM (*n=3*)

4.4 Discussion

In the present study, the antioxidant activity of the three extracts of *M. serrata* leaves (ethanol, hydroalcohol and water extract) was assessed against DPPH, ABTS, nitric oxide, reducing power, hydroxyl radicals, hydrogen peroxide and metal chelating assay. The free radical scavenging and antioxidant activity of the extracts was compared to that of a standard antioxidant drug, ascorbic acid. The antioxidant capacity of the extract is influenced by various factors (Gupta, 2010). Each assay in the present study employs a unique mechanism of action of scavenging or antioxidant activity (Alam *et al*., 2013). Therefore, measuring the antioxidant capacity of the extracts through different assays will ensure to cover different mechanisms (Gan *et al*., 2010). The antioxidant potential of the extracts was measured using the IC_{50} value which is 50% of the amount of extract needed to inhibit or quench free radicals at a specified time. IC_{50} value for each extract was calculated from the linear calibration curve graphs of concentrations versus percentage inhibition. Lower IC_{50} value indicates greater antioxidant activity (Raghavendra *et al*., 2013). The DPPH antioxidant activity of *M, serrata* extracts increased with an increase in concentration. The DPPH scavenging activity of the extracts strongly correlates with the extract phenolic content (Khan *et al*., 2012; Konan *et al*., 2014). Furthermore, an excellent linear correlation between the Folin-Ciocalteu reagent assay for total phenolic content and DPPH assay is also a contributing factor on the scavenging of free radicals (Karadag *et al*. 2009). Accordingly, in the present study maximum DPPH scavenging activity of *M. serrata* leaves was displayed by the ethanol extract which contained the highest phenolic content. These findings are in line with the findings of Aksoy *et al*. (2013) who observed that the radical scavenging effect of the extracts was positively correlated with their total amount of phenolic compounds. The ability of an extract to act as free radical scavenger is due to its ability to neutralise free radicals to non-radical molecules. The antioxidant activity of phenolics is confined to their ability to donate hydrogen, accept free radicals, and interrupt chain oxidation reaction or chelate metals (Viuda-Martos *et al*., 2010), thus terminating the chain of lipid peroxidation during oxidative stress (Dutta *et al*., 2012).

In addition to this, the DPPH free radical scavenging activity displayed by the ethanol extract might be due to the detected phytochemicals in the ethanol crude extract. The ethanol extract showed presence of phytochemicals such as tannins, flavonoids, steroids, phenols and resins. In literature, these phytochemicals have been reported to possess

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antioxidant activity (Mooradian, 1993; Assimopoulou *et al*., 2005; Sekar *et al*., 2012; Aksoy *et al*., 2013; Saxena *et al*., 2013).

Mukhtar *et al*. (2009), states that hydrophilic extracts have much higher antioxidant capacity as compared to hydrophobic extracts. This may be due to the properties of water as a universal solvent as it is capable of extracting more substances than any other solvent. This is the observed case for the water extract in the ABTS, Hydroxyl radical and hydrogen peroxide and metal chelating assays. The maximum scavenging activity displayed by the water extract may be attributed to the high flavonoid and flavonol contents. According to Andarwulan *et al*. (2010), the intake of flavonoids is implicated in reduced risk of oxidative stress related disorders. Evidence from Gates *et al* (2007), revealed that increased consumption of broccoli and spinach which are both rich in a type of flavonoid called kaemferol is associated with reduced risk of ovarian cancer. The role of flavonoids as an antioxidant is due to their ability to quench, up-regulate free radicals or protect antioxidant defences and chelate radical intermediate compounds (Ndhlala *et al*., 2010). Morales *et al.* (2012) reported that flavonoids are efficient singlet oxygen quenchers. They are thus regarded as potential antioxidants which could act against free oxidative stress in a system. This probably explains the destruction of active oxygen species in the hydrogen peroxide and hydroxyl radical scavenging assays. In addition to this, Yilmaz and Toledo (2004) reported flavonoids to contain polyvalent phenolics in their molecular structure which allows chelating of metal ions. Water extract detected one unique phytochemical saponin which possesses antioxidant activity (Chen *et al*., 2014). Saponins may have worked together in combination with other antioxidants substances present in the water extract to fight against free radicals and oxidative stress. High consumption of plants and their products is associated with a low risk of free radical implicated diseases which is attributed to the presence of antioxidant vitamins and the phytochemicals within them (Ames *et al*., 1993; Prior, 2003). Due to the observed antioxidant activity of the extracts to scavenge free radicals, this may support the use of the plant among South Africans in treating asthma and diabetes which are associated with excess free radicals.

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NO which is produced by the endothelial cells (Inda, 2014), is a potent opleitropic mediator of physiological processes such as smooth muscle relaxation, neuronal signalling, inhibition of platelet aggregation, and regulation of cell mediated toxicity

(Hagerman *et al*., 1998). It is primarily responsible for regulating blood pressure and blood flow in arteries (Huang *et al*., 1995) which during erection is the requirement for relaxation of the penis muscles. Adequate blood flow supply is needed to relax smooth muscles in the penis arteries during erection, thus making NO responsible for endothelial contraction. The lack of NO production plays a role in the pathogenesis of erectile dysfunction (Sullivan *et al*., 1999). The role of excess NO production in diseases has also been documented (Luiking *et al*., 2010). *M. serrata* ethanolic extract showed high capacity to quench NO production by donating a proton to nitrite radical. The use of *M. serrata* as a sex stimulator may be explained by the mechanism that the extract act as a donor thus stimulating the enzyme called nitric oxide synthase in the epithelium to produce adequate nitric oxide necessary for erection. The ethanol extract was observed to contain high antioxidant compounds especially, phenolics followed by flavonols and flavonols. According to Shahidi *et al*. (1994) synergism of polyphenolic compounds in the plant extract may contribute to its antioxidant activity. Thus, the antioxidant compounds quantified in the ethanol extract can also be attributed to the observed NO scavenging activity of the ethanol extract. A study by Lakhanpal and Rai (2007) found flavonoids to directly scavenge nitric oxide. Report by Vanacker *et al*. (1995) also highlighted NO scavenging ability of flavonoids. Thus, this explains the traditional use of the plant *M. serrata* by the *Basotho* people of the Eastern Free State to enhance male sexual performance.

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4.5 Conclusions

Based on the findings from the study, it could be concluded that *M. serrata* can be used as a natural antioxidant to treat ailments where excess free radicals and oxidative stress are implicated due to the displayed antioxidant activity. The effective antioxidant activity of *M. serrata* leaf extracts may be linked to the detected phytochemicals, which are reported to possess antioxidant activity. Based on the varios antioxidant methods employed, the water extract was the most potent antioxidant. Thus, the study justifies the folkloric use of the plant in the management diseases such as asthma, diabetes and sexual dysfunction where oxidative stress is implicated by the people of South Africa. The study also confirms that the antioxidant activity of the plant is highly correlated with its high total phenolic content observed.

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CHAPTER 5

Hepatoprotective activity of *Morella serrata* leaf aqueous-ethanol extract against Carbon tetrachloride (CCl_4) - induced liver injury in Wistar rats

5.1 introduction

In the previous chapter, the *in vitro* antioxidant activity of the three crude extracts (ethanol, hydroalcohol and water) of *M. serrata* was in the order of water> ethanol> hydroalcohol. Although the finding was clinically significant, it was only able to justify the folkloric use of the plant in treating oxidative stress related diseases. This prompts a research into practical possible ability of the extracts in protecting the liver from oxidative stress. The carbon tetrachloride (CCl_4) model of hepatic injury is a widely used model (Jiang *et al*., 2004) and the effect caused by Carbon tetrachloride in the liver is comparable to that of acute viral hepatitis in the liver (Rubinstein, 1962). Therefore, carbon tetrachloride was chosen as an experimental model referee to induce damage to the liver in the present study. The animal model of *Rattus norvegicus* has extensively answered human questions from different scientific fields such as pharmacology, toxicology, nutrition, behaviour, immunology and neoplasia (Jacob, 1999). Research on rat models have extensively demonstrated related symptoms of human diseases in variety of studies (Aitman *et al*., 2008). For this reason, the species was chosen to demonstrate the injury caused by CCl_4 in the liver in the present study. A study by Handa *et al*. (1986) reported that 170 phytoconstituents extracted from 110 plants had liver protection activity. The study further revealed that an estimated 600 commercial herbal formulations worldwide which are claimed to have hepatoprotective activity are being marketed. Recent review on plants with hepatoprotective activity shows the extensiveness of the use of medicinal plants in treating liver diseases (Kumar *et al*., 2011; Jannu *et al*., 2012; Asadi-Samani *et al*., 2015). However, in the absence of scientifically valid application of these medicinal plants in liver disease, they cannot be recommended (Jain *et al*., 2013). Even in the presence of modern medicine, there is hardly any drug that can protect or restore the liver functions from oxidative damage (Roy *et al*., 2014). Therefore, it is necessary to search for herbal drugs that may be used in the management of liver diseases to replace current drugs of doubtful efficacy and safety.

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5.2 Material and methods

5.2.1 Collection, identification and authentication of plant material

Fresh leaves of *Morella serrata* were collected in February 2014 around Phuthaditjhaba area, Witsishoek (28°39'31.3"S and 28°52'47.4"E; altitude 2047 m), Free State Province, South Africa. The plant was identified, authenticated (Voucher specimen No: MbhMed/02/2014/QHB) and deposited at the University of the Free State Herbarium, Qwaqwa Campus. The leaves were washed under running tap water to remove all contaminants, shade-dried to constant weight and pulverized to fine powder using Waring Commercial Laboratory electric blender. The powdered leaves were then stored in airtight glass containers at 5°C in a refrigerator until further use.

5.2.2 Chemicals and reagents

Silymarin, carbon tetrachloride, halothane, TBA (thiobarbituric acid) and TCA (trichloroacetic acid) were products of Sigma – Aldrich. All other chemicals were of analytical grade. Distilled water was prepared in the Department of Plant Sciences, University of the Free State, Qwaqwa Campus.

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5.2.3 Preparation of aqueous-ethanol leaf extract of *M. serrata* and pre-treatments

5.2.3.1 Preparation of *M. serrata* leaf aqueous-ethanol extract

Two portions of powdered leaf sample weighing 100 g each were respectively extracted with 1 L ethanol and water in separate volumetric flasks and placed in a Labcon shaker (Laboratory Consumables, PTY, Durban, South Africa) for 24 h with agitation. The resulting infusions were filtered using Whatsman No. 1 filter paper. The ethanol extract was concentrated in a rotary evaporator (Cole Parmer SB 1100, Shanghai, China) under reduced pressure while the water extract was concentrated in a water bath at 40°C. The ethanol extract yielded 15.43 g (52.57% w/w) while the water extract yielded 13.23 g (44.13% w/w). Equal portions, 12 g each from the two extracts were mixed together and used to prepare the pre-treatment concentrations.

5.2.3.2 Pre-treatments of *M. serrata* leaf aqueous-ethanol extract

The study investigated the effect of three different dosages (100, 200 and 400 mg/kg, body weight) of *Morella serrata* leaf aqueous-ethanol extract (MSLAEE) against CCl_4 induced hepatotoxicity in Wistar rats. The standard used in the study was silymarin at 100 mg/kg body weight (b.w) while CCl_4 (1 ml/kg b.w) was used to induce hepatotoxicity in

rats. The pre-treatments of MSLAEE (100, 200 and 400 mg/kg body weight) were prepared in distilled water and serial dilution method was used to obtain respective doses.

5.2.4 Experimental design: Hepatoprotective study

5.2.4.1 Experimental animals and housing

Ten week old male and female Wistar rats, species *Rattus norvegicus* weighing 157.1 ± 20.60 g were obtained from the experimental animal facility of the University of the Free State, Bloemfontein, South Africa. The present study was carried out at the Zoology Department animal house situated at UFS, Qwaqwa campus. A total of 24 animals were used for the study. The rats were divided into 6 groups each containing 4 animals (*n=4*) and were housed sex-wise in clean metabolic cages with soft wood shaving as their bedding (**Figure 5.1**). The cages were placed in a well-ventilated house condition (temperature: 22±2 ℃; photoperiod: 12 h light and 12 h dark cycle; humidity: 40-45%). The animals were allowed free access to water (freed of contaminants) and standard pellets (Pioneer Food (Pty) Ltd, Huguenot, South Africa). Cleaning of the cages was done on a daily basis to maintain a clean and comfortable environment for the animals. Fourteen days prior to experiment, the animals were acclimatized to the animal house conditions, environment and to one another.

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Figure 5.1 Grouping and housing of rats in cages at the Zoology animal house, UFS, QwaQwa campus

5.2.4.2 Examination of animals' body weight, feed intake and water consumption during experimental study

To monitor the change in body weight, animals weights were taken at the start of the hepatoprotective study (day 1), day 7, day 14 and on day 21 (last day of experimental study). Mean body weights were calculated for each group for the overall study period (day 1-21). Body weight gain percentages for each group of animals were calculated following the method of Chapman *et al*. (1959) using the following equation:

$$\text{Body weight gain (\%)} = \frac{Final\ \ body\ \ weight - Initial\ \ body\ \ weight}{Initial\ \ body\ \ weight} \times 100 \ldots\ldots\ldots\ldots\ldots\ldots\ldots (1)$$

On daily basis, each animal group was given 200 g of feed and 200 ml of water. The feed and water consumed daily by each group was recorded.

5.2.4.3 Acute toxicity studies

A study conducted by Saheed and Ashafa (2016) on acute toxicity of *Morella serrata* found that the plant extract had no treatment-induced mortality or toxicity in rats at doses of 5-5000 mg/kg. Based on these findings, doses 100, 200 and 400 mg/kg b.w were chosen for the present study.

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5.2.4.4 Animal grouping and treatments

Hepatoprotective study was done according to a method described by Nasiruddin *et al.* (2013) with few modifications. The animals received oral administration (**Figure 5.2**) with respective treatments twice a day (Daily double dose), at 12 h interval. Treatments were in accordance with international ethical guidelines. The study was carried out following approval from the University of the Free State Ethic Committee, clearance number: UFS-AED 2015/002.

Figure 5.2 Oral administration of respective pre-treatments to rat groups

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Group I: Normal control: (Female)

The animal received distilled water (1 mg/kg) body weight (b.w.) orally for 21 days, twice a day.

Group II: Negative control: CCl_4 group (Male)

The animal received distilled water (1 ml/kg) body weight orally for 20 days, twice a day.

Group III: Positive control: Silymarin group (Male)

The animal received silymarin (1 ml/kg, 100 mg/kg b.w.) orally for 20 days, twice a day.

Group IV: Test Group A: (Female)

The animal received *Morella serrata* aqueous-ethanol leaf extract (1 ml/kg, 100 mg/kg b.w.) orally for 20 days, twice a day.

Group V: Test group B: (Female)

The animal received *Morella serrata* aqueous-ethanol leaf extract (1 ml/kg, 200 mg/kg b.w.) orally for 20 days, twice a day.

Group VI: Test group C: (Female)

The animal received *Morella serrata* aqueous-ethanol leaf extract (1 ml/kg, 400 mg/kg b.w.) orally for 20 days, twice a day.

5.2.4.5 Anaesthesia, blood collection and liver excision

After the last administration of treatments on day 20, animals were fasted for 12 h. In the morning of day 21, animals in all the groups except group I (Normal control) received a single dose of carbon tetrachloride (1 ml/kg b.w) prepared in olive oil (1:1), intraperitoneal (Bhandarkar and Khan, 2004). Immediately after the CCl_4 administration, animals were given water and food to eat. The rats were anesthetized by halothane anaesthesia 24 hr after CCl_4 administration. Anaesthesia was applied by placing the rats in a large glass jar containing cotton wool soaked with halothane. Immediately, through cardiac puncture, blood samples were withdrawn and collected in labelled tubes with/without EDTA. The animals were then immediately excised and the liver was collected, trimmed of fat and other connective tissues, washed with cold sucrose (2 M), blotted with clean paper towel and weighed for evaluation of liver-body weight ratio using the formula:

$$\text{Liver-body weight ratio} = \frac{liver\ weight}{Final\ body\ weight} \times 100 \quad \text{.........} (3)$$

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The liver was cut in to two equal portions. One half were preserved in 10% formalin for proper fixation and later used for histopathological studies and the other was used for biochemical studies.

5.2.4.6 Preparation of serum and liver homogenate

The blood samples collected were thereafter centrifuged at 3 000 rpm for 15 min. The serum supernatants were drawn using a pipette and kept in the freezer at – 80°C for biochemical studies. Liver homogenate was prepared by weighing 0.5 g of liver and homogening in 10 mL ice-cold 0.1 mol/L tris-HCl buffer (pH 7.2). This was further centrifuged at 10,000 rpm for 15 min and supernatant obtained was kept in the freezer at – 80 °C to be used for biochemical studies.

5.2.4.7 Assessment of haematological and liver function parameters

The automated analyser (Sysmex, KX-21, Japan) was used to analyse haematological parameters such as red blood count (RBC), haemoglobin (Hb), haematocrit (Hct), white blood count (WBC), neutrophils, and platelets and serum activities of alkaline phosphatase (ALP), alanine transaminase (ALT) and aspartate transaminase (AST) as markers of hepatic function.

5.2.4.8 Assay of liver homogenate antioxidant enzyme markers

Assay of Catalase (CAT) activity

The method described by Aebi (1984) was used to measure catalase activity in the liver homogenate. Briefly, 1.9 ml of 50 mM phosphate buffer (pH 7.4) was added to 1 mL of liver homogenate in a test tube. H_2O_2 (1 mL, 30 mM) was added to the mixture to initiate the reaction mixture. A mixture of 2.9 ml of phosphate buffer and 1 ml of H_2O_2 (30 mM) without liver homogenate served as a blank. The reduction in absorbance due to the decomposition of H_2O_2 was read at 240 nm against the blank using spectrophotometer. Units of catalase were expressed as the amount of enzyme that decomposes 1 µM of H_2O_2 per min at 25°C. The activity was expressed in terms of units per mg of protein.

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Determination of Thiobarbituric acid reactive species (TBARS)

Lipid peroxidation in the liver homogenate was determined by colorimetric measurement of thiobarbituric acid reactive substances (TBARS) as described by Niehaus and Samuelsson (1968). Briefly, 100 µl of liver homogenate in a test tube was treated with 2 ml thiobarbituric acid (TBA)-tricarboxylic acid (TCA)-hydrochloric acid (HCl) reagent (0.37% TBA, 15% TCA and 0.25 N HCl (1:1:1 ratio). The tube was placed in a boiling water bath for 30 min and allowed to cool. The amount of malondialdehyde formed was evaluated by taking the absorbance of the clear supernatant at 535 nm against blank using spectrophotometer. The percentage lipid inhibition was calculated using the equation:

Lipid inhibition (%) = $[(A_0-A_1)/A_0] \times 100$ where A_0 is the absorbance of the sample and A_1 is the absorbance of sample extract.

5.2.4.9 Histopathological examination of the liver

Histological analysis was done on livers fixed in 10% formalin. Liver tissues were dehydrated in graded (50-100%) alcohol, embedded in paraffin, cut onto 4-5 µm tick

sections and stained with haematoxylin-eosin for examination. Sections of stained liver tissues were evaluated for the pathological/ rejuvenative changes in the hepatocytes under light microscope and photomicrographs were taken.

5.2.5 Statistical analysis

Statistical analysis was performed using a Graph Pad Prism version 5.00 statistical package (Graph Pad software®, San Diego, CA, USA). Results were expressed as mean of four determinations ± standard error of mean (SEM) and subjected to one way analysis of variance (ANOVA) followed by Bonferroni comparison test to determine the significant difference in all parameters among different treatments. P value < 0.05 was considered statistically significant.

5.3 Results

5.3.1 Effect of CCl_4 on feed and water intake of rats

All groups (II-VI) intoxicated with CCl_4 except normal control (group I) experienced a disturbance in their normal routine of feeding. The animals experienced loss of appetite which was manifested by a decrease in both feed and water intake on day 21 as shown in **Figure 5.3** and **Figure 5.4.** The feed and water intake pattern of MSLAEE groups was more similar to that of normal control compared to silymarin and CCl_4 treatments groups which fed at a higher rate than the normal control.

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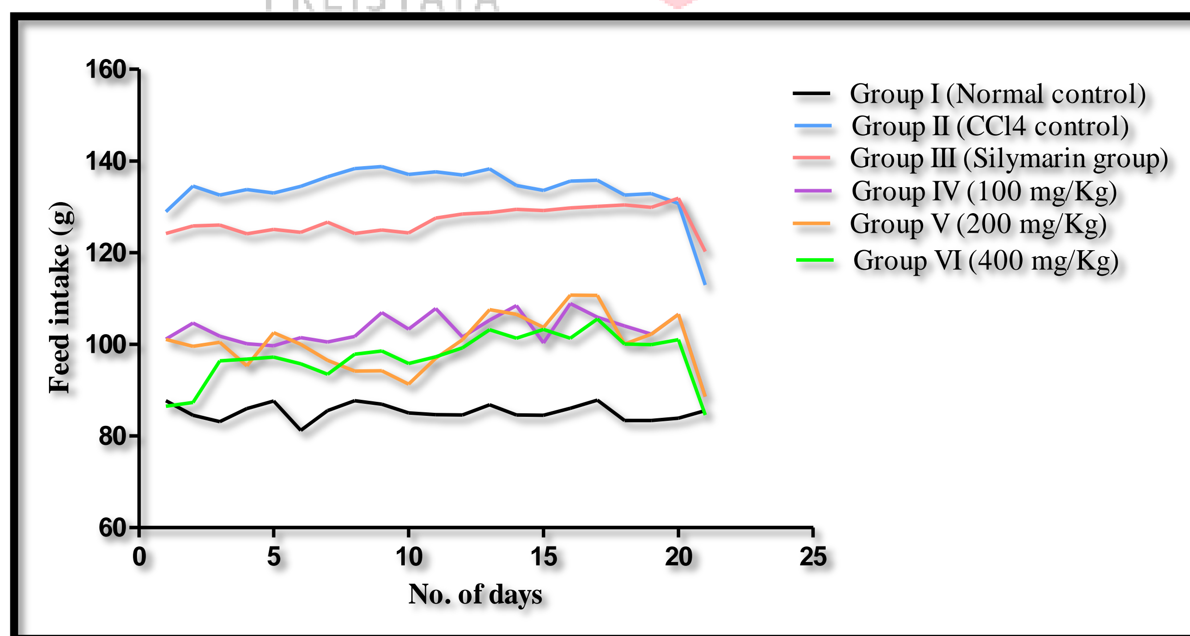

Figure 5.3 Effect of oral administration of MSLAEE on feed intake in CCl_4 intoxicated Wistar rats

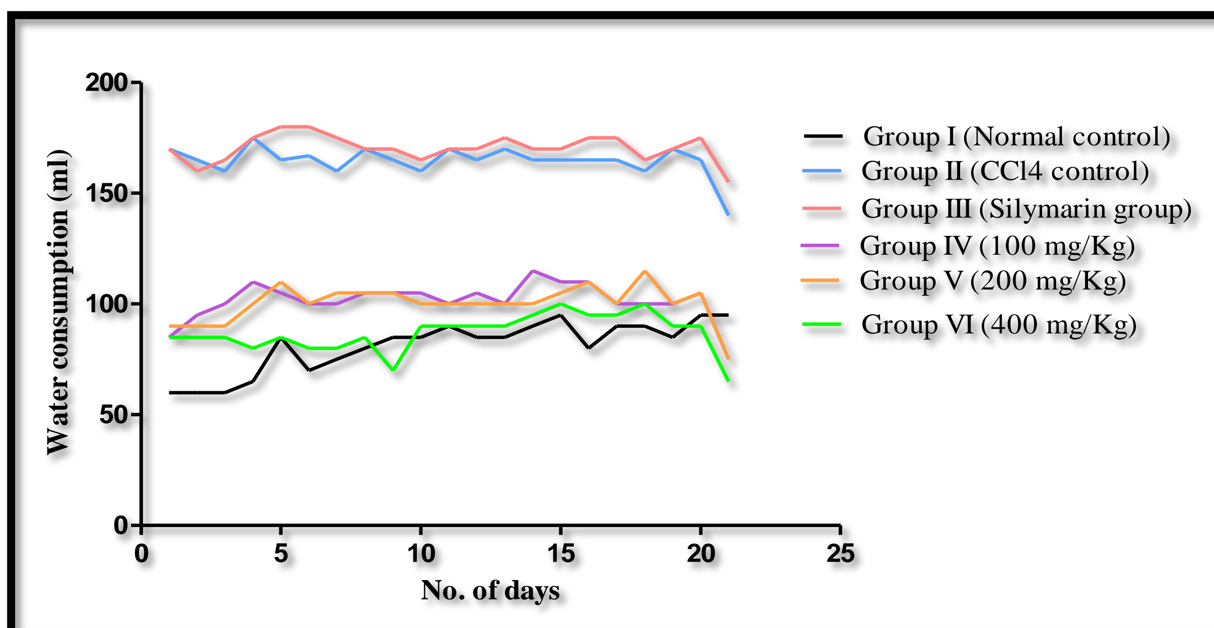

Figure 5.4 Effect of oral administration of MSLAEE on water consumption in CCl_4 intoxicated Wistar rats

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5.3.2 Effect of aqueous-ethanol extract and silymarin treatments on body weight of CCl_4-intoxicated rats

The effect of the treatments on body weight gain in normal and hepatotoxic rats are represented in **Table 5.1**. A significant reduction ($p<0.05$) in body weight was observed in the extracts treated groups after the 21 day study period. There was a significant increase in weight gain in the hepatotoxic and silymarin treated rats when compared to the normal control and MSLAEE treated groups. The weight gain in the MSLAEE pre-treated groups (100, 200 and 400 mg/kg b.w) was significantly ($p<0.05$) reduced in comparison to the normal control. Although all the animals gained weight up to day 21, there was a loss in body weight in all groups administered with CCl_4 as observed on day 22. The loss in body weight was in order of CCl_4-alone > 200 mg/kg MSLAEE> 400 mg/kg MSLAEE> 100 mg.kg MSLAEE > silymarin group with values of -1.2 g> -0.39 g> -0.36 g> -0.25 g> -0.12 g as shown in **Figure 5.5** and **Table 5.2.**

Table 5.1 Effect of MSLAEE, silymarin and CCl_4 on body weight of Wistar rats

Treatment groups	Body weight (g) Initial (Day 1)	Final (Day 22)	Weight difference	Percentage weight gain (%)
Normal control	127.40±1.25	172.10±0.04	44.70±0.04[a]	35.09±0.01[a]
CCl_4 group	182.40±8.60	316.60±0.02	134.20±0.04[b]	73.57±0.01[b]
Silymarin group	178.80±9.52	285.40±0.04	106.60±0.11[c]	59.62±0.01[c]
100 mg/kg MSLAEE	155.00±10.87	187.20±0.08	32.20±0.11[d]	20.77±0.01[d]
200 mg/kg MSLAEE	151.40±4.51	198.60±0.02	47.20±0.11[e]	31.18±0.01[e]
400 mg/kg MSLAEE	147.50±1.64	196.90±0.01	49.40±0.04[f]	33.49±0.01[f]

Values are presented as mean ± SEM (*n=4*).
Means sharing the same letter superscript in the same column are not significantly different from each other ($p>0.05$)

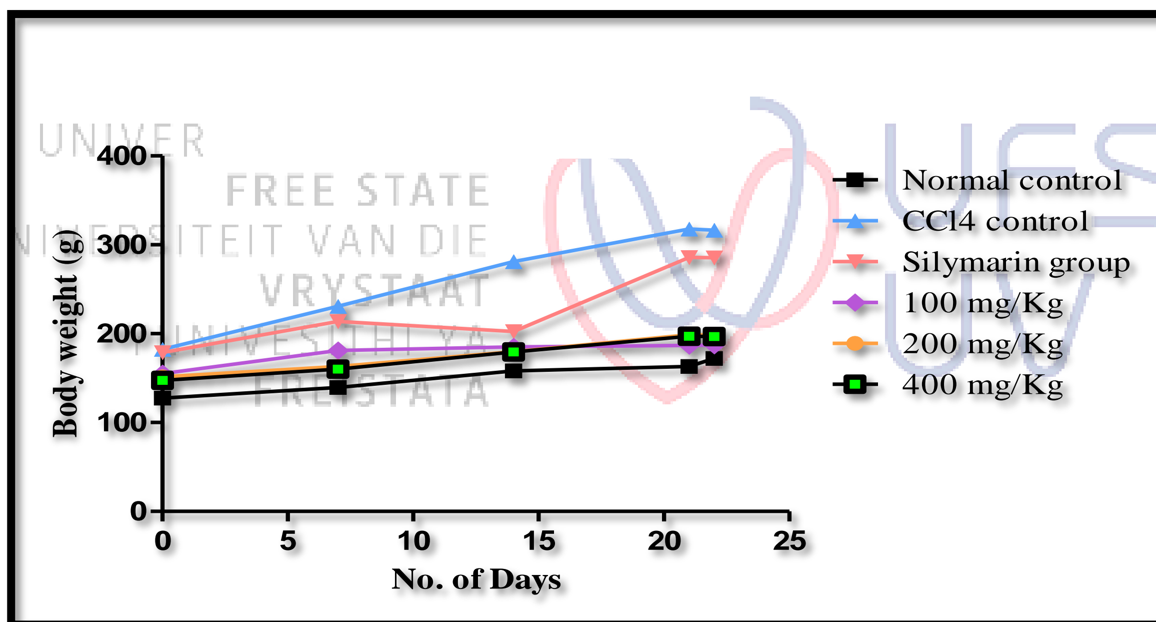

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Figure 5.5 Effect of MSLAEE, silymarin and CCl_4 on body weight of Wistar rats

Table 5.2 Body weight difference of Wistar rats on day 7, 14, 21 and 22 of experimental study

No. of Days	Body weight (g)					
	Normal control	CCl_4 control	Silymarin group	100 mg/kg MSLAEE	200 mg/kg MSLAEE	400 mg/kg MSLAEE
0	127.40	182.40	178.80	155.00	151.40	147.50
7	139.39	230.94	213.93	181.03	163.00	160.00
14	158.00	281.00	202.66	185.29	179.90	179.50
21	163.16	317.80	285.78	186.95	198.99	197.26
22	172.10	316.60	285.40	187.20	198.60	196.90
Weight gain/loss (g) between day 21-22	**+ 8.94**	**-1.2**	**-0.12**	**-0.25**	**-0.39**	**-0.36**

-: Weight loss; +: weight gain

5.3.3 Effect of MSLAEE on liver and liver- body weight in CCl_4 intoxicated in Wistar rats.

Liver and liver-body weight results are shown in **Table 5.3**. Liver weight of normal control was significantly ($p<0.05$) lower than CCl_4 intoxicated groups. Hepatotoxic control (group II) liver weight was significantly ($p<0.05$) higher than MSLAEE and silymarin pre-treated groups. Twenty-four hours after CCL_4 administration, a significant ($p<0.05$) increase in liver-body weight ratio was observed in CCl_4-alone treated group. This increase was also seen in rats treated with 100 mg/kg MSLAEE. This was however reduced following pre-trement with Silymarin, 200 and 400 mg/kg MSLAEE.

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Table 5.3 Effect of oral administration of MSLAEE on liver and liver-body weight of Wistar rats treated for 21 days

Parameter	Normal control	CCL_4 group	Silymarin group	MSLAEE (mg/kg body weight)		
				100	200	400
Liver	6.12 ± 0.22^{a}	11.87 ± 0.13^{b}	8.75 ± 0.93^{b}	7.78 ± 0.31^{a}	7.35 ± 0.41^{a}	7.32 ± 0.10^{a}
Liver-body weight (%)	3.56 ± 0.01^{a}	3.75 ± 0.01^{b}	3.07 ± 0.01^{c}	4.16 ± 0.01^{d}	3.70 ± 0.01^{e}	3.72 ± 0.02^{e}

Values are presented as mean ± SEM (*n=4*)
Means sharing the same letter superscript in the same row are not significantly different from each other ($p > 0.05$)
MSLAEE: *Morella serrata* leaf aqueous-ethanol extract

5.3.4 Effect of MSLAEE on haematological parameters in CCL_4-treated rats

Table 5.4 shows some of haematological parameters such as red blood cells (RBC), haemoglobin (Hb), haematocrit (Hct), white blood cells (WBC) and neutrophils (N). A significant ($p<0.05$) decrease in CCl_4-alone treated group was observed in the RBC content when compared to normal control. Silymarin and MSLAEE (400 mg/kg) pre-treated groups showed a marked recovery in RBS content that is not significant ($p>0.05$) different from that of a normal control. The haemoglobin content of treatment groups was significantly ($p<0.05$) higher from the normal control. The CCl_4-alone treated group showed a significant ($p<0.05$) decrease in haemoglobin content when compared to normal control group. This decrease was however elevated in silymarin and MSLAEE pre-treated groups. The Hct content of CCl_4-alone treated group was significantly ($p<0.05$) lower when compared to normal control whilst the silymarin and MSLAEE pre-treated groups indicated an increase in Hct content when compared to CCl_4-alone treated group. The WBC and N content of CCl_4-alone treated group increased significantly ($p<0.05$) when compared to that of normal control. This increase was however ameliorated in silymarin and MSLAEE pre-treated groups. The platelets count of all treatment groups were significantly ($p<0.05$) different from each other and to that of normal control. A significant ($p<0.05$) decreased in platelets count in CCl_4-alone treated group was observed when compared to normal control. This decrease was however ameliorated in MSLAEE and silymarin pre-treated groups. Better amelioration of platelets count was seen in silymarin, followed by MSLAEE doses 100 mg/kg and 400 mg/kg whilst least amelioration of platelets was seen on MSLAEE dose 200 mg/kg.

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Table 5.4 Effect of daily double dose of MSLAEE on haematological profile in CCl_4-induced hepatotoxicity in Wistar rats

	RBC ($x10^{12}$/L)	Hb (g/dL)	Hct (L/L)	WBC ($x10^9$/L)	N ($x10^9$/L)	P ($x10^9$/L)
Normal control	8.62±0.01[a]	18.00±0.01[a]	0.62±0.01[a]	6.90±0.01[a]	0.33±0.01[a]	1405±1.08[a]
CCl_4 group	7.63±0.02[b]	16.40±0.01[b]	0.51±0.01[b]	10.20±0.01[b]	2.25±0.01[b]	865±1.08[b]
Silymarin group	8.62±0.01[a]	17.20±0.01[c]	0.60±0.01[ac]	8.80±0.01[c]	0.66±0.01[c]	1345±0.41[c]
100 mg/kg MSLAEE	8.49±0.01[c]	17.30±0.02[d]	0.58±0.01[ac]	6.20±0.01[d]	1.71±0.01[d]	1159±1.08[d]
200 mg/kg MSLAEE	7.88±0.01[d]	16.50±0.01[e]	0.56±0.01[c]	5.60±0.01[e]	1.83±0.01[e]	897±0.82[e]
400 mg/kg MSLAEE	8.58±0.01[a]	16.90±0.01[f]	0.56±0.01[c]	8.50±0.01[f]	1.80±0.01[e]	1016±0.71[f]

Values are presented as mean ± SEM (*n=4*)
Means sharing the same letter superscript in the same column are not significantly different from each other ($p > 0.05$)
RBC= Red blood cell, haemoglobin= Hb, haematocrit= Hct, WBC=white blood cell, neutrophils=N, platelets=P, MSLAEE= *Morella serrata* leaf aqueous-ethanol extract

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5.3.5 Effect of MSLAEE on liver serum biochemical parameters in CCl_4-treated rats

The damage on the plasma membrane caused by CCl_4 was assessed by monitoring the levels of ALP, ALT and AST in the serum. **Figure 5.6** shows the levels of ALP, ALT and AST in hepatotoxic rats and those treated with MSLAEE or silymarin followed by intraperitoneal administration of CCl_4 as compared to normal control. Rats treated with CCl_4-alone demonstrated a significant ($p<0.05$) increase in ALP activity when compared to normal control. Rats treated with MSLAEE at doses 100 and 200 mg/kg b.w, also indicated ALP levels which are significantly ($p<0.05$) higher than the normal control and CCl_4-alone treated groups. Only MSLAEE at dose 400 mg/kg and silymarin were able to ameliorate the effect of CCl_4 on ALP as both treatments displayed ALP activity lower than that of CCl_4-alone treated group. The activity of ALP in MSLAEE pre-treated groups was dose dependent. The lower the dose, the higher the ALP activity observed.

CCl_4-intoxicated rats exhibited a significant ($p<0.05$) increase in ALT activity when compared to normal control. Silymarin was able to significantly ($p<0.05$) reduce the activity towards that of a normal control. MSLAEE at doses 100 and 200 mg/kg b.w showed a significant ($p<0.05$) reduction in ALT activity when compared to normal content while MSLAEE at dose 400 mg/kg b.w presented activity which is comparable to CCl_4-alone treated group and MSLAEE at dose 100 mg/kg. The activity of ALT in MSLAEE pre-treated groups was dose dependent. The lower the dose, the lower the ALT activity observed.

There was no significant difference in AST activity in hepatotoxic rats when compared to normal control. However, the elevated AST activity caused by exposure to CCl_4 was significantly ($p<0.05$) reduced by MSLAEE at doses 100 and 200 mg/kg b.w. but not for MSLAEE at 400 mg/kg b.w. Silymarin on the other hand also significantly (<0.05), prevented the rise of AST activity caused by CCl_4 intoxication. The activity of AST in MSLAEE treated groups was dose dependent. The lower the dose, the lower the AST level observed.

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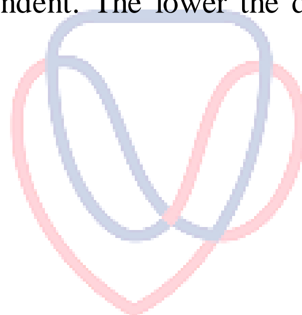

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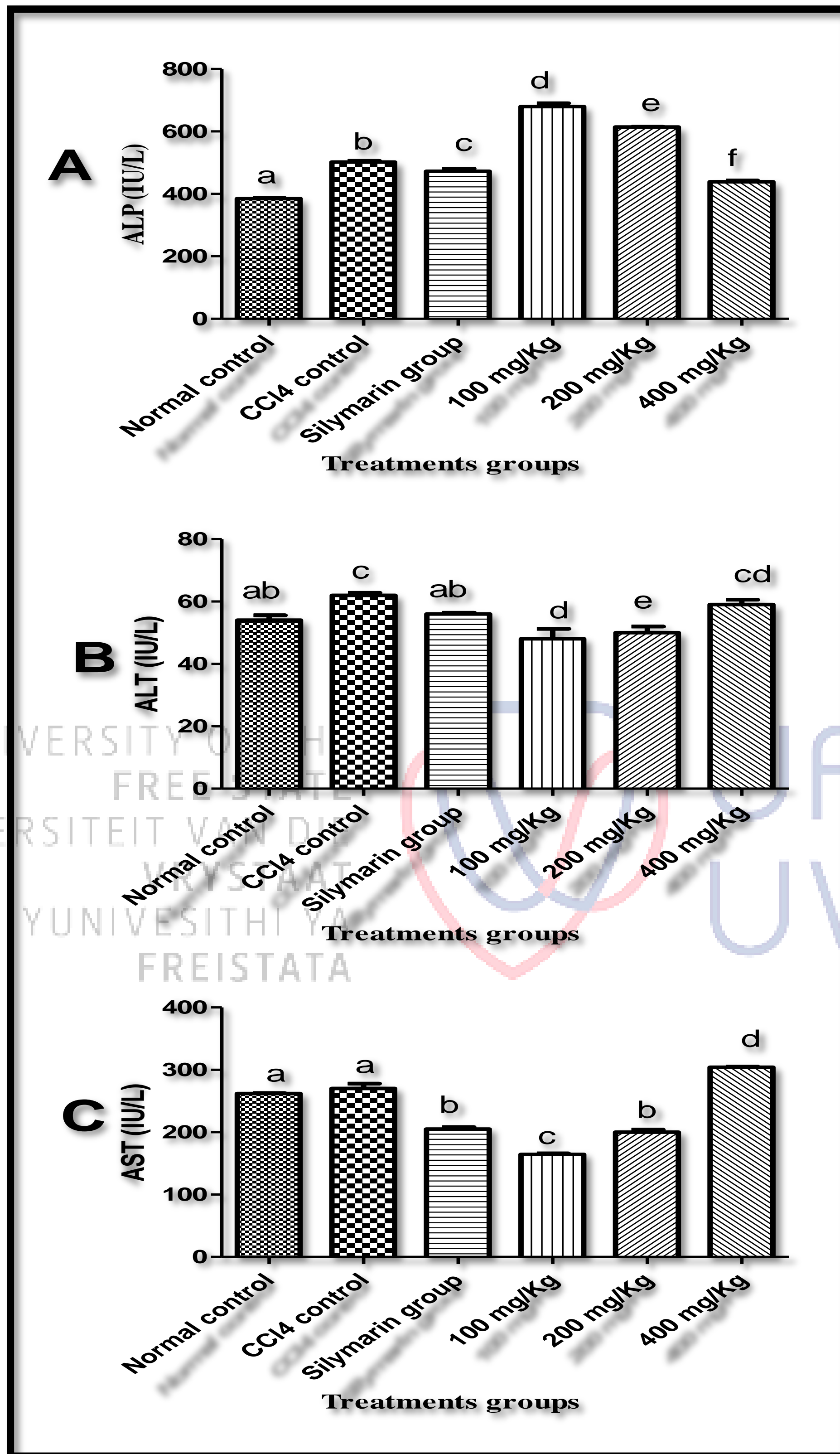

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Figure 5.6 Effects of pre-treatment with MSLAEE on liver serum marker enzymes (A) ALP, (B) ALT and (C) AST in Wistar rats intoxicated with CCl_4. Values are presented as mean ± SEM (*n=4*). Means sharing the same letter are not significantly different from each other ($p > 0.05$)

5.3.7 Effect of MSLAEE on antioxidant marker enzymes in CCl_4-intoxicated rats

The effect of CCl_4 on Wistar rats was assessed on *in vivo* antioxidant indices namely CAT and TBARS as shown in **Figure 5.7.** Administration of CCl_4 in pre-treated rats resulted in a significant ($p<0.05$) reduction in CAT activity of hepatotoxic rats when compared to that of normal control. The observed reduced CAT activity following CCl_4 intoxication was however elevated by silymarin and MSLAEE treatment in a dose dependent manner. CAT activity increased with an increase in MSLAEE dose as shown in **Figure 5.7 (A)**.

A significant ($p<0.05$) increase in TBARS concentration was observed in rats intoxicated with CCl_4 when compared to normal control. Pre-treatments with silymarin or MSLAEE significantly ($p<0.05$) reduced the elevated TBARS level caused by intoxication of CCl_4. Lowest TBARS level was observed in silymarin treatment treated group, followed by 100, 200 and 400 mg/kg MSLAEE treatments in that order as shown in **Figure 5.7 (B)**.

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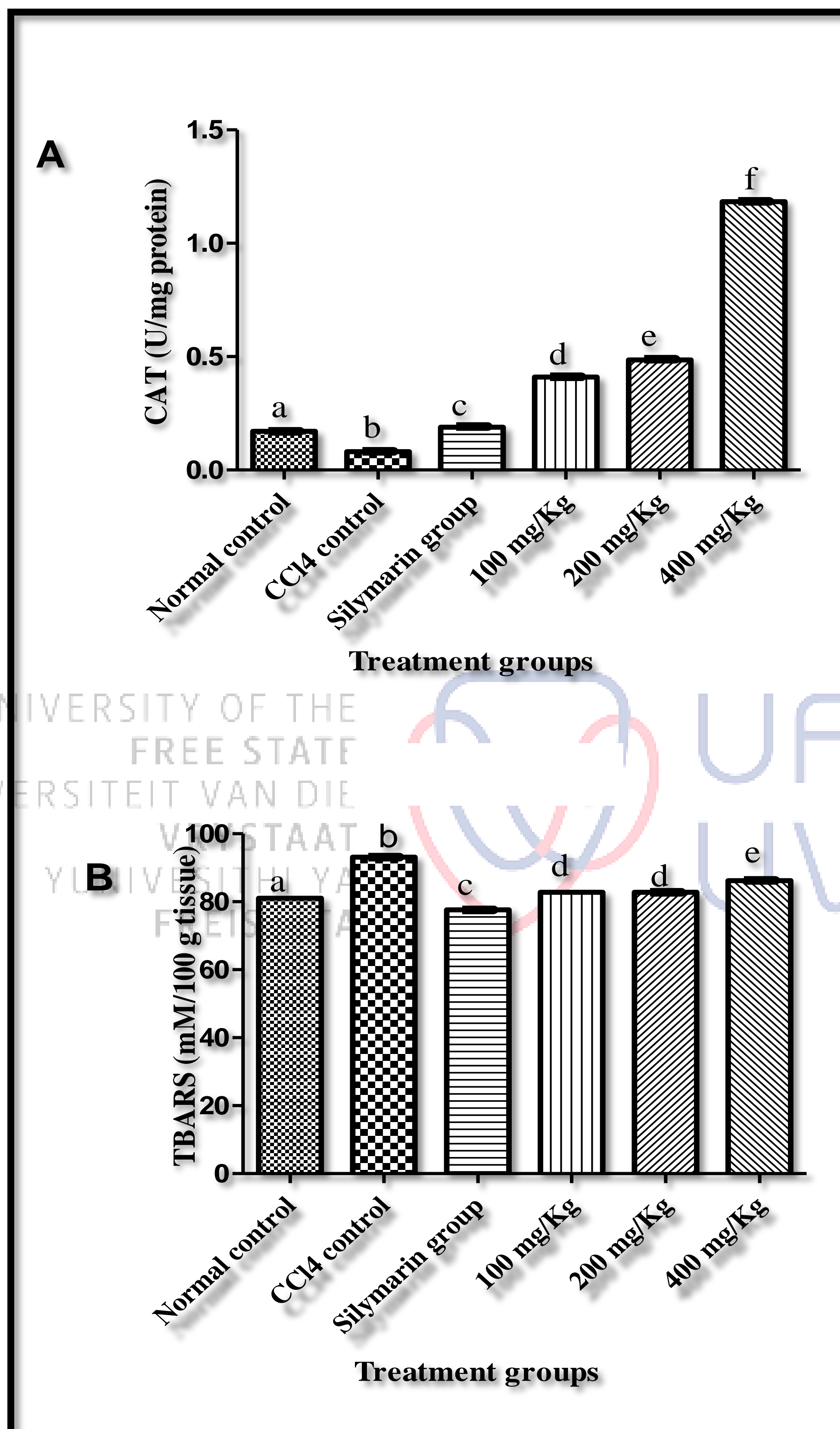

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Figure 5.7 Effect of silymarin and MSLAEE on (A) CAT activity and (B) TBARS concentration in CCl_4 intoxicated rats. Values are presented as mean ± SEM (*n=4*). Means sharing the same letter are not significantly different from each other ($p > 0.05$)

5.3.8 Histological findings

The hepatoprotective effect of MSLAEE was further confirmed by histological examination of the liver (**Figure 5.8**).

Group I: Normal control rats

Liver sections of normal control rats showed liver parenchyma with intact structure. The hepatocytes appeared normal as shown in **Figure 5.8 (A)**.

Group II: CCl_4-intoxicated untreated rats

Liver sections of CCl_4 intoxicated untreated rats displayed abnormal parenchyma cells. Most of the hepatocytes showed severe necrosis displayed by scattered mononuclear inflammation, cytoplasm degeneration and karryolysis as shown in **Figure 5.8 (B)**.

Group III: Silymarin pre-treated rats

Liver sections of silymarin pre-treated rats showed liver parenchyma with partially abnormal structure. Mild inflammations of hepatocytes were observed as shown in **Figure 5.8 (C)**.

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Group IV: 100 mg/kg b.w MSLAEE

Liver sections of rats pre-treated with 100 mg/kg b.w of MSLAEE showed partially abnormal structure characterised by mild inflammation of hepatocytes and mild cytoplasmic degeneration as shown in **Figure 5.8 (D)**.

Group V: 200 mg/kg b.w MSLAEE

Liver sections of rats pre-treated with 200 mg/kg b.w of MSLAEE showed severe necrosis with nuclear karryolysis and cytoplasmic degeneration. However, moderate vacoular degeneration was present **Figure 5.8 (E)**.

Group VI: 400 mg/kg b.w MSLAEE

Liver sections of rats pre-treated with 400 mg/kg b.w of MSLAEE displayed partial abnormal hepatocytes. This was characterised by the presence of moderate inflammation with intra-haemorrhage and mild vacoular degeneration. Nuclear integrity was intact **Figure 5.8 (F)**.

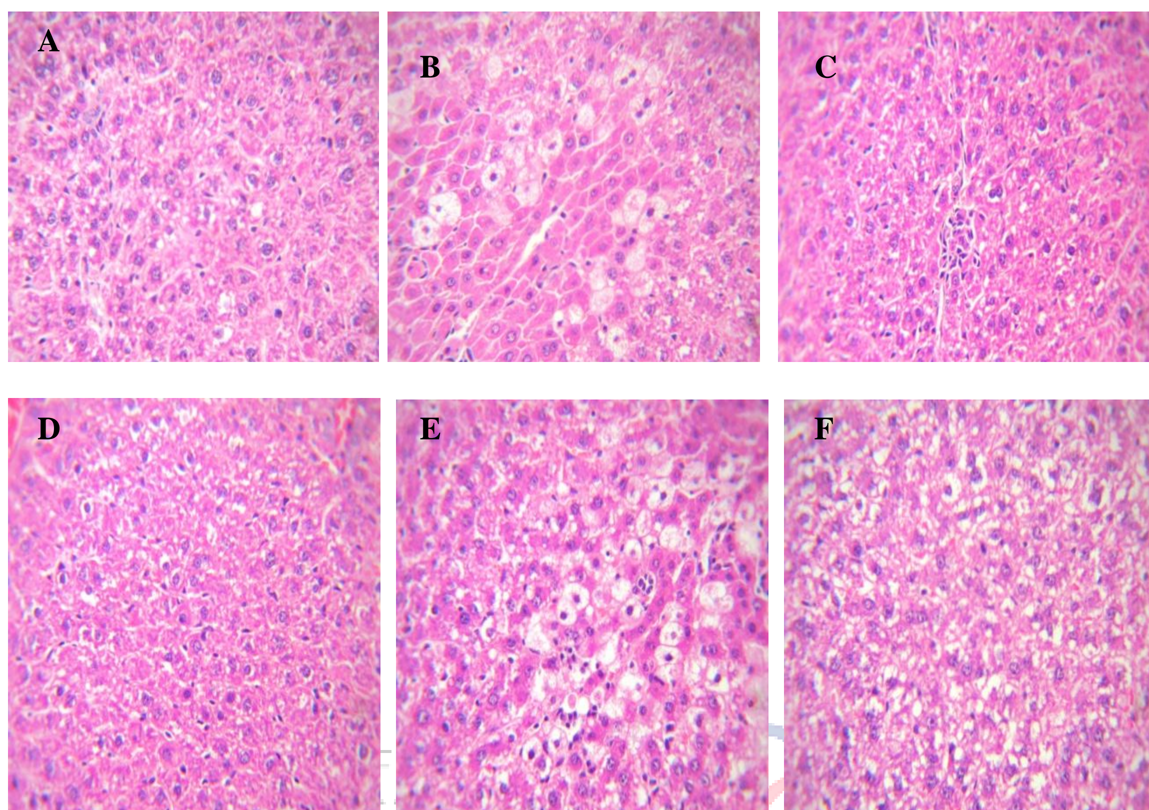

Figure 5.8 Histopathological micrographs of the normal and CCl_4 intoxicated animals (Haematoxylin and eosin staining, x400). (A) Liver section of normal control; (B) Liver section of CCl_4-alone treated rats; (C) Liver section of CCl_4-treated group pre-dosed with silymarin (100 mg/kg b.w); (D) Liver section of CCl_4-treated rats pre-dosed with 100 mg/kg b.w MSLAEE; (E) Liver section of CCl_4-treated rats pre-dosed with 200 mg/kg b.w MSLAEE; (F) Liver section of CCl_4-treated rats pre-dosed with 400 mg/kg b.w MSLAEE

5.4 Discussion

The present study was designed to assess the protective effect of MSLAEE at doses 100, 200 and 400 mg/kg b.w upon CCl_4-induced liver injury in Wistar rats by investigating feed and water intake, body and relative liver weight, haematological parameters (RBC, WBC, Hb, Hct, Neutrophils and platelets), serum antioxidant marker enzymes (ALP, ALT and AST) and liver histology. Pre-treatments of rats with MSLAEE before intoxication with CCl_4 was aimed at preparing the hepatocytes and their contents for the CCl_4- mediated oxidative stress.

A first worthy statement in the present study is that none of the animals died naturally nor showed abnormal physical appearances due to intake of extracts during the experimental period. This is an indication that the extract did not have any negative effect on the animals. Anorexia is a consequence of liver inflammation (Robin *et al*., 2012) which results in loss of weight due to a disturbance in carbohydrates, protein or fat metabolism functions of the liver (Klaassen *et al*., 2001). Dubey and Mehta (2014) further defined anorexia as a kind of liver diseases. All groups of rats intoxicated with CCl_4 experienced a loss of appetite which was manifested by a deviation from the animals' normal routine in water and feed intake. This work is in agreement with work carried out by Balogun and Ashafa (2016) which reported that CCl_4 intoxicated groups of rats displayed a loss of appetite after CCl_4 administration. Anorexia resulted in a decrease in body weight of these groups of animals. From the present study, it is thus clear that CCl_4 did indeed induce hepatotoxicity in rats treated with CCl_4.

Assessment of organ weight is an accepted way of evaluating the toxic effect of drugs in a system (Pfeiffer, 1968). According to Uemitsu *et al*. (1986), liver intoxicated with CCl_4 is expected to enlarge and increase in weight. This is due to a disturbance in normal functioning of the liver which then leads to hepatocytes cytoplasm retaining water which eventually leads to enlargement (Blanco *et al*., 1990). Accordingly, a significant increase in liver weight of CCl_4-alone treated group was observed in the present study. The results are in line with findings from various reports (Ohta *et al*., 2000, Osman *et al*., 2011; Adu-Nti, 2011) which found an increase in liver weight of rats intoxicated with CCl_4. Another possible explanation of increased liver weight by Dadzeasah (2012) was that CCl_4 intoxication results in cell membrane damage which then allows fatty acids and glycerols into the hepatocytes thus, leading to a significant increase in liver weight. This is an indication of failure of the liver to detoxify toxins and waste products. Pre-treatments of rats with MSLAEE at doses 100, 200 and 400 mg/kg b.w reduced the weight of liver caused by intoxication with CCl_4 towards normal control better than silymarin.

A loss in body weight in CCl_4 intoxicated rats was observed in the present study. Usually, CCl_4 treatment is expected to present a reduced percentage weight gain as compared to normal control due to the toxic nature of CCl_4 (Kanter *et al*., 2015). However, in the present study instead of less percentage weight gain, the CCl_4-alone treated rats' percentage weight exceeded that of normal control. Similar findings were observed by

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Bukhsh *el al*. (2014) and Al-Seeni *et al*. (2016) where a net gain instead of net loss was observed in the hepatic group. Bukhsh *et al*. (2014) reported that a possible mechanism for this is that CCl_4 was not given enough time to affect the system metabolism. There are two possible explanations why in the present study a net gain was seen. Firstly CCl_4 was not given enough time as it was administered on the second to the last day (21st day) of the study and animals were sacrificed 24 hours after CCl_4 administration. It is insignificant to measure the overall percentage weight loss in the prophylactic study as the CCl_4 is only intoxicated on day 21. Thus, the weight loss can only be seen between the day of CCl_4 administration (day 21) and the day of sacrifice (day 22). Another explanation may be that CCl_4-alone treated group and silymarin group were male rats and throughout the experiment these two male groups were on top of the graph in terms of daily feed and water intake. Thus, making the loss caused by CCl_4 on the last day impossible or slightly seen. The increase in liver to body weight ratio may be due to a loss in body weight caused by administration of CCl_4 (Mahli *et al*., 2015) in CCl_4 intoxicated rats. Accordingly, a significant increase in liver to body weight ratio was observed in untreated hepatotoxic rats in the present study.

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Haematology is the study of morphology and physiology of blood which involves inspecting the blood and its related disorders (Yilmaz, 2015). Waugh and Grant (2001) defined haematological parameters as those parameters that are related to blood and blood-forming organs. The assessment of haematological parameters such as RBC, Hb, Hct, WBC, Neutrophils and platelets in the present study is regarded as a practical diagnostic tool to assess physiological status (Metin *et al*., 2008; Yasini *et al*., 2012). RBC, WBC and platelets are constituents contained in blood (Owolabi *et al*, 2013). Red blood cells main function is to transport haemoglobin which in turn supplies oxygen from the lungs to the tissues (Mayne *et al*., 1994; Etim *et al*., 2014). Lutz *et al*. (1975) concluded that supply of oxygen in the liver was dependent on blood flow. The significant reduction observed in RBC of CCl_4-alone treated rats is an indication of poor transportation of haemoglobin which then results in reduction in oxygen supply in the animals' body. Similarly, the findings of the present study are parallel to those obtained by Mandal *et al*. (1998). Thus, this explains the dysfunctional of liver in rats treated with CCl_4-alone. A low content of haematocrit, red blood cells and haemoglobin in blood is an indication of anaemia (Howard and Hamilton, 2013). Anaemia is a result of destruction in blood cells. According to Poli (1993), failure of the immune system is a result of excess

free radicals in the system. White blood cells mainly fight against infections and protect the body against foreign particles. In the presence of a foreign invader, white blood cells distribute antibodies to boost the immune system (Etim *et al*., 2014). A study by Bamishaiye *et al*. (2009) further confirms that the elevation in white blood cells and neutrophils in blood is due to anti-infection properties of the extract. Accordingly, in the present study, in the normal control, silymarin and MSLAEE pre-treated groups a high content of WBC and neutrophils was observed. A measure in these two parameters thus highlights the protective and boosting effect of the extract in question in antigens of the immune system. Free radicals alter the blood constituents due to oxidative stress. Francis *et al*. (2014) study observed that Malaria-infected patients had reduced platelets count than normal uninfected patients which was attributed to immune response. Similarly, the present study observed that CCl_4 intoxicated rats platelets count was lower than that of normal control. This highlights that CCl_4 mediated free radicals to activate the immune system in rats. Thus high platelets count in normal control indicated a normal immune system without the presence of a suspected foreign particle whilst lower platelets count indicated infected immune system.

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Damage to the cells, cell membrane and dysfunction of its content is an indication of lipid peroxidation (Dianzani, 1987). ALP, ALT and AST are liver marker enzyme located in the cell cytoplasm (Lin and Huang, 2000) in higher concentrations (Bairwa *et al*., 2010). Upon cytoplasm damage, the enzymes are leaked into circulation (Zimmerman and Seeff, 1970; Paduraru *et al*., 1996; Cullen, 2005) leading to cellular necimmune sysrosis which is a diagnostic measure of damage to the liver (Alexander and Griffiths, 1993; Rajkapoor *et al*., 2008). ALT is the most sensitive of them all (Bairwa *et al*., 2010) because it is more specific to the liver, thus can be used as parameter to measure liver damage (Rajkapoor *et al*., 2008). Based on these facts, these enzymes are sensitive indicators of liver injury (Molander *et al*., 1955; Ozer *et al*., 2008) and their quantification is a significant tool to identify the level and type of hepatocellular damage (Mitra *et al*., 1998). CCl_4 acute exposure to the liver can cause liver damage (Singh and Rao, 2008). A single intraperitoneal administration of CCl_4 caused a significant liver damage as evidenced by altered biochemical parameters in the present study. Elevated serum levels of these marker enzymes as well as alteration of several liver parameters may indicate liver or kidney disease (Sharpe *et al*., 1996). Accordingly, the elevation of these enzymes in the serum of CCl_4 treated rats corresponds to the extensive liver damage. Similar trends

in CCl_4 intoxicated rats were observed by various investigators (Saba *et al*., 2010; Adewale *et al*., 2014; Rajalingam *et al*., 2016). These authors reported that rats treated with CCl_4 displayed an elevation in these marker enzymes which indicated damage to the hepatocytes. This elevation in serum marker enzymes is due to loss and functional failure of antioxidant system to neutralise free radicals, a decrease in antioxidant defence and an increase in free radicals thus more damage to the liver.

According to Adewale *et al*. (2014), serum levels of ALT and AST return to normal when hepatic cells are repaired and regenerate from the damaged caused by the toxicant CCl_4. This is an indication of stabilization of plasma membrane as well as repair of hepatic tissue damage caused by CCl_4 (Ranawat *et al*., 2010). MSLAEE pre-treatments attenuated the levels of ALT, ALP and AST which were elevated by CCl_4 intoxication. This is an indication of the extracts ability to prevent the damage caused by CCl_4 in the liver. The extracts may have attenuated the oxidative stress and suppressed inflammation caused by CCl_4 intoxication. Work by various investigators (Ozturk *et al*., 2012; Adewale *et al*., 2014; Saba *et al*., 2010) observed that a decrease in ALT, ALP and AST indicated a prevention or restoration of CCl_4-induced damage to the liver by the test extracts. The reduction in elevated liver marker enzymes in MSLAEE pre-treated rats observed in the present study might probably be due to the presence of some phytochemicals in the extract such as flavonoids which possesses stronger antioxidant activity. Roy *et al*. (2014) concluded that hepatoprotective activity in extracts may be linked to the presence of flavonoids which have the ability to decrease the hepatic marker enzymes ALT, ALP and AST in the serum.

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Lipid peroxidation is the focal point of oxidative damage to cell structure and cell death during the process of toxicity (Repetto *et al*., 2012) and TBARS is the consequence of elevated free radical production from CCl_4 damage (Recknagel *et al*., 1989). CAT is an enzyme responsible for decomposing hydrogen peroxide and protects the liver tissues from highly reactive hydroxyl radicals (Chance and Greenstein, 1952). From the findings of the present study, it was observed that TBARS level in rats group intoxicated with CCl_4-alone was elevated which is an indication of production of free radicals. This remarkable increase in TBARS levels and a decrease in CAT activity is in confirmation with previous reports on the hepatotoxicity of CCl_4 (Loki and Rajamohan, 2003; Adewale *et al*., 2014). These authors reported that CCl_4 intoxicated rats have increased TBARS

and reduced CAT activity as a result of stress due to free radicals. Reduction in CAT activity thus explains the observed damage caused by CCl_4 in. Reduction in CAT activity disables or reduces its role to quench free radicals which then leads to accumulation of excess free radicals leading to oxidative stress (Rajalingam *et al*., 2016). This was however ameliorated by pre-treatments with MSLAEE. MSLAEE pre-treated rats displayed a significant reduction and elevation in TBARS levels and CAT activity respectively which is indicative of reduced oxidative stress caused by CCl_4 in the liver and a boost in antioxidant capacity. This also indicates that the extracts of *M. serrata* leave have the ability to restore the enzymes towards normalization in CCl_4-damaged liver. These findings are in line with previous findings of Adewole *et al*. (2007) and Sahreen *et al*. (2015). The observed action of MSLAEE pre-treatment may also be due to the presence of some detected phytochemicals including flavonoids. According to Asadi-Samani *et al*. (2015), flavonoids help elevate the activity of CAT.

Hepatocytes are the main components of the liver responsible for performing major processes taking place in the liver. Thus, they are a focal point of attack by free radicals and reactive oxygen species. According to Inoue (1994), enzymatic and non-enzymatic antioxidants provide a well-organized and competent way of functioning for the liver. Damage to the liver is indicated by malfunctioning of liver cells, tissues, structure and its functions (Maher, 1997). A fatty liver, cirrhosis and necrosis are the most remarkable pathological characteristics of CCl_4 induced hepatotoxicity (Khurelbat *et al*., 2014). Accordingly, CCl_4 intoxicated rats exhibited various histological changes in the liver, ranging from cell necrosis, inflammation of the cells to cytoplasm degeneration and karrolysis. Similar characteristics have been reported in previous studies (Stachura *et al*., 1981; Uskoković-Marković *et al*., 2007; Adu-Nti, 2011; Balogun and Ashafa, 2016). A biological agent with hepatoprotective effect protects and restores liver damage caused by toxins (Lin *et al*., 2008; Adesanoye and Farombi, 2010; Adewale *et al*., 2014). A marked recovery from necrosis was observed in MSLAEE and silymarin pre-treated animal. Histology observations of liver sections from MSLAEE pre-treated rats showed a marked recovery from the injury caused by CCl_4 in the liver. This was evidenced by a reduction in inflammation, necrosis and cytoplasm degeneration of the liver structure. Observed recovery from these groups, display the protective effect of *M. serrata* extracts which may be explained by the extracts ability to decrease the production of reactive oxygen species (Pérez-Garcia, 2000). The observed protection displayed by MSLAEE treatment

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may be due to free radical scavenging activity of the detected phytochemicals in *M. serrata* leaves. It has been found that ethanol and water extract of *Morella serrata* leaves contain tannins, saponins, flavonoids, steroids, terpenoids, phenols and resins, thus confirming the ability of MSLAEE in prevention or reducing the impact caused by CCl_4 in the liver. Earlier investigators (Krishnamurthy *et al*., 2010; Huang *et a*l., 2012; Yahya *et al*., 2013; Tong *et al*., 2015) have reported hepatoprotective activity of these phytochemicals which were also detected in *M. serrata* leaves.

5.5 Conclusions

The present study clearly demonstrated that extract of *M. serrata* leave at different doses have excellent hepatoprotective activity. The extract further exhibited ability to boost and protect the overall antioxidant system of the body cells against oxidative stress caused by CCl_4. These findings show that this plant could substitute expensive synthetic antioxidants available in the market which are out of reach of the poor.

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CHAPTER 6

Summary and further recommendation for research

6.1 Summary

This study reports on the phytochemical screening, quantitative analysis and *in vitro* antioxidant activity of *M serrata* leaves in order to explain its folkloric use by the South African communities to treat oxidative stress related conditions such as asthma, diabetes and male sexual dysfunction.

- Equal portions (30 g) of powdered leaf material of *M. serrata* plant were extracted with three different extracts namely, ethanol hydroalcohol and water ending up with three different crude extracts. The use of different extractants during the study resulted in a variation in the extraction yield of extracts where the highest percentage yield was observed in the hydroalcohol extract followed by ethanol and water extracts respectively.

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- During the phytochemical screening of the extracts, variations of phytochemicals were detected in the three extracts of *M. serrata* leaves. The detected phytochemicals in *M. serrata* leaves include tannins, saponins, flavonoids, steroids, terpenoids, phenols and resin. The phytochemicals detected in this study have a wide range of biological activities, one of which is antioxidant activity which is found to help with treating oxidative stress related conditions caused by excess free radicals in the system as reported in the present study.

- Antioxidant compounds such as phenolics, flavonoids and flavonols were quantified in each of the extracts of *M. serrata*. During the study, it was observed that ethanol extract of *M. serrata* leaves gave highest phenolic content, whilst water extract expressed highest content of both flavonoids and flavonols. The hydroalcohol extract was observed to have the lowest phenolic content as compared to other antioxidant compounds.

- The antioxidant capacity of the three extracts of *M. serrata* leaves was tested against a range of well-known *in vitro* antioxidant assays and compared to that of

a standard ascorbic acid (Vitamin C). The water extract showed maximum antioxidant activity in the ABTS, hydroxyl radical, hydrogen peroxide and metal chelating activity assays compared to other extracts. This was followed by the ethanol extract which showed good antioxidant activity in both the DPPH and nitric oxide scavenging assays, whilst the hydroalcohol extract was only active in the reducing power capacity assay. The antioxidant activity displayed by *M. serrata* leaves extracts in the study supports the continuous traditional use of this plant by the South African communities to treat asthma, diabetes and male sexual dysfunction, thus highlighting the potential use of this plant in developing a novel drug against such oxidative stress related disorders.

- Our findings clearly demonstrate that a single dose of carbon tetrachloride caused a significant damage in liver tissue as evidenced by increased lipid peroxidation, weakened antioxidant activity of enzymes which lead to disturbance on biochemical processes and necrosis in sections of liver tissues. The findings showed that *M. serrata* has antioxidant, antifibrotic, anti-inflammatory, immunomodulating and liver regenerating properties as evidenced by liver weights, body weight, haematological parameters, biochemical parameters, liver antioxidant marker enzymes and liver histology of MSLAEE pre-treated rats.

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6.2 Recommendation

- Further studies using methods such as Thin-layer Chromatography (TLC), High Performance Liquid Chromatography (HPLC) and Liquid Chromatography-Mass-Spectrometry (LC-MS) are essential as they will provide enough information for confirmation, purification and profiling of *M. serrata* leaves active phytoconstituents. This will highlight the fingerprint of the mechanism of action of *M. serrata* active principles.

- A combined extract may not be better understood in a plethora of substrates present within the extract. Therefore, the analysis of antioxidant activity of individual purified active principles to better elucidate their role as an antioxidant is of importance.

- Studies on chemical studies which will focus on seasonal and geographic variation of *M. serrata* species to identify the best season to harvest the plant and an environment with good outcomes on antioxidant activity of the active principles is also of importance.

- Studies on essential oils of *M. serrata* leaves are also important as essential oils are also reported to have outstanding medicinal properties.

- Ultimately, research feedback should be given to the communities of South Africa on the outcomes of the studies involving the plant which they use for treating ailments. This will not only contribute on the conservation aspect of the plant species, but will also benefit and enrich the community on other uses of the plant.

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