Antioxidant properties of *Lippia javanica* (Burm.f.) Spreng.

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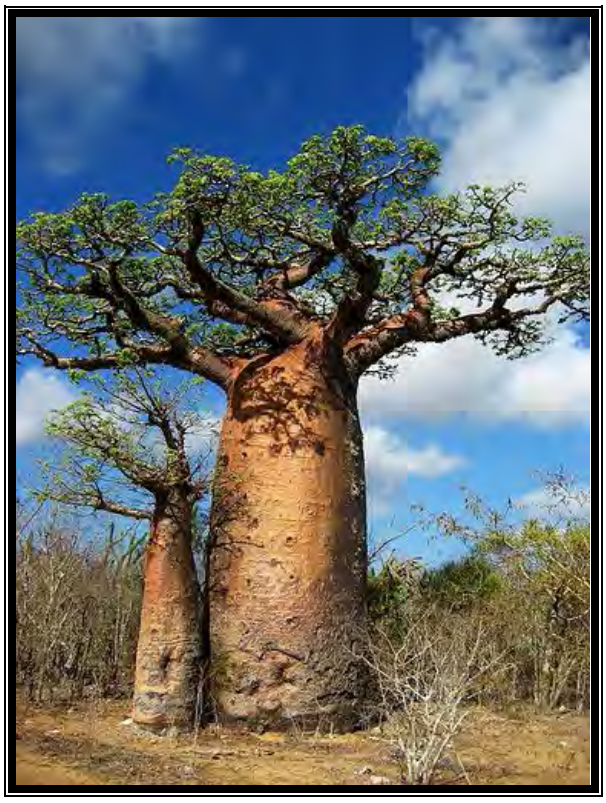
Knowledge and wisdom are like the trunk of a Baobab tree.
No one person's arm span is great enough to encompass them.

- *Saying from the Volta region of Ghana.*



ABSTRACT

The evolution of aerobic metabolic processes unavoidably led to the production of reactive oxygen species (ROS). ROS have the ability to cause harmful oxidative damage to biomolecules. Increased ROS generation and subsequent oxidative stress have been associated with aging and neurodegenerative disorders such as Parkinson's and Alzheimer's diseases as a result of the extreme sensitivity of the central nervous system to damage from ROS. Antioxidant defence systems have co-evolved with aerobic metabolic processes to counteract oxidative damage inflicted by ROS. The impact of neurodegenerative disorders on society is increasing rapidly as the life expectancy of the global population increases. In this day and age, a much younger group of the population is also experiencing neurodegenerative symptoms as a result of the harmful effect of the human immunodeficiency virus (HIV) on the central nervous system.

Plants are an invaluable source of medicinal compounds. The use of plants for their healing properties is rooted in ancient times. The aim of this study was to select from twenty one plants, the plant with the most promising antioxidant activity and to determine whether extracts of this plant could act as free radical scavengers, comparing the results to Trolox, a known free radical scavenger. The next step was to isolate and characterize a compound from an extract exhibiting promising antioxidant activity. Bioassay-guided fractionation was followed to achieve this.

During screening trials, twenty one plants, namely *Berula erecta, Heteromorpha arborescens, Tarchonanthus camphoratus, Vernonia oligocephala, Gymnosporia buxifolia, Acacia karroo, Elephantorrhiza elephantina, Erythrina zeyheri, Leonotis leonurus, Plectranthus ecklonii, P. rehmanii, P. venteri, Salvia auretia, S. runcinata, Solenostemon latifolius, S. rotundifolius, Plumbago auriculata, Clematis brachiata, Vangueria infausta, Physalis peruviana* and *Lippia javanica* were selected from literature, based on reported antioxidant activity within the plant families, for screening of their antioxidant activity. One hundred and ten extracts were prepared from the leaves, using Soxhlet extraction and the solvents petroleum ether (PE), dichloromethane (DCM), ethyl acetate (EtOAc) and ethanol (EtOH), consecutively.

The focus during initial screening trials was on chemistry-based assays. The oxygen radical absorbance capacity (ORAC) and ferric reducing antioxidant power (FRAP) assays were employed for the primary screening of the one hundred and ten leaf extracts. The ORAC assay was used to determine whether the plant extracts were able to scavenge peroxyl radicals and the FRAP assay was used to determine the reducing abilities of the extracts. Quantification of the peroxyl radical scavenging activity by the ORAC assay revealed that activity was observed for most of the extracts, with the ethyl acetate and ethanol extracts of *L. javanica* exhibiting the most promising activity. This pattern of activity was also found with

the reducing capacity evaluated by the FRAP assay in which the EtOAc and EtOH extracts of *L. javanica* also exhibited the most promising activity.

L. javanica was selected for further study by screening for biological activity, employing the nitro-blue tetrazolium (NBT) assay and thiobarbituric acid reactive substances (TBARS) assay. Using a cyanide model to induce neurotoxic effects in rat brain homogenate, the neuroprotective properties of the extracts of *L. javanica* leaves were examined using the NBT assay and compared to that of Trolox. The NBT assay determines the level of superoxide anions. All the extracts of *L. javanica* significantly reduced superoxide anion generation at all concentrations used. The petroleum ether and ethyl acetate extracts, at all concentrations, reduced superoxide anion generation to values lower than that of the control, suggesting that these extracts may be able to attenuate normal free radical processes in the brain. The petroleum ether extract exhibited the most promising activity at a concentration of 1.25 and 2.5 mg/ml and also exhibited similar results as the ethyl acetate extract at a lower concentration than the ethyl acetate extract (2.5 mg/ml compared to 5 mg/ml).

A toxin-solution consisting of hydrogen peroxide (H_2O_2), iron(III)chloride ($FeCl_3$) and ascorbic acid was used to induce lipid peroxidation and the ability of the extracts of the leaves of *L. javanica* to attenuate lipid peroxidation was investigated in rat brain homogenate and compared to that of Trolox. All of the extracts of *L. javanica* significantly attenuated toxin-induced lipid peroxidation at all concentrations used. All of the extracts were also able to significantly attenuate toxin-induced lipid peroxidation to values lower than that of the control. These results suggest that all of the extracts of *L. javanica* possess the ability to attenuate not only toxin-induced lipid peroxidation, but also lipid peroxidation that occurs during normal processes in the brain.

The petroleum ether extract was subjected to bioassay-guided fractionation using column and thin-layer chromatography and the NBT and TBARS assays. Fraction DD1 was investigated by means of nuclear magnetic resonance, infrared and mass spectrometry. The exact structure of fraction DD1 was not elucidated.

Considering all the results, it is clear that *L. javanica* shows great potential as a medicinal plant with antioxidant activity and may therefore be beneficial in diminishing the destructive oxidative effects inflicted by free radicals. There are however still many compounds to be isolated from *L. javanica.*

Key words: Verbenaceae, *Lippia javanica,* antioxidant, neurodegeneration, oxygen radical absorbance capacity (ORAC), ferric reducing antioxidant power (FRAP), nitro-blue tetrazolium assay (NBT), thiobarbituric acid reactive substances assay (TBARS).

UITTREKSEL

Vryradikale is die onvermydelike gevolg van die evolusie van aerobiese prosesse. Vryradikale besit die vermoë om molekules in die liggaam te beskadig deur oksidatiewe stres. Verhoogde vorming van vryradikale en die oksidatiewe stres wat daarop volg, speel moontlik ‘n rol by veroudering en neurodegeneratiewe siektes soos Alzheimer- en Parkinson se siektes. Die rol van vryradikale en oksidatiewe stres by die bogenoemde is as gevolg van die sensitiwiteit van die sentrale senuweestelsel vir beskadiging deur vryradikale. Antioksidantsisteme het gelyktydig met aerobiese prosesse verbeter om die oksidatiewe skade wat deur vryradikale veroorsaak word, teen te werk. Die impak van neurodegeneratiewe siektes op die samelewing is besig om te verhoog aangesien die gemiddelde ouderdom van die wêreldwye populasie besig is om te verhoog. Neurodegeneratiewe simptome word deesdae ook by ’n baie jonger deel van die samelewing opgemerk as gevolg van die nadelige effek van die menslike immuniteitsgebreksvirus (MIV) op die sentrale senuweestelsel.

Plante is ‘n belangrike bron van medisinale verbindings en word al vir eeue as geneesmiddels gebruik. Die doel van hierdie studie was om die plant te kies wat die mees belowende antioksidant aktiwiteit besit en om te bepaal of die ekstrakte van die blare van die plant as vryradikaalopruimers kan optree deur die resultate met Trolox, ‘n bekende vryradikaalopruimer, te vergelyk. Die volgende stap was om ‘n verbinding uit ‘n ekstrak met belowende aktiwiteit te isoleer en te identifiseer deur gebruik te maak van ’n bio-toetsing-geleide fraksioneringsmetode.

Na ‘n literatuursoektog na die antioksidant aktiwiteit reeds gevind in sekere plant families, is daar besluit om te bepaal of *Berula erecta, Heteromorpha arborescens, Tarchonanthus camphoratus, Vernonia oligocephala, Gymnosporia buxifolia, Acacia karroo, Elephantorrhiza elephantina, Erythrina zeyheri, Leonotis leonurus, Plectranthus ecklonii, P. rehmanii, P. venteri, Salvia auretia, S. runcinata, Solenostemon latifolius, S. rotundifolius, Plumbago auriculata, Clematis brachiata, Vangueria infausta, Physalis peruviana* en *Lippia javanica* antioksidant aktiwiteit besit. Een honderd en tien ekstrakte van die blare is berei deur middel van Soxhlet ektraksie met opeenvolgend petroleumeter (PE), dichloormetaan (DCM), etielasetaat (EtOAc) en etanol (EtOH) as oplosmiddels.

Die aanvanklike siftings-toetse was gebasseer op chemiese metodes. Die antioksidant aktiwiteit van die een honderd en tien ekstrakte van die blare is bepaal deur gebruik te maak van die ORAC en FRAP sisteme. Die ORAC sisteem het die vermoë van die ekstrakte om suurstofradikale op te ruim bepaal en die FRAP sisteem het die vermoë van die ekstrakte om yster te reduseer bepaal. Kwantifisering van die suurstofradikaal opruimings aktiwiteit deur middel van die ORAC sisteem het aktiwiteit van die verskillende ekstrakte getoon wat gewissel het van swak tot baie belowend. Die etielasetaat- en etanolekstrakte van die blare

van *L. javanica* het die mees belowende aktiwiteit getoon. Die patroon van aktiwiteit is ook waargeneem by die reduserende kapasiteit deur middel van die FRAP sisteem.

Daar is dus besluit om *L. javanica* vir verdere studie te gebruik. Die biologiese aktiwiteit van *L. javanica* is bepaal deur gebruik te maak van die nitrobloutetrasolium (NBT) toets asook die tiobarbituursuur (TBARS) toets. 'n Sianiedmodel in rotbreinhomogenaat is gebruik om die mate van superoksiedanioon opruiming van die ekstrakte van *L. javancia* te bepaal en te vergelyk met die van Trolox. Die superoksiedanioonkonsentrasie is betekenisvol verminder by alle konsentrasies van al die ekstrakte van die blare van *L. javanica* wat gebruik is. Alle konsentrasies van die petroleumeter- en etielasetaatekstrakte van die blare van *L. javanica* het waardes beter as die kontrole gehad. Dit dui daarop dat hierdie ekstrakte in staat is om die geïnduseerde radikale te verminder asook radikale wat vrygestel word deur normale metaboliese prosesse in die brein. Die petroleumeterekstrak het die mees belowende aktiwiteit getoon by konsentrasies van 1.25 en 2.5 mg/ml en die aktiwiteit is vergelykbaar met die van die etielasetaatekstrak deurdat dit effektief was by 'n laer konsentrasie as die etielasetaatekstrak (2.5 mg/ml teenoor 5 mg/ml).

Lipiedperoksidase is geïnduseer deur 'n toksien oplossing wat bestaan het uit waterstofperoksied (H_2O_2), yster(III)chloried ($FeCl_3$) en askorbiensuur. Die vermoë van die ekstrakte van die blare van *L. javanica* om lipiedperoksidase te inhibeer is ondersoek en vergelyk met die van Trolox. Al die ekstrakte van *L. javanica* het die toksien-geïnduseerde lipiedperoksidase verminder na waardes beter as die kontrole. Die resultate dui daarop dat al die ekstrakte in staat is om toksien-geïnduseerde lipiedperoksidase te inhibeer asook lipiedperoksidase wat tydens normale metaboliese prosesse in die brein plaasvind.

Met behulp van kolom- en dunlaagchromatografie en 'n bio-toetsing-geleide fraksioneringsmetode (deur middel van die NBT en TBARS toetse), is 'n fraksie vanuit die petroleumeterfraksie verkry. Fraksie DD1 is met behulp van massaspektrometrie, infraroospektrometrie en kernmagnetiese-resonans-spektrometrie ondersoek. Die struktuur van fraksie DD1 is egter nie opgeklaar nie.

Indien die gevolgtrekkings uit die resultate van die onderskeie analises in aanmerking geneem word, kan afgelei word dat *L. javanica* wel oor die potensiaal beskik om as 'n medisinale plant wat antioksidant aktiwiteit besit, gebruik kan word. Daar is wel nog baie verbindings wat uit *L. javanica* geïsoleer kan word.

Sleutelwoorde: Verbenaceae, *Lippia javanica,* suurstofradikaal-opruimings aktiwiteit (ORAC), yster-reduserende antioksidant aktiwiteit (FRAP), nitrobloutetrasolium toets (NBT), tiobarbituursuur reaktiewe substanse toets (TBARS).

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$^{\circ}C$	-	Degrees celsius
%	-	Percentage
µg/ml	-	Microgram per millilitre
µg	-	Mictrogram
µl	-	Microliter
µM	-	Micromolar
µM/ml	-	Micromole per millilitre
^{13}C	-	13-carbon
^{1}H	-	1-hydrogen
$^{1}O_2$	-	Singlet oxygen
$A^{\cdot}$	-	Antioxidant radical
AAPH	-	2,2'-azo-bis-(2-amidinopropane)hydrochloride
ADP	-	Adenosine diphosphate
AE	-	Ascorbic acid equivalents
AE/10 mg	-	Ascorbic acid equivalents per 10 milligram
AH	-	Antioxidant
$AH^{\cdot+}$	-	Oxidized antioxidant
AIDS	-	Acquired immunodeficiency syndrome
ALA	-	Alpha-lipoic acid
ANOVA	-	One-way analysis of variance
$Asc^{\cdot}$	-	Ascorbyl radical
$AscH^{\cdot}$	-	Ascorbate monoanion
ATP	-	Adenosine triphosphate
AUC	-	Area under the curve
BHT	-	Butylated hydroxytoluene
BSA	-	Bovine serum albumin
Ca^{2+}	-	Calcium (II) cation
CAT	-	Catalase
CC	-	Column chromatography
$CDCl_3$	-	Deutirated chloroform
Cl^{-}	-	Chloride ion
cm	-	Centimetre
cm^{-1}	-	Per centimeter
CO_2	-	Carbon dioxide
CoA	-	Coenzyme A
Cu	-	Copper
$CuSO_4$	-	Copper (II) sulphate
$CuSO_4.5H_2O$	-	Copper (II) sulphate pentahydrate

DCM - Dichloromethane/dichloormetaan

DHLA - Dihydrolipoic acid

DMSO - Dimethyl sulfoxide

DNA - Deoxyribonucleic acid

e^- - Electron

EI - Electron impact

Eq - Equation

ESI - Electronspray ionization

ETC - Electron transport chain

EtOAc - Ethyl acetate/etielasetaat

EtOH - Ethanol/etanol

eV - Electron volt

f / F - Fluorescence

f_0 - Fluorescence at time 0/initial fluorescence

f_5; f_{10}; f_{15}; f_{175}; f_{180} - Fluorescence at 5, 10, 15, 175 and 180 minutes

f_1; f_2 - Fluoresence at time 1 and 2

f_n - Fluorescence at time n

FAD - Flavin adenine dinucleotide (oxidized form)

$FADH_2$ - Flavin adenine dinucleotide (reduced form)

Fe^{2+} - Ferrous [Iron (II)]

Fe^{2+}-TPTZ/ $[Fe\ (II)\ (TPTZ)_2]^{2+}$ - Ferrous tripyridyltriazine

Fe^{3+} - Ferric [Iron (III)]

Fe^{3+}-TPTZ/ $[Fe\ (III)\ (TPTZ)_2]^{3+}$ - Ferric tripyridyltriazine

$FeCl_3$ - Iron (III) chloride

$FeCl_3.H_2O$ - Iron (III) chloride hexahydride

FL - Fluorescein

Fluorescein - 3’6’-dihydrospiro[isobenzofuran-1-[3H],9’[9H]-xanthe]-3-one

FRAP - Ferric reducing antioxidant power/yster-reduserende antioksidant aktiwiteit

g - Relative centrifuge force (rcf)

g - Gram

g/L - Gram per litre

G-6PD - Glucose-6-phosphate dehydrogenase

GPX/ GSH-PX - Glutathione peroxidase

GRed - Glutathione reductase

GSH - Reduced glutathione

GSSG - Oxidized glutathione/ Glutathione disulphide

H^+ - Hydrogen ion

H_2O	-	Water
H_2O_2	-	Hydrogen peroxide
H_2SO_4	-	Sulphuric acid
HAT	-	Hydrogen atom transfer
HCl	-	Hydrochloric acid
HIV	-	Human immunodeficiency virus
HNO_2	-	Nitrous acid
HOCl	-	Hypochlorous acid
IR	-	Infrared
K^+	-	Potassium cation
K_2HPO_4	-	Potassium dihydrogen orthophosphate
KBr	-	Potassium bromide
KCl	-	Potassium chloride
KCN	-	Potassium cyanide
L	-	Litre
$L^{\cdot}$	-	Lipid radical
LC/MS	-	Liquid chromatography mass spectrometry
LH	-	Lipid substrate/polyunsaturated fatty acid
Lippia javanica$_{DCM}$/ LJDCM	-	Dichloromethane extract of *Lippia javanica*
Lippia javanica$_{EtOAc}$/ LJEtOAc	-	Ethyl acetate extract of *Lippia javanica*
Lippia javanica$_{EtOH}$/ LJEtOH	-	Ethanol extract of *Lippia javanica*
Lippia javanica$_{PE}$/ LJPE	-	Petroleumether extract of *Lippia javanica*
L. javanica	-	*Lippia javanica*
LMWA	-	Low molecular weight antioxidants
$LO^{\cdot}$	-	Lipid alkoxyl radical
LOH	-	Lipid alcohol
$LOO^{\cdot}$	-	Lipid peroxyl radical
LOOH	-	Lipid hydroperoxide
m	-	Meter
M	-	Molar concentration (mole per litre)
M^{n+}	-	Metal ion
MA	-	Malonic acid
MDA	-	Malondialdehyde
mg/ml	-	Milligram per millilitre
MHz	-	Mega hertz
MIV	-	Menslike immuniteitsgebreksvirus
ml	-	Millilitre
ml/min	-	Millilitre per minute

mg	-	Milligram
mm	-	Millimeter
mM	-	Millimolar
MPTP	-	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MS	-	Mass spectroscopy
$_{mt}$DNA	-	Mitochondrial DNA
m/z	-	Mass to charge ratio
n	-	Number of replicates
NA	-	Not available
Na^{2+}	-	Sodium (II) cation
NaCl	-	Sodium chloride
Na_2CO_3	-	Disodiumcarbonate solution
NAD^+	-	Nicotinamide adenine dinucleotide
NADH	-	Reduced nicotinamide adenine dinucleotide
$NADP^+$	-	Nicotinamide adenine dinucleotide phosphate (oxidized form)
NADPH	-	Nicotinamide adenine dinucleotide phosphate (reduced form)
Na_2HPO_4	-	Di-sodium hydrogen orthophosphate anhydrous
NaOH	-	Sodium hydroxide
NBD	-	Nitro-blue diformazan
NBT	-	Nitro-blue tetrazolium/nitrobloutetrasolium
NBT^{2+}	-	Nitro-blue tetrazolium chloride
nm	-	Nanometer
nM	-	Nanomolar
nmoles/L	-	Nanomoles per litre
NMDA	-	*N*-methyl-D-aspartate
NMR	-	Nuclear magnetic resonance
$NO^{\cdot}$/NO	-	Nitric oxide
NO^+	-	Nitrosyl cation
NO^-	-	Nitroxyl anion
$NO_2^{\cdot}$	-	Nitrogen dioxide
NO^{2+}	-	Nitronium ion
NO_2Cl	-	Nitryl chloride
NO_2O_3	-	Dinitrogen trioxide
NOS	-	Nitric oxide synthase
NWU	-	North-West University
O_2	-	Oxygen
$O_2^{\cdot-}$	-	Superoxide anion
O_3	-	Ozone

$^{\cdot}OH/HO^{\cdot}$	-	Hydroxyl free radical
^{-}OH	-	Hydroxide
$ONOO/ONOO^{\cdot}$	-	Peroxynitrate
$ONOO^{-}$	-	Peroxynitrate anion
ONOOH	-	Peroxynitrous acid
ORAC	-	Oxygen radical absorbance capacity/suurstofradikaal-opruimings aktiwiteit
p	-	Probability
PBS	-	Phosphate buffered saline
PE	-	Petroleum ether/petroleumeter
Pi	-	Phosphate
ppm	-	Parts per million
$R^{\cdot}$	-	Free radical
R^2	-	Correlation coefficient
RH	-	Substrate
RFU	-	Relative fluorescence units
RMCD	-	Randomly methylated β-cyclodextrin
RNS	-	Reactive nitrogen species
$RO^{\cdot}$	-	Substrate free radical
ROH	-	Substrate alcohol
$ROO^{\cdot}$	-	Perhydroxyl radical
ROOA	-	Non-radical product
ROOH	-	Substrate hydroperoxide
ROS	-	Reactive oxygen species
$RS^{\cdot}$	-	Thiyl radical
SEM	-	Standard error of the mean
SET	-	Single electron transfer
SH	-	Thiol group
SOD	-	Superoxide dismutase
T	-	Time cycle
T_1, T_2, T_3, T_n	-	Time at fluorescence 1, 2, 3 and n
t/T	-	Time
TBA	-	Tiobarbituursuur
TBARS	-	Thiobarbituric acid reactive substance assay/tiobarbituursuur reaktiewe substance toets
TCA (figure 2.3)	-	Tricarboxylic acid
TCA	-	Trichloroacetic acid
TE	-	Trolox® equivalents

TE/10 mg	-	Trolox® equivalents per 10 milligram
THC	-	α-Tetrahydrocannabinol
TLC	-	Thin-layer chromatography
TMP	-	1,1,3,3-tetramethoxypropane
T-O$^{\cdot}$/α-TO$^{\cdot}$	-	Vitamin E radical
T-OH/ α-TOH	-	Vitamin E (active form)
TPTZ	-	2,4,6-tripyridyl-*s*-triazine
Trolox	-	6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid
UV	-	Ultraviolet
v/v	-	Volume by volume
v/v/v	-	Volume by volume by volume
w/v	-	Weight by volume
WHO	-	World health orginasation
α	-	Alpha
β	-	Beta
γ	-	Gamma

CHAPTER 1

Introduction, aim and objectives

1.1 Introduction

Free radicals or reactive oxygen species (ROS) are generated as a part of the body's normal metabolic processes. Although oxygen is essential for respiration, it is also toxic due to the partially reduced forms of oxygen (Gerschman *et al.,* 1954).

Antioxidant systems have evolved to counteract the deleterious effects of ROS (Halliwell & Gutteridge, 1990b; Prior & Cao, 1999) by keeping the pro-oxidant/antioxidant relationship in check (Gilgun-Sherki *et al.,* 2001). A disruption in the oxidant/antioxidant equilibrium due to a decrease in the body's antioxidant defence system or over-production of free radicals, leads to oxidative stress (Gilgun-Sherki *et al.,* 2001). Considerable evidence has accumulated to imply that oxidative stress arising from the cellular damage inflicted by ROS plays a central role in the mechanism and pathogenesis of aging and age related neurodegenerative disorders, such as Parkinson's and Alzheimer's diseases (Aruoma, 2003) as well as the oxidative insult by the human immunodeficiency virus (HIV) on patients (Famularo *et al.,* 1997). It is believed that if the body's natural antioxidant defence systems are overwhelmed by an increase in ROS, the consumption of antioxidants may be very effective in diminishing the effect of ROS-induced oxidative stress (Halliwell, 1994).

Due to the fact that oxidative stress is implicated in aging and age related neurodegenerative disorders, strategies aimed at inhibiting oxidative stress caused by free radicals may be beneficial in slowing down the progression of these diseases. Antioxidants can therefore be used to combat oxidative stress by scavenging free radicals and thereby preventing the chain reactions that play a major role in aging and neurodegenerative diseases (Singh *et al.,* 2004).

The interest in the use of medicinal plants is expanding throughout the world (WHO, 1998). Because of South Africa's remarkable biodiversity and the fact that up to 80% of South Africans make use of traditional medicine, it is reasonable to presume that beneficial drugs can be found in the plant kingdom (Van Wyk *et al.,* 1997). Plant based antioxidants can play an invaluable role in providing protection against oxidative stress induced by ROS (Benzie, 2003).

Naturally occurring substances from plants include a wide variety of molecules, such as flavonoids, anthocyanins, carotenoids, dietary glutathionine, vitamins and endogenous

metabolites which have been documented to possess antioxidant activities (Cao *et al.,* 1996).

1.2 Aim and objectives of this study

The aim of this study was to investigate the antioxidant activity of South African plants, selecting the plant with the most promising antioxidant activity and to isolate and characterize a compound from an extract exhibiting promising antioxidant activity employing a bio-assay guided approach.

To achieve the aim of the study, the following objectives were set:

- To conduct a literature survey and identify South African plants from plant families that may possess antioxidant activity.
- To prepare plant extracts from the leaves using solvents with different polarities.
- To chemically screen the leaf extracts prepared from the selected South African plants for antioxidant activity using the oxygen radical absorbance capacity (ORAC) and ferric reducing antioxidant power (FRAP) assays.
- To biologically screen the crude leaf extracts of the most promising plant (*Lippia javanica*) for the ability to reduce superoxide anions *in vitro* using the nitro-blue tetrazolium (NBT) assay as well as the ability to attenuate induced lipid peroxidation *in vitro* using the thiobarbituric acid reactive substances (TBARS) assay.
- To do preliminary chromatographic analysis of the petroleum ether extract using thin layer chromatography and then use several consecutive steps of chromatographic separation with samples of ample amount followed by the screening of these fractions for activity (bio-assay guided fractionation).
- To isolate a compound from an extract exhibiting promising antioxidant activity (petroleum ether) of the chosen plant (*Lippia javanica)* employing bio-assay guided fractionation and using chromatographic techniques.
- To verify the purity of and structural elucidation of the isolated compound using spectrophotometric techniques.

CHAPTER 2

Literature review

2.1 Free radicals and reactive oxygen- and nitrogen species

2.1.1 Introduction

The evolution of aerobic lifestyles is advantageous in several aspects. Oxygen is essential for respiration and ultimately for energy and is also utilized by cells for many of their biochemical reactions (Gerschman *et al.,* 1954; Di Mascio *et al.,* 1991; Packer, 1991; Halliwell & Gutteridge, 1999).

Atmospheric oxygen, in its ground or inactive state, is usually present as dioxygen. Dioxygen is a biradical that contains two unpaired electrons that have parallel spins (Green & Hill, 1984). As a rule, molecular oxygen undergoes univalent reduction since spin restrictions arise when reduction with electron pairs is attempted (Marklund & Marklund, 1974).

ROS are toxic by-products that occur as a consequence of aerobic metabolism where molecular oxygen is reduced by oxidative phosphorylation that occurs in mitochondria (figures 2.1 & 2.3) (Coyle & Puttfarcken, 1993; Lenaz, 1998; Halliwell & Gutteridge, 1999). At physiological levels, ROS perform useful purposes in the human body such as in signal transduction and gene transcription (Lander, 1997; Zheng & Storz, 2000). Another example is the pivotal role that free radicals play in the destruction of microbes by specialized blood cells called phagocytes. In addition to these useful purposes, free radicals may also act as highly deleterious and cytotoxic oxidants at pathological levels (Freidovich, 1999). Free radicals are not always harmful (De Lamirande & Gagnon, 1992). For that reason, there exists an oxygen paradox in that the free radicals formed from oxygen perform rather contradictory actions in biology.

The production of free radicals is an integral part of human metabolism and as a result, all living organisms are exposed to ROS on a continuous basis (Halliwell & Gutteridge, 1999). Free radicals are unstable and highly reactive molecules or chemical species that is capable of independent existence (Halliwell & Gutteridge, 1999; Gilbert, 2000; McCord, 2000). ROS have a high potential to damage vital biological systems. ROS have therefore been implicated in the aging process as well as in a variety of pathological conditions (Chance *et al.,* 1979; Halliwell *et al.,* 1992; Stadtman, 1992; Ames *et al.,* 1993). ROS are hypothesized to be mainly responsible for progressive and specific neuronal degeneration (Hensley *et al.,* 1994).

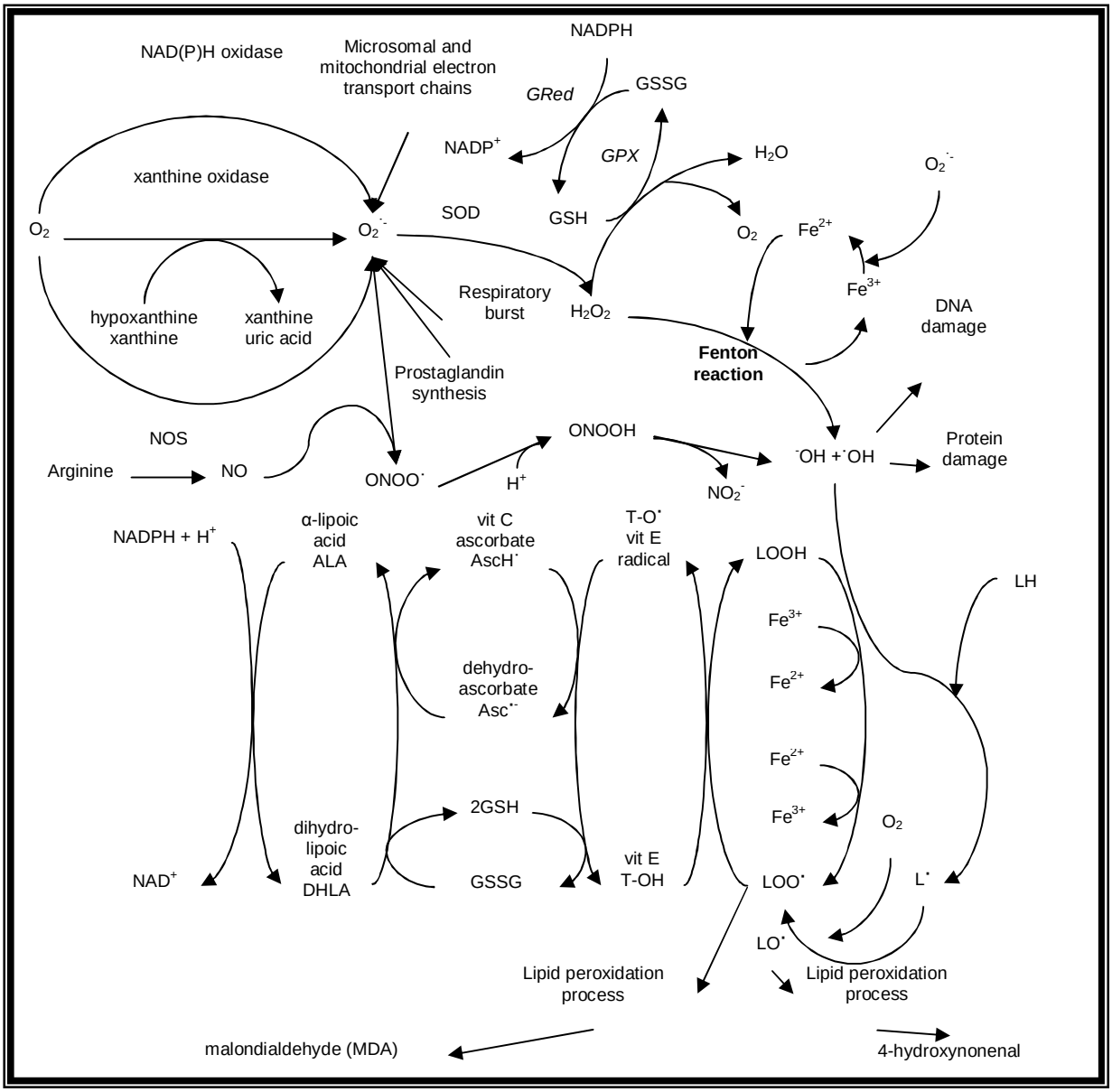

Figure 2.1 Pathway of ROS formation, the lipid peroxidation process and the role of glutathione (GSH) and other antioxidants (vitamin E, vitamin C and lipoic acid) in the management of oxidative stress (Valko *et al.,* 2007).

2.1.2 Generation of free radicals

In figure 2.2 reaction 1 depicts the reaction where sufficient energy is absorbed to reverse the spin on one of the unpaired electrons from triplet oxygen (ground state) to singlet oxygen ($^1O_2$) (highly activated). Reaction 2 depicts monovalent reduction where singlet oxygen accepts a single electron to form the superoxide anion ($O_2^{\cdot -}$). In reaction 3, the superoxide anion undergoes further monovalent reduction to form hydrogen peroxide (H_2O_2). In reaction 4, hydrogen peroxide is further reduced to hydroxyl radicals ($HO^{\cdot}$) in the presence of ferrous ions (Fe^{2+}). This reaction was first described by Fenton and later developed by Haber and Weiss. Reaction 5 depicts the production of water and oxygen from the reaction

of hydrogen peroxide with hydroxyl radicals. Reaction 6 depicts the production of hydroxyl radicals from the reaction of hydrogen peroxide with superoxide anions. Reaction 5 and 6 are collectively known as the Haber-Weiss reaction (Apel & Hirt, 2004; Agarwal & Prabakaran, 2005). ˙OH radicals are the most reactive ROS and are capable of oxidizing a variety of biomolecules, such as enzymes, carbohydrates, proteins, deoxyribonucleic acid (DNA) and unsaturated fatty acids (Halliwell & Gutteridge, 1999; Janisch *et al.,* 2002).

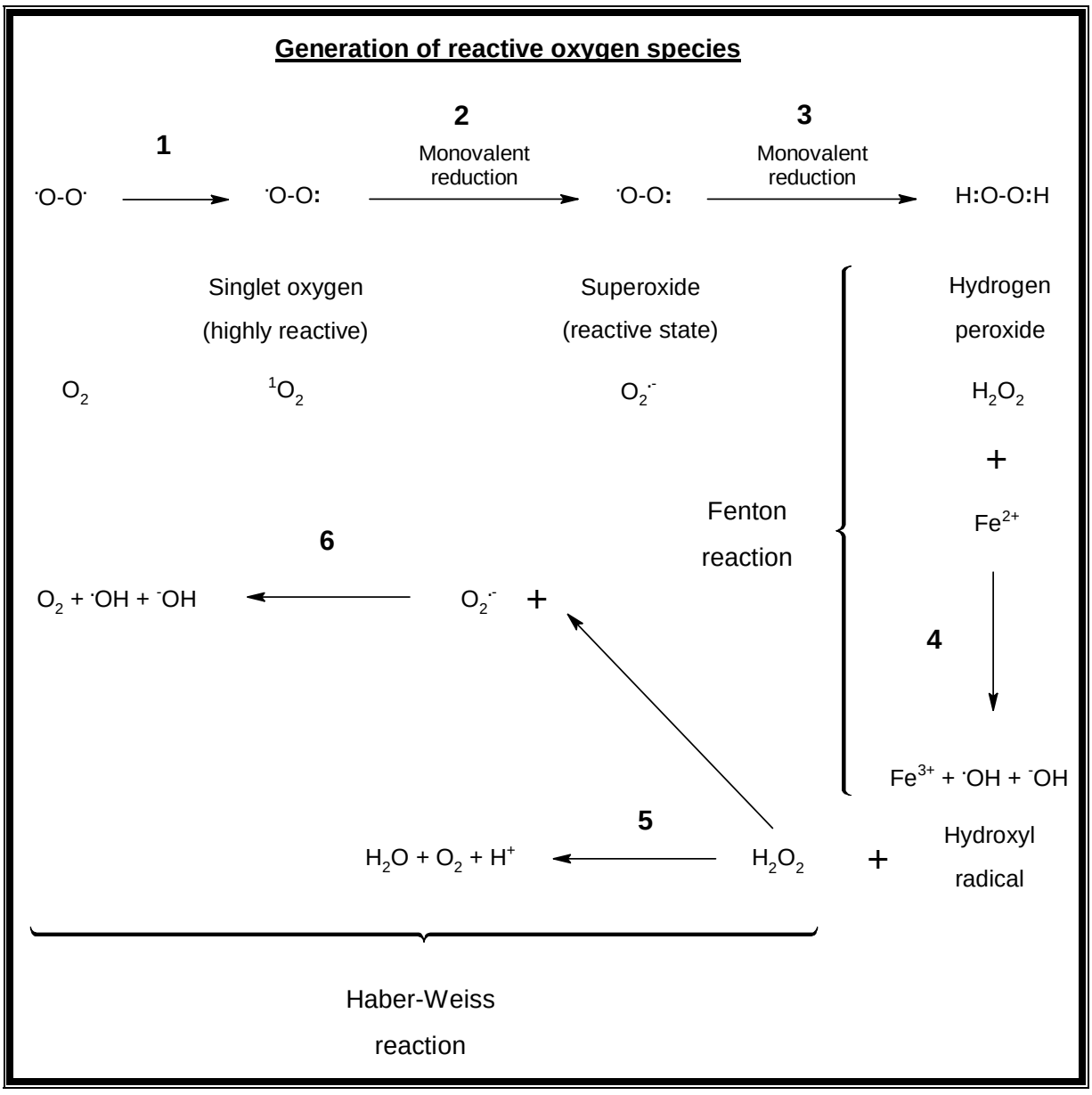

Figure 2.2 Activation of oxygen into ROS (Apel & Hirt, 2004; Agarwal & Prabakaran, 2005).

Endogenously, ROS are also generated by enzymatic reactions such as cytochrome P450 in the endoplasmic reticulum, lipoxygenases, cylooxygenases, xanthine oxidase activated in ischemia/reperfusion and NADPH oxidase of activated leukocytes; outo-oxidation; exercise and inflammation. Apart from the endogenous sources, ROS are also formed by several

different exogenous sources. Exogenous sources include ionizing radiation, environmental pollutants, cigarette smoke, ultraviolet (UV) light, xenobiotics and ozone (Young & Woodside, 2001).

Mitochondria are found in the cytoplasm of just about all eukaryotic cells (Chance *et al.,* 1979; Boveris, 1984; Finkel & Holbrook, 2000). Mitochondria utilize approximately 85 – 90% of oxygen. The mitochondrion is therefore the major endogenous source of ROS ($O_2^{\cdot -}$, H_2O_2 and $HO^{\cdot}$) production (Loschen *et al.,* 1973). The residual 10 – 15% of oxygen is used by other cellular oxidative enzymes (Halliwell & Gutteridge, 1999). Prolonged exposure to free radicals, even at low concentrations, may result in the damage of biologically important molecules and potentially lead to DNA mutation, tissue injury and disease (McCord, 2000; Freidovich, 1999).

During cellular respiration the energy of oxidation drives the synthesis of adenosine triphosphate (ATP) which is the energy currency of the cell. All of the oxidative steps in the degradation of carbohydrates, fats and amino acids converge at this final stage of cellular respiration (Finkel & Holbrook, 2000). .

The source of electrons in oxidative phosphorylation is initiated with the entry of electrons into the respiratory chain and the energy is transferred by passing along a pair of electrons. The electron-transporting protein complexes are embedded in the inner membrane and are collectively called the electron transport system (figure 2.3). The components of the electron transport system include complexes I, II, III, IV and V. It also contains two individual molecules, coenzyme Q and cytochrome C (Alexi *et al*., 2000; Papucci *et al*., 2003).

Since ATP is an ubiquitous store of energy needed for transport across membranes for all processes of the cell, energetically compromized mitochondria may have detrimental effects on the survival of the cell, potentially leading to apoptosis. Mitochondrial respiratory chain defects have been implicated in the pathogenesis of Alzheimer's disease (Grünewald & Beal, 1999) and mitochondrial dysfunction has been associated with the neurodegeneration of Parkinson's disease (Berman & Hastings, 1999).

When the electron transport chain (ETC) is inhibited, the generation of free radicals is induced. Apart from producing free radicals, oxidative phosphorylation is itself vulnerable to damage by free radicals. A possible cause of the susceptibility to injury by free radicals is most likely the lack of protective histones, limited repair ability and the close proximity to the ETC. Complex I is particularly sensitive to $^{\cdot}OH$ and $O_2^{\cdot -}$. This entire process may also reduce ATP levels and lead to an excessive release and reuptake of mitochondrial calcium. The mitochondrion is thus both a target and source of free radicals (Cadenas, 2004).

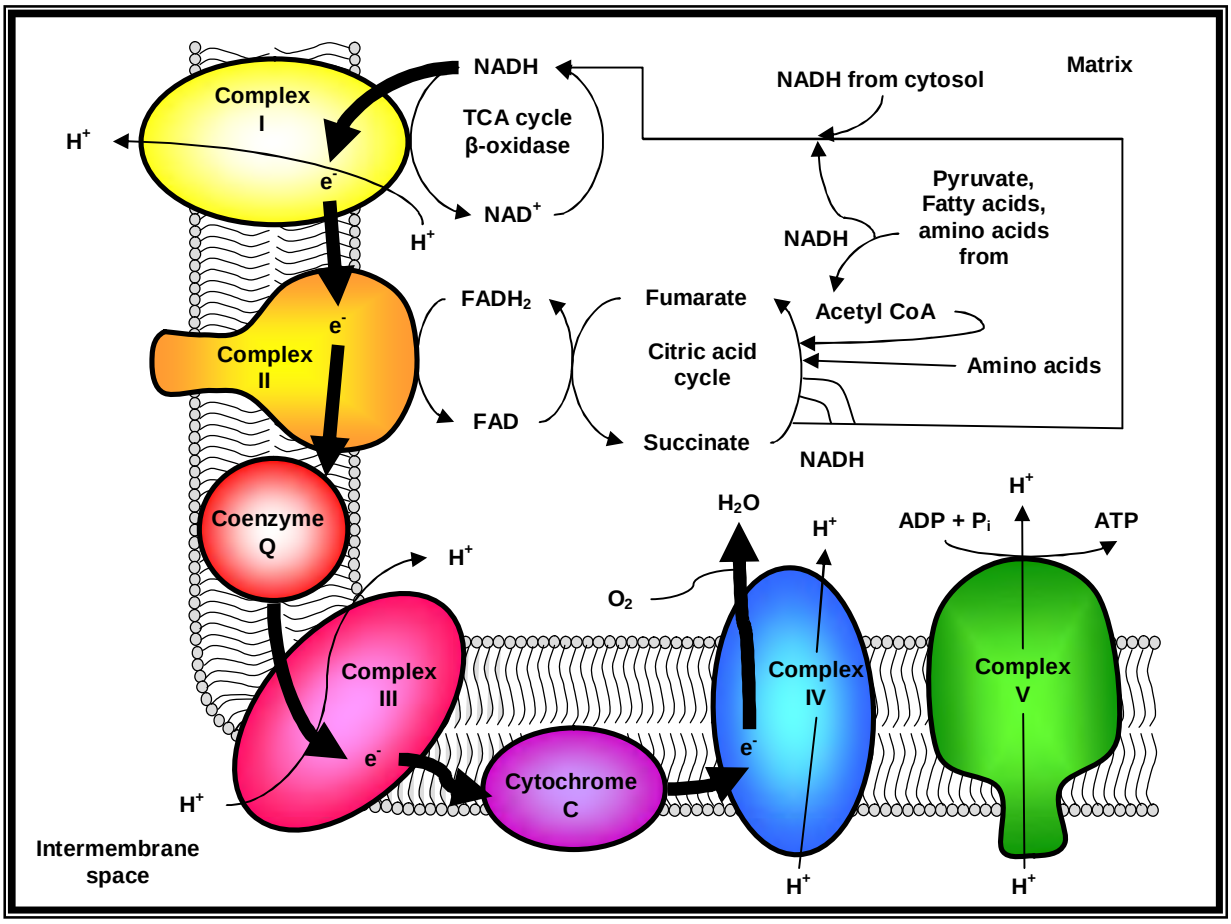

Figure 2.3 The mitochondrial electron transport complexes (Kidd, 2000; Foster *et al.,* 2006).

2.1.3 Types of free radicals

ROS represent a broad category of molecules that include radicals (that contain unpaired electrons) and non-radical oxygen derivatives (that possess the ability to pull electrons from other molecules). Table 2.1 lists the types of ROS that exist as non-radicals and free radicals in living organisms (Agarwal & Prabakaran, 2005).

Table 2.1 Types of ROS that exist as non-radicals and free radicals.

Radicals		Non-radicals	
Hydroxyl	$HO^{\cdot}$	Peroxynitrite	$ONOO^{-}$
Superoxide	$O_2^{\cdot -}$	Hypochloric acid	HOCl
Nitric oxide	$NO^{\cdot}$	Hydrogen peroxide	H_2O_2
Thiyl	$RS^{\cdot}$	Singlet oxygen	$^1O_2$
Peroxyl	$RO_2^{\cdot}$	Ozone	O_3
Lipid peroxyl	$LOO^{\cdot}$	Lipid peroxide	LOOH

In addition to ROS, there is another class of free radicals that are nitrogen derived. This class is called reactive nitrogen species (RNS). RNS are considered a subclass of ROS (Darley-Usmar *et al.,* 1995; Sikka, 2001). They are listed in table 2.2:

Table 2.2 Types of RNS that induce oxidative stress.

RNS	
Nitrous oxide	$NO^{\cdot}$
Peroxynitrite	$ONOO^{-}$
Peroxynitrous acid	ONOOH
Nitroxyl anion	NO^{-}
Nitryl chloride	NO_2Cl
Nitrosyl cation	NO^{+}
Nitrogen dioxide	$NO_2^{\cdot}$
Dinitrogen trioxide	NO_2O_3
Nitrous acid	HNO_2

2.1.3.1 Superoxide anion ($O_2^{\cdot -}$)

The reduction of oxygen by its acceptance of a single electron produces the first product of univalent reduction, the superoxide anion ($O_2^{\cdot -}$). $O_2^{\cdot -}$ is generated in many biological processes (Werns & Lucchesi, 1990). It has been estimated that at physiological levels of O_2, 1 – 3% of molecular oxygen reduced in mitochondria may form superoxide (Halliwell & Gutteridge, 1999). Superoxide itself is not highly reactive (Sawyer & Valentine, 1981) and superoxide toxicity appears to be through an indirect action on living cells as $O_2^{\cdot -}$ is capable of producing the more powerful and damaging hydroxyl radical ($^{\cdot}OH$) in the presence of hydrogen peroxide (Haber-Weiss reaction) and peroxynitrite ($ONOO^{-}$) through its rapid reaction with nitric oxide ($NO^{\cdot}$). Superoxide anion radicals can also react with each other to produce hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) (Fridovich, 1975; Halliwell & Gutteridge, 1984; Fridovich, 1989). In environments with a pH of approximately 7.4, superoxide is partially protonated to form the perhydroxyl radical ($HO_2^{\cdot}$). $HO_2^{\cdot}$ is also a more powerful and damaging reactive oxidizing species that is estimated to inflict more than five times the damage that the hydroxyl radical is capable of (Fahn & Cohen, 1992).

2.1.3.2 Hydrogen peroxide (H_2O_2)

Hydrogen peroxide will be present whenever $O_2^{\cdot -}$ is formed, because the dismutation of $O_2^{\cdot -}$ generates molecular oxygen plus H_2O_2:

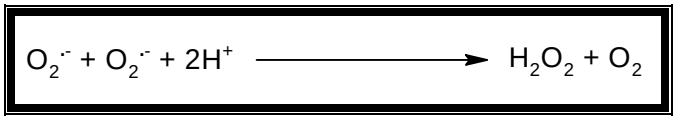

Figure 2.4 The generation of H_2O_2 from $O_2^{\cdot-}$.

Hydrogen peroxide is a weak oxidizing agent and generally poorly reactive but can act as an intermediate in the formation of highly reactive free radicals such as hydroxyl radicals (Fridovich, 1997).

Much of the damage that has been attributed to $O_2^{\cdot-}$ may actually have been caused by H_2O_2 that was formed from the $O_2^{\cdot-}$ that was initially generated. DNA is damaged by H_2O_2 in the presence of metal ions (Uchida *et al.,* 1965; Massie *et al.,* 1972; Keller & Pollard, 1977; Meneghini & Hoffmann, 1980).

2.1.3.3 Hydroxyl radical ($^{\cdot}OH$)

The hydroxyl radical ($HO^{\cdot}$) is formed from the slow decomposition of hydrogen peroxide (Halliwell & Gutteridge, 1999). This formation of $HO^{\cdot}$ from H_2O_2 is accelerated in the presence of reduced metal ions, such as ferrous ions, Fe^{2+} (Fenton reaction).

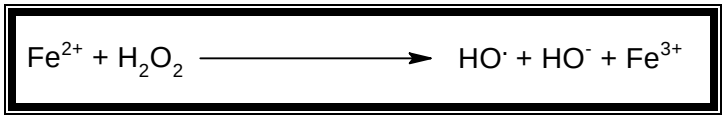

Figure 2.5 The formation of $HO^{\cdot}$ from Fe^{2+} and H_2O_2 through the Fenton reaction.

The hydroxyl radical ($^{\cdot}OH$) is the most reactive ROS and is capable of oxidizing a variety of biomolecules, such as enzymes, carbohydrates, proteins, DNA and unsaturated fatty acids. $^{\cdot}OH$ can also induce radical chain reactions with a multitude of organic molecules (Janisch *et al.,* 2002).

The hydroxyl radical can also be formed from $O_2^{\cdot-}$. $O_2^{\cdot-}$ can react with hydrogen peroxide to form $^{\cdot}OH$ (Haber & Weiss, 1934):

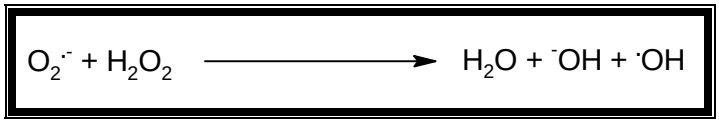

Figure 2.6 The formation of $^{\cdot}OH$ from the reaction of $O_2^{\cdot-}$ and H_2O_2.

The reaction in figure 2.6 is known as the Haber-Weiss reaction. The Haber-Weiss reaction is also known as superoxide-driven Fenton chemistry.

The hydroxyl radical can also be formed through the reaction of HOCl with $O_2^{\cdot-}$ (Candeias *et al.,* 1993):

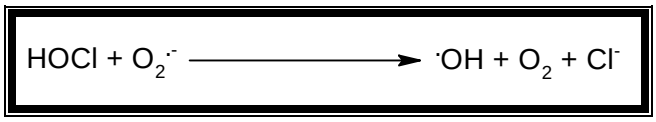

Figure 2.7 Reaction of HOCl with $O_2^{\cdot-}$ to form $^{\cdot}OH$, O_2 and Cl^-.

HOCl is a strong oxidant that is generated from H_2O_2 and Cl^- by myeloperoxidase (a heme enzyme) particularly in immunologically activated phagocytes (Cadeias *et al.,* 1993).

2.1.3.4 Singlet oxygen ($^1O_2$)

Singlet oxygen is produced by photosensitization reactions. It can also be formed when ozone (O_3) reacts with human body fluids; when $ONOO^-$ reacts with H_2O_2 and the reaction of peroxyl radicals with themselves during lipid peroxidation (Halliwell, 1995). Oxygen has two singlet states but the $^1O_2$ state is probably the most important. Singlet oxygen ($^1O_2$) is a powerful oxidizing agent even though it is not a free radical. $^1O_2$ is able to rapidly attack several molecules, including polyunsaturated fatty acids (Watabe *et al.,* 2007).

2.1.3.5 Peroxynitrite (ONOO)

Peroxynitrite ($ONOO^-$) is a very potent oxidant formed from the rapid reaction of the superoxide anion ($O_2^{\cdot-}$) with the nitric oxide radical ($NO^{\cdot}$) (Beckman *et al.,* 1994; Halliwell & Gutteridge, 1999).

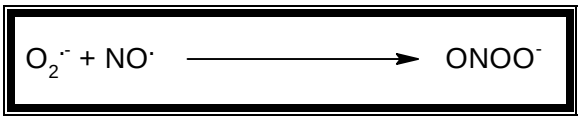

Figure 2.8 Reaction of $O_2^{\cdot-}$ with $NO^{\cdot}$ to form $ONOO^-$ (Ischiropoulos & Al-Mehdi, 1995).

$ONOO^-$ is believed to be directly cytotoxic and can decompose at physiological pH to several noxious products, including the nitronium ion (NO^{2+}), nitrogen dioxide ($NO_2^{\cdot}$), and some $^{\cdot}OH$ (Beckman *et al.,* 1994; Van der Vliet *et al.,* 1994)

Because of the highly reactive nature of peroxynitrite, it can cause oxidative damage to proteins, lipids and DNA (Mecocci *et al.,* 1993).

2.1.3.6 Nitric oxide (NO)

Nitric oxide ($NO^{\bullet}$) is formed from L-arginine by nitric oxide synthase (NOS) (Wu & Morris, 1998). NO is one of the most widespread signaling molecules. It participates in practically every cellular and organ function in the body (Ignarro *et al.,* 1999). NO is essential for regulating the relaxation and proliferation of vascular smooth muscle cells. At physiologic levels, NO is produced by endothelial cells and is responsible for leukocyte adhesion, platelet aggregation, angiogenesis, thrombosis, vascular tone, and hemodynamics (Ignarro *et al.,* 1999). In addition, neurons can also produce NO, which then serves as a neurotransmitter. Activated macrophages also generate NO, an important mediator of the immune response (Freidovich, 1999). Nitric oxide ($NO^{\bullet}$) can react with $O_2^{\bullet-}$ or H_2O_2 to form peroxynitrite ($ONOO^{\bullet}$), whose oxidant potential is greater than that of $O_2^{\bullet-}$ or H_2O_2 alone (Freidovich, 1999; McCord, 2000).

2.1.4 Free radical chain reaction

A free radical chain reaction occurs in initiation, propagation and termination steps (see figure 2.9). During the initiation step, hydrogen is removed from a carbon atom to produce a carbon free radical ($R^{\bullet}$). In the presence of diatomic oxygen, a peroxyl radical ($ROO^{\bullet}$) is formed. The peroxyl radical is able to abstract hydrogen from carbon to form another carbon radical and hydroperoxide during the propagation step. The newly formed carbon radical is capable of reacting with molecular oxygen to continue the propagation step. The chain reaction can be terminated when two radicals react with each other to form stable products (non-radical products) (Jain & Sharma, 2010).

2.2 Defence mechanisms against free radicals

2.2.1 Antioxidants

The existence of harmful free radicals is considered to be the reason why it was essential for living organisms to develop various complex antioxidant strategies to protect themselves against the noxious effects of oxygen and its partially reduced species (Halliwell & Gutteridge 1990b; Prior & Cao, 1999). An antioxidant can be defined as a substance that significantly prevents or delays oxidation of a substance initiated by a pro-oxidant with the prerequisite of being present at lower concentrations compared to that of the oxidizable substance (Halliwell & Gutteridge 1990b). A pro-oxidant can be defined as a toxic substance capable of causing oxidative damage to lipids, proteins and nucleic acids that result in various pathological events or disease (Prior & Cao, 1999).

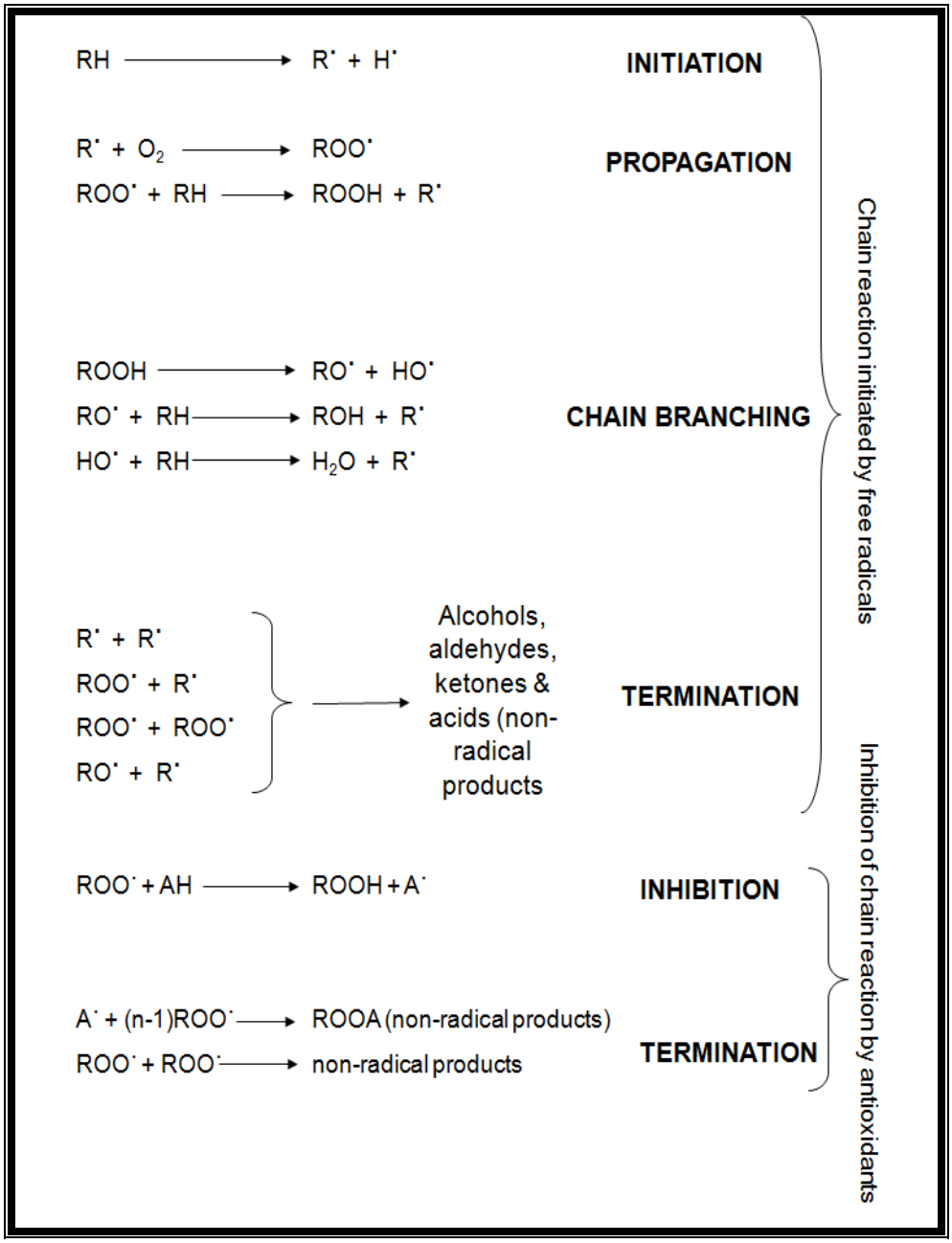

Figure 2.9 General pathway in a free radical chain oxidation (Diaby *et al.,* 2009) and the actions of inhibitors (antioxidants) (assuming one antioxidant scavenges two radicals and oxygen in large excess; $ROO^{\bullet}$ = free radical; AH = antioxidant (Huang *et al.,* 2005).

Antioxidant reactions involve multiple steps including the initiation, propagation, branching and termination of free radicals. This whole process is termed a chain reaction (see figure 2.9). Therefore antioxidants fall into two mechanistic groups: the first group includes antioxidants that inhibit or retard the formation of free radicals from their unstable precursors (initiation) and are called the “preventative” antioxidants and the second group includes antioxidants that interrupt the radical chain reaction (propagation and branching) and are called the “chain-breaking” antioxidants (Wright *et al.,* 1997; Barclay *et al.*, 2000).

The driving force behind the antioxidant mechanism in figure 2.9 is the formation of a delocalized stable radical that does not continue the chain reaction or continues it with low efficiency. A chain-breaking antioxidant donates its labile hydrogen atom to ROO˙ much more rapidly than ROO˙ reacts with substrate. The radical (A˙) is stable and is not able to continue the autoxidation of the chain. Antioxidants that follow this mechanism of action, follows the hydrogen atom transfer (HAT) mechanism. The HAT mechanism has been extensively studied and has been widely accepted as the predominate mechanism that antioxidants follow (Wright *et al.,* 1997; Barclay *et al.*, 2000).

Taking the basis of the chemical reactions involved into consideration, antioxidant capacity assays can generally be divided into two categories: (1) hydrogen atom transfer (HAT) reaction based assays and (2) single electron transfer (SET) based assays. SET-based assays involve a redox reaction with an oxidant. The oxidant also acts as the probe for monitoring the reaction and is the indicator of the reaction endpoint. HAT-based assays monitor competitive reaction kinetics. The quantitation is derived from the kinetic curves. HAT-based methods involve a synthetic free radical generator, an oxidizable molecular probe and antioxidant. HAT and SET-based assays are set to measure the radical (or oxidant) scavenging capacity and not the preventative antioxidant capacity of a sample (figure 2.10) (Huang *et al.,* 2005).

Antioxidants can also be physically classified according to their solubility into two groups, namely hydrophilic antioxidants (such as vitamin C and the majority of polyphenolic compounds) and lipophilic antioxidants (mainly including vitamin E and carotenoids). Hydrophilic antioxidants do not accumulate in the body and are excreted in the urine. Lipophilic antioxidants on the other hand, penetrate the lipoprotein cell membrane more easily and therefore reach a higher level of bioavailability (Huang *et al.,* 2002a).

A diverse group of antioxidant systems protect cells from oxidative damage (Halliwell & Gutteridge, 1990b; Halliwell & Gutteridge, 1992; Ames *et al.,* 1993; Halliwell & Gutteridge, 1999). These defence mechanisms include the antioxidant enzymes, free radical scavengers (chain breaking antioxidants) and metal chelating agents (figure 2.10). The antioxidant enzymes include catalase (CAT), glutathione peroxidase (GSH-PX) and superoxide dismutase (SOD) and are the primary line of defence against ROS. The antioxidant enzymes are able to effectively remove superoxide anions and peroxides. The free radicals scavengers (chain breaking antioxidants) are non-enzymatic antioxidants able to trap free radicals. The free radical scavengers include ascorbate, α-tocopherol, glutathione (GSH), albumin, β-carotene, uric acid, bilirubin and flavonoids (Prior & Cao, 1999). The metal binding (chelating) agents are antioxidants that remove transition metal

ions and as a result, remove the precursors of ROS to prevent their chain reactions (figure 2.10) (Halliwell, 1995).

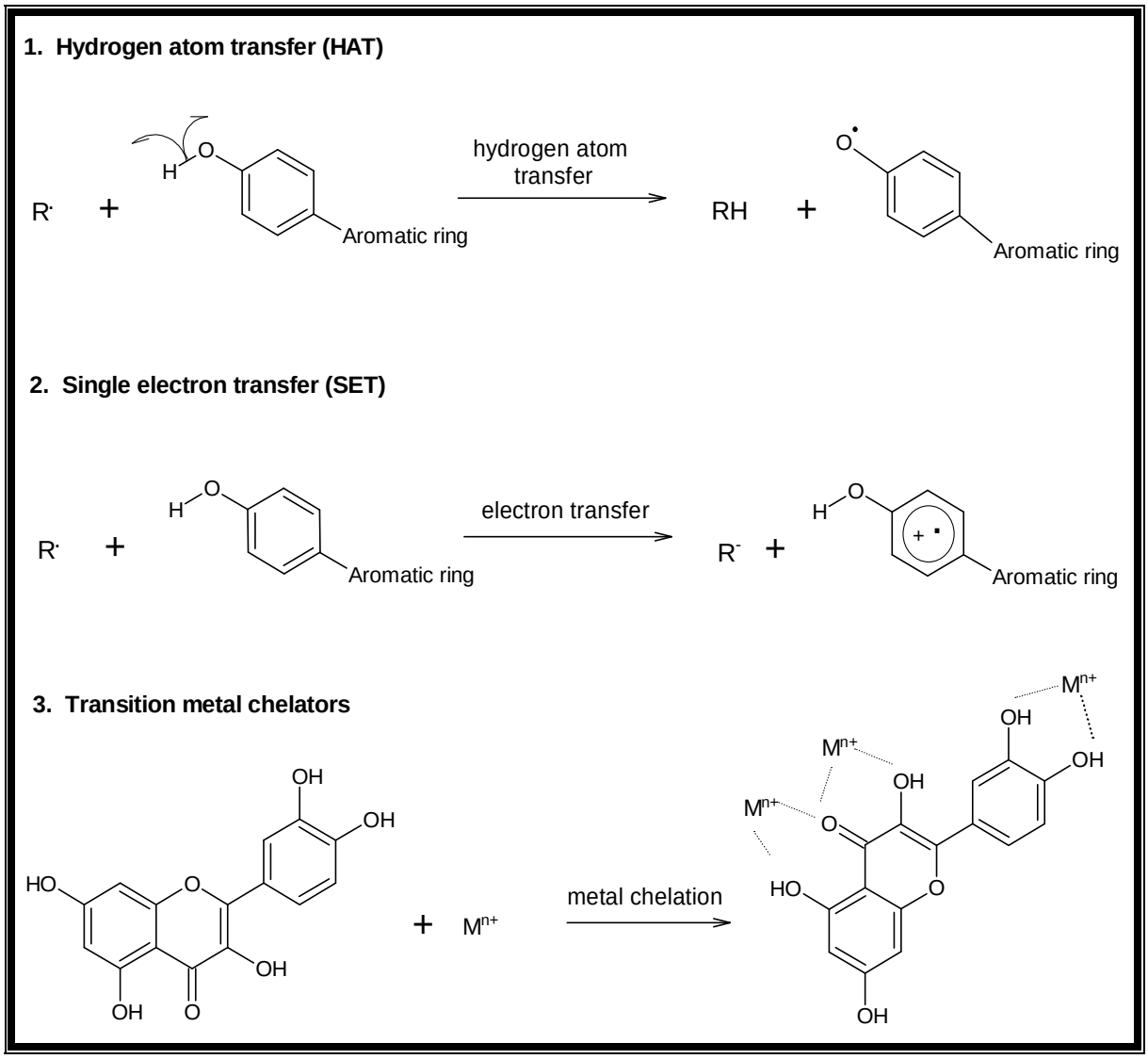

Figure 2.10 Mechanisms of actions that antioxidants follow (Leopoldini *et al.,* 2010).

Small-molecular-weight compounds can also act as antioxidants. These small-molecular-weight compounds react with oxidizing chemicals, reducing their capacity for exhibiting damaging effects. Some, such as glutathione (GSH), ubiquinol and uric acid, are produced during normal metabolism. Ubiquinol is the only known fat soluble antioxidant synthesized by animal cells. It is believed to play an important role in cellular defence against oxidative damage. Other small-molecular-weight antioxidants are found in the diet, the best known being vitamin E, vitamin C and carotenoids (Halliwell & Gutteridge, 1999).

2.2.1.1 Endogenous antioxidant systems

Glutathione peroxidase, catalase and superoxide dismutases are the primary antioxidant enzymes, which directly eliminate toxic oxidative intermediates (hydroxyl radical, superoxide

radical and hydrogen peroxide). They require micronutrients as cofactors such as selenium, iron, copper, zinc, and manganese for optimum catalytic activity (table 2.3) and effective antioxidant defence mechanisms (Halliwell, 2001). Glutathione reductase and glucose-6-phosphate dehydrogenase are the secondary enzymes (Vendemiale *et al.,* 1999; Singh *et al.,* 2003).

2.2.1.1.1 Superoxide dismutase (SOD)

The superoxide dismutases are a family of antioxidant enzymes which are important in the catalytic decomposition of superoxide radicals to hydrogen peroxide and oxygen (Delibas *et al.,* 2002; Viggiano *et al.,* 2003). The superoxide dismutases have evolved to inactivate both intracellular and extracellular $O_2^{\bullet -}$. Since SOD scavenges both intracellular and extracellular superoxide radicals, it prevents the lipid peroxidation of plasma membranes. Therefore, SOD plays a prominent role in the protection from superoxide anions and against lipid peroxidation. SOD should however be conjugated with catalasse or glutathione peroxidase to prevent the action of H_2O_2, which is responsible for the formation of hydroxyl radicals (Jeulin *et al.,* 1989).

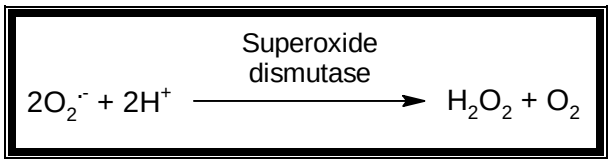

Figure 2.11 The decomposition of $O_2^{\bullet -}$ to H_2O_2 and O_2 by the catalytic action of superoxide dismutase (SOD) (Agarwal & Prabakaran, 2005).

2.2.1.1.2 Catalase (CAT)

The catalses belong to the family of enzymes that contain the hydroperoxidases and peroxidases. Catalase specifically catalyzes the intracellular and extracellular decomposition of hydrogen peroxide to water and oxygen (Baker *et al.,* 1996).

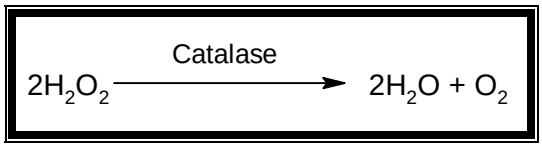

Figure 2.12 The decomposition of H_2O_2 to H_2O and O_2 by the catalytic action of catalase (CAT) (Agarwal & Prabakaran, 2005).

Table 2.3 ROS, their corresponding neutralizing antioxidants and additional antioxidants (Singh *et al.,* 2004).

ROS	Antioxidants (endogenous)		Antioxidant (exogenous)
	Direct role	**Indirect role**	
Hydroxyl radical ($^{\cdot}OH$)	Glutathione peroxidase (GPX) (co-factor selenium)	-	Vitamin C Lipoic acid
Lipid peroxide	Glutathione peroxidase (GPX) (co-factor selenium)	-	Vitamin E B-carotene
Superoxide radical ($O_2^{\cdot -}$)	Superoxide dismutase (SOD) (co-factor Cu/Zn/Mn)	Ceruloplasmin (Cu) Metalloghionin (Cu) Albumin (Cu)	Vitamin C
Hydrogen peroxide (H_2O_2)	Catalase (co-factor iron)	Transferin (iron) Ferritin (iron) Myoglobin (iron)	Vitamin C B-carotene Lipoic acid
Pro-oxidant/antioxidant equilibrium	Thiols (GSH, Lipoic acid, N-acetylcysteine) NADPH and NADH Ubiquinone	Bilirubin Uric acid	Flavonoids

2.2.1.1.3 Glucose-6-phosphate dehydrogenase (6GPD)

Glucose-6-phosphate dehydrogenase is one of the secondary enzymes. The secondary enzymes help in the detoxification of ROS by decreasing peroxide levels or maintaining a steady supply of metabolic intermediates like glutathione and nicotinamide adenine dinucleotide phosphate (NADPH) necessary for optimum functioning of the primary antioxidant enzymes (Vendemiale *et al.,* 1999; Singh *et al.,* 2003).

2.2.1.1.4 Glutathione peroxidase

The glutathione peroxidases are a family of selenium containing antioxidant enzymes that are important in the breakdown of H_2O_2 as well as the reduction of toxic hydroperoxides. Hydroperoxides usually result from lipid peroxidation. The glutathione peroxidase/glutathione reductase system is very important for its antilipoperoxidative defence. Glutathione peroxidase (GSH-PX) reacts with peroxides and requires reduced glutathione (GSH) as the reductive substance that donates an electron (Delibas *et al.,* 2002). Glutathione reductase stimulates the reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH). This ensures a steady supply of the reductive substrate, NADPH, to glutathione peroxidase (Agarwal & Prabakaran, 2005).

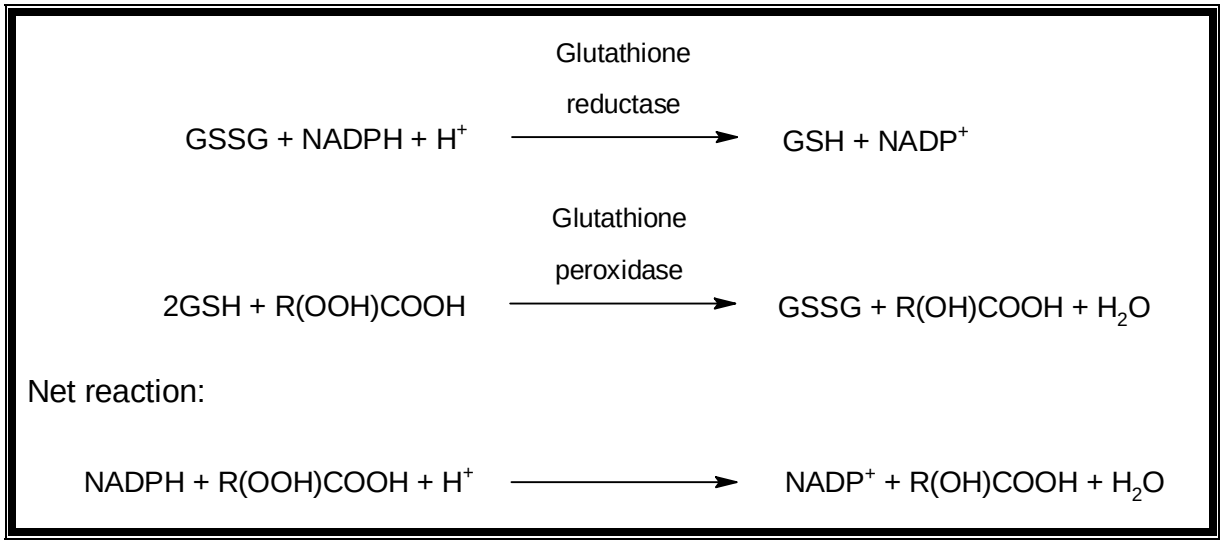

Figure 2.13 The reactions of the glutathione peroxidase/glutathione reductase system as well as the net reaction of this system (Agarwal & Prabakaran, 2005).

2.2.1.2 Exogenous antioxidant systems

Apart from the antioxidant enzymes, there are also various small-molecule antioxidants that play important roles in the antioxidant defence system. These small-molecule antioxidants can be divided into compounds made *in vivo*, and compounds obtained from the diet (exogenous antioxidants). Vitamins such as α-tocopherol (vitamin E), β-carotene (vitamin A), and ascorbic acid (vitamin C) and micronutrient elements such as zinc and selenium are examples of these exogenous antioxidants (Halliwell & Gutteridge, 1999). Antioxidants obtained from the diet are known as dietary antioxidants. Dietary antioxidants can scavenge ROS or RNS to stop radical chain reactions sacrificially (Huang *et al.,* 1996). They can also inhibit the reactive oxidants from being formed in the first place (preventative). Dietary antioxidants often broadly include radical chain reaction inhibitors, metal chelators, oxidative enzyme inhibitors and antioxidant enzyme cofactors (Huang *et al.,* 2005).

2.2.1.2.1 Vitamin E

Vitamin E is a major lipid-soluble chain-breaking antioxidant that is present in all cellular membranes and in plasma where it serves to protect membrane fatty acids from lipid peroxidation both *in vitro* and *in vivo* (Di Mascio *et al.,* 1991; Halliwell, 1994; Ottino & Duncan, 1997; Wang *et al.,* 2002). Vitamin E reacts directly with free radicals such as the peroxyl radical ($ROO^{\cdot}$) by transferring its phenolic hydrogen to the peroxyl free radical, yielding lipid hydroperoxides and tocopheroxyl radicals. These lipid hydroperoxides and tocopheroxyl radicals can be removed by the glutathione peroxidase/glutathione reductase system (Burton *et al.,* 1982). The glutathione peroxidise/glutathione reductase system breaks the radical chain reaction and also prevents the peroxidation of polyunsaturated fatty

acids in cellular and subcellular membrane phospholipids. Vitamin E effectively scavenges all three important types of ROS, namely superoxide, hydrogen peroxide, and hydroxyl radicals (Agarwal *et al.,* 2004). Although the radicals formed from the reaction of vitamin E with the free radicals, may under certain conditions also mediate further lipid peroxidation, cooperative interaction between vitamin E and other antioxidants like vitamin C can reduce the tocopheroxyl radicals to regenerate tocopherol (Thomas *et al.,* 1995; Stocker & Bowry, 1996).

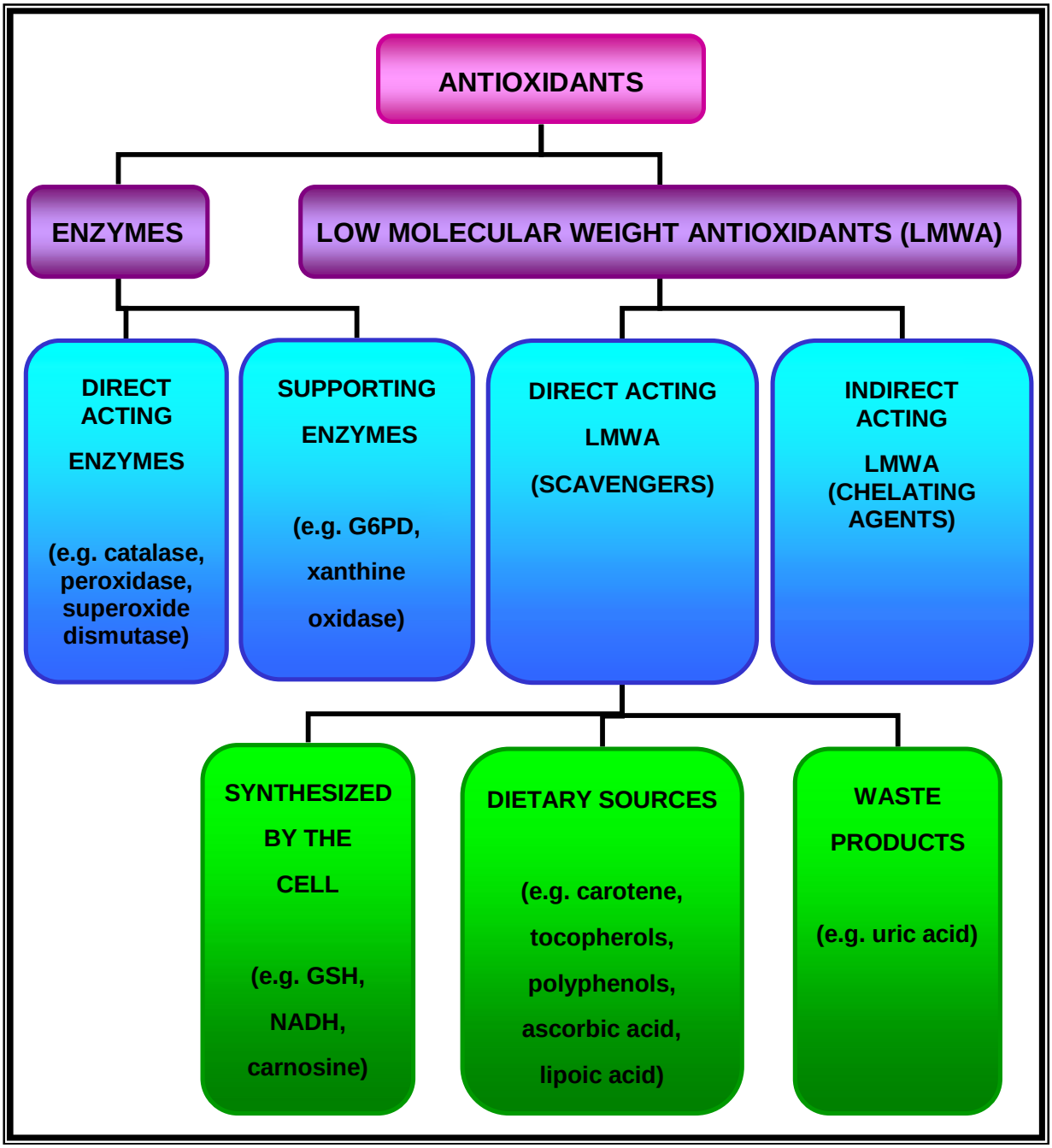

Figure 2.14 Broad scope of the antioxidant defence system (Granot & Kohen, 2004).

2.2.1.2.2 Vitamin C

Vitamin C (ascorbic acid) is the most important water-soluble chain-breaking antioxidant and is found both intracellularly and extracellularly (Wang *et al.,* 2002). Vitamin C prevents lipid peroxidation by neutralizing the peroxyl radicals in the aqueous phase before lipid peroxidation is initiated (Block *et al.,* 1992; Halliwell, 1994; Jacob, 1995). Vitamin C also recycles vitamin E and protects DNA against damage induced by H_2O_2. Therefore vitamin C works directly by the reaction with aqueous radicals and indirectly by restoring the antioxidant properties of vitamin E (Bendich *et al.,* 1986). Vitamin C also has a paradoxical effect (Lutsenko *et al.,* 2002) in that it can also produce ROS by its reaction with transition metal ions (Block *et al.,* 1992; Halliwell, 1994; Jacob, 1995).

2.2.1.2.3 Glutathione

Glutathione is a peptide that consists of glutamate, cysteine and glycine. Glutathione exists in thiol-reduced glutathione (GSH) and oxidized glutathione (GSSH) (Sies, 1999) and is the most abundant non-thiol protein present in mammalian cells (Irvine, 1996). Glutathione can be partly absorbed from the small intestines as well as synthesized in the body. It is therefore an endogenous and exogenous antioxidant. Glutathione plays a vital role in reducing oxygen toxicity by interrupting the reaction leading to $O_2^{\cdot-}$ formation (Yoda *et al.,* 1986). Glutathione directly quenches lipid peroxides and plays a major role in xenobiotic metabolism (Meister, 1994; Sies & Stahl, 1995; Anderson, 1996). In the reduced form, glutathione metabolizes H_2O_2 and $^{\cdot}OH$ formed during normal cellular metabolism. The glutathione radical that is formed from the oxidation of GSH is a pro-oxidant radical but these glutathione radicals can react with each other to yield oxidized glutathione (GSSG) which is then reduced to glutathione by the NADPH-dependent glutathione reductase (figure 2.13) (Sies, 1999). Glutathione also converts vitamin C radical to vitamin C and is therefore an important antioxidant that functions directly in eliminating toxic peroxides and aldehydes and indirectly in regenerating vitamins C and E in their reduced and functional forms (Meister, 1994; Sies & Stahl, 1995; Anderson, 1996).

2.2.1.2.4 Phytonutrients or phytochemicals

A number of other dietary antioxidants exist beyond the traditional vitamins. They are collectively known as phytonutrients or phytochemicals which are being increasingly appreciated for their antioxidant activity (Zheng & Wang, 2001; Huang *et al.,* 2005).

2.2.1.2.4.1 Flavonoids

An example of the phytochemicals is the flavonoids which are a group of polyphenolic compounds (also see section 2.9.1).

2.3 Oxidative stress

Under physiological conditions, free radicals and ROS are produced in cells and organisms (Guaiquil *et al.,* 2001) and the antioxidant defence mechanisms are quite capable of protecting neurons from ROS initiated oxidative insult. Oxidative stress results whenever an excess of ROS overwhelms the ability of the antioxidant defence systems of the cell. This alters the redox homeostasis and produces an imbalance between prooxidants and antioxidants that favours the prooxidants (Sikka *et al.,* 1995; Sharma & Agarwal, 1996). Oxidative stress leads to damage of important macromolecules (Tseng *et al.*, 1997; Marnett, 2000; Stadtman & Levine, 2000) and is inflicted on cells as a result of three factors. These factors include increased oxidant generation, decreased antioxidant protection and a failure to repair the oxidative damage.

The nervous system which includes the brain, spinal cord, and peripheral nerves, consumes a large amount of the total body oxygen and has relatively low concentrations of antioxidant enzymes (superoxide dismutase, catalase and glutathione reductase) compared to other organs in the body. Glutathione peroxidase is the major enzyme responsible for detoxification of H_2O_2 in the brain since the brain has very low catalase activity (Reiter, 1995; Dringen *et al.,* 2000). In addition, the nervous system is rich in iron and unsaturated fatty acids. Unsaturated fatty acids are the target of lipid peroxidation. High levels of iron leads to oxidative stress via the iron-catalyzed formation of ROS (Andorn *et al.,* 1990; Bauer & Bauer, 1999). The high lipid content of nervous tissue, coupled with its high aerobic metabolic activity, makes it especially susceptible to high levels of free radical formation and thus oxidative damage (Halliwell, 1992; Coyle & Puttfarcken, 1993; Dawson & Dawson, 1996).

There exists substantial evidence that the generation of free radicals and subsequent oxidative stress play a central role in both the mechanism and pathogenesis of the undesirable effect of the aging process as well as age related neurodegenerative diseases such as Alzheimer's and Parkinson's diseases (Ebadi *et al.,* 1996; Markesbery & Carney, 1999; Cheng *et al.*, 2001; Gilgun-Sherki *et al.*, 2001).

With the acceptance of the role that oxidative stress plays in aging and neurodegenerative diseases, the significance of antioxidant strategies become very important (Mariani *et al.,* 2005). Various *in vitro* studies have shown that endogenous and exogenous antioxidants are able to protect nervous tissue from damage by oxidative stress (Contestabile, 2001). Examples include uric acid, an endogenous antioxidant that was found to prevent *in vitro* and *in vivo* neuronal damage in rats from the metabolic stresses of ischaemia, oxidative stress and exposure to the excitatory amino acid glutamate and the toxic compound, cyanide. Vitamin E was also found to prevent cell death (apoptosis) in rat neurons following hypoxia/oxygen reperfusion. Vitamin E was also found to prevent neuronal damage from

RNS (Yu *et al.,* 1998). Vitamin E and β-carotene were both found to protect rat neurons against oxidative stress following exposure to ethanol (Copp *et al.,* 1999).

2.3.1 Molecular targets of oxidative stress

2.3.1.1 Lipids

A particularly important consequence of free radical induced oxidative damage, is the peroxidation of polyunsaturated fatty acids. Polyunsaturated fatty acids are most susceptible to lipid peroxidation, presumably because of the presence of carbon-carbon double bonds that weaken the carbon-hydrogen bond on the adjacent hydrogen atom, making it susceptible to cleavage (Geva *et al.,* 1998; Lenzi *et al.,* 1998). Lipid peroxidation occurs in a two-step process of initiation and propagation (see figure 2.15). The initiation process involves a free radical interaction which leads to the oxidation of unsaturated lipid components (Blaylock, 1998). Once this process is initiated, continuation of the oxidation of lipid tail groups within the membrane occurs in three dimensional waves from the point of impact. This is referred to as propagation (Blaylock, 1998). The oxidation of polyunsaturated fatty acids results in the formation and propagation of lipid peroxides, the uptake of oxygen, the rearrangement of double bonds in unsaturated lipids and the eventual destruction of membrane lipids which produces breakdown products such as malondialdehyde (Curtis *et al.,* 1984; Burton & Ingold, 1989; Cheeseman & Slater, 1993) and 4-hydroxynonenal (Valko *et al.,* 2007).

Essential reactions that occur in lipid peroxidation are catalysed by iron and copper ions (Halliwell & Gutteridge, 1990a). It is believed that lipid peroxidation may be controlled by limiting the levels of $O_2^{\cdot-}$ and H_2O_2; limiting the availability of metal catalysts and by rapidly interrupting the reaction once it occurs. Therefore, intra- and extracellular scavenger systems may serve to prevent the potential toxic effects of lipid peroxidation caused by the superoxide anion and other ROS (Jeulin *et al.,* 1989). The function of water-soluble antioxidants is to block the initiation phase of lipid peroxidation (Halliwell & Gutteridge, 1999), whereas the function of lipid soluble antioxidants is to inhibit the propagation phase of lipid peroxidation (Blaylock, 1998).

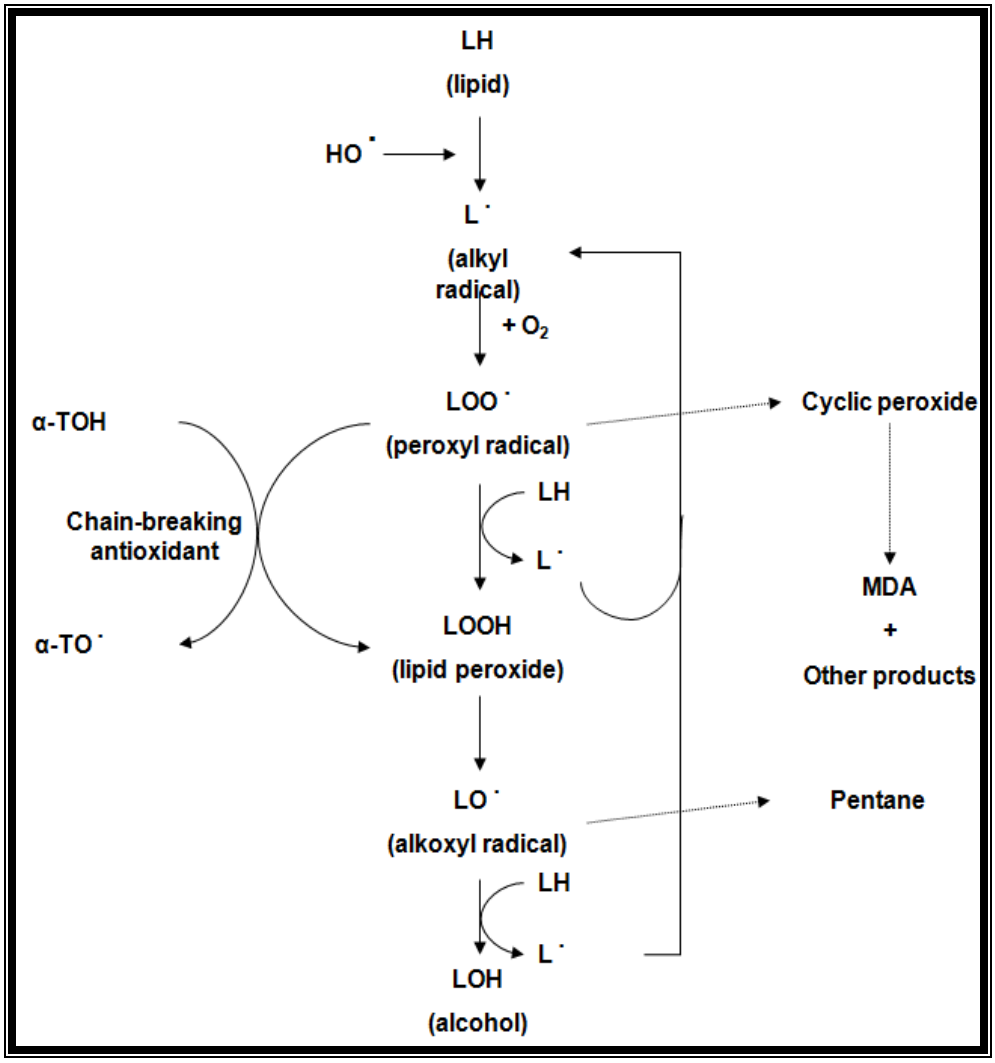

Figure 2.15 Lipid peroxidation process (Sachdev & Davies, 2008).

2.3.1.2 Deoxyribonucleic acid (DNA)

DNA undergoes constant oxidative damage especially if hydroxyl radicals are generated close to DNA. The estimated number of oxidative hits to human DNA per cell per day is believed to be as high as 10 000 and oxidative lesions of DNA accumulates with age (Sen, 2001). Mitochondria have their own DNA, mitochondrial DNA (mtDNA), which is highly susceptible to oxidative damage. The reason for this being that mtDNA is in close proximity to the inner mitochondrial membrane and thus the flux of ROS. mtDNA differs from nuclear DNA in that the complex repair mechanisms that is present in nuclear DNA is absent from mtDNA. The factors mentioned above increase the susceptibility of mtDNA to oxidative damage (Van Remmen & Richardson, 2001). Oxidative DNA damage is considered to be an important promoter of aging and neurological diseases (Poulsen, 2005; Singh, 2006). It therefore seems reasonable to propose that dietary antioxidants (vitamin E, vitamin C, β-carotene and flavonoids) are capable of diminishing oxidative damage to DNA *in vivo* (Halliwell, 1994).

2.3.1.3 Protein

Oxidative protein damage occurs throughout the body at rest. 0.9% of the total oxygen consumed by cells contributes to protein oxidation at rest. Most of the oxidative damage to protein is irreparable and therefore ROS damaged proteins are highly susceptible to proteolytic degradation. Increased protein oxidation is associated with disease and aging (Sen, 2001). Free radicals react with proteins leading to enzyme inactivation and disruption of cellular function (Ebadi *et al.,* 2001). Oxidative damage to proteins is initiated by reaction with aldehydes, which is formed during lipid peroxidation. An example is the reaction of malondialdehyde with the amino group of proteins (Csallany *et al.,* 1984).

Due to the close proximity of lipids and proteins in membranes, oxidative damage of mitochondrial proteins occur as a direct result of oxidative stress or as a consequence of lipid peroxidation. A number of the mitochondrial electron transport chain enzymes are sensitive to inactivation by oxidative stress. An example is ATPase. The inactivation of these proteins can therefore lead to impaired mitochondrial function (Van Remmem & Richardson, 2001).

2.4 Mechanisms of neurodegeneration

Neurodegeneration is a pathological state beyond normal aging that is caused by three major mechanisms of neuronal cell death. These three mechanisms are metabolic compromize, excitotoxicity and oxidative stress and can act either separately or cooperatively to cause neuronal cell death. These three mechanisms form the lethal triplet (Blaylock, 1998; Alexi *et al.,* 2000).

Neurodegenerative diseases are associated with impaired mitochondrial function (Szeto, 2006) and therefore it has been hypothesized that antioxidants might be helpful in ameliorating the symptoms or in slowing the progression of some neurological disorders.

At present, there are no real treatments for any of the neurodegenerative diseases. Treatment is usually aimed at alleviating the symptoms of these diseases.

2.4.1 The lethal triplet

2.4.1.1 Metabolic compromize

Metabolic compromize of neurons results in a depletion of ATP as well as dysregulation of mitochondrial function. This loss of mitochondrial function results in the loss in the ionic integrity and an accumulation of intracellular calcium (Stout *et al.,* 1998). The accumulated intracellular calcium induces additional mitochondrial strain, the production of free radicals as well as a multitude of subsequent neurotoxic processes such as activating Ca^{2+}-dependent proteases and lipases (Alexi *et al.,* 2000).

2.4.1.2 Excitotoxicity

A dysfunction of the neurotransmission of excitatory amino acids results in excitotoxicity. This process is usually triggered by the stimulation of glutamate receptors that turns them pathological (Olney *et al.,* 1971). A plethora of evidence exists for the toxic effects of excitatory amino acids. This evidence shows that the toxic effects of excitatory amino acids are for the most part due to activation of the *N*-methyl-D-aspartate (NMDA) receptor which leads to an influx of Ca^{2+} and thereby toxic overloading (Choi, 1987). The overloading of Ca^{2+} causes the indiscriminate activation of calcium-dependent signals as well as oxidative stress via ROS and RNS (Alexi *et al.,* 2000).

2.4.1.3 Oxidative stress

Oxidative stress is the result of the actions of highly reactive free radicals such as the ROS, superoxide anion ($O_2^{\cdot -}$) and hydroxyl radical ($^{\cdot}OH$) and the reactive nitrogen species, peroxynitrite ($ONOO^-$). (Also see section 2.3).

2.5 Biological aging and age-related neurodegenerative diseases

2.5.1 Biological aging

The aging process is the accumulation of oxidative changes that is responsible for the sequential alterations that accompany advancing age and the associated progressive increase in the chance of disease and death (Harman, 1956; Harman, 1996; Beckman & Ames, 1998). These changes can be attributed to disease, environment, immune dysfunction, and to an inborn process (Harman, 1996). This inborn process is the aging process that produces aging changes at an apparently unalterable and exponentially increasing rate with advancing age (Harman, 1991).

Harman first proposed the free radical theory of aging in 1956. This theory was based on the basis that a single common process was responsible for the aging and death of all living beings (Harman, 1981). This single common process is the initiation of free radical reactions (Harman, 1996). The theory is based on the chemical nature of free radical reactions as well as their ubiquitous and prominent presence in living beings (Holmes *et al.,* 1992).

Aging is accompanied by progressively higher rates of superoxide radical and hydrogen peroxide production and decreased formation of ATP with age (Sohal & Sohal, 1991; Levine & Stadtman, 2001).

The advances in medicine have affected the growth rate of the population aged 65 and older in industrialized countries. Due to this increase in life expectancy, there is an increase in the proportions of people suffering from neurodegenerative disorders. Neurodegenerative

disorders place an enormous emotional and financial burden on these patients and the society as a whole (Przedborski *et al.,* 2003).

2.5.2 Alzheimer's disease

Alzheimer's disease is the most common neurodegenerative disease associated with aging. It affects nearly 20 – 30 million people worldwide. Most Alzheimer's disease cases are sporadic (late-onset) with an age of onset of symptoms occuring in individuals older than 65 years. Some cases of Alzheimer's disease have a genetic component and are inherited with an onset relatively early in life, typically 40 – 60 years of age. Alzheimer's disease is a progressive disorder characterized by memory loss, spatial and temporal disorientation and impaired judgement (Wang *et al.,* 1996).

There exists a proposed "two-hit hypothesis" that states that both oxidative stress and mitogenic dysregulation are necessary and sufficient to cause Alzheimer's disease (Perry *et al.,* 2002). Given that the aging process is associated with an increase in the production of ROS together with a simultaneous decrease in the body's ability to defend against these ROS, oxidative stress and its resulting damage have been recognized as a cause of Alzheimer's disease (Markesbery, 1997). As a result, antioxidant therapies for the prevention and treatment of neurodegenerative diseases currently appear to be a promising field of research. The association between the level of serum antioxidants (vitamins E, C, A, carotenoids, selenium) and memory performance in an elderly sample of 4809 subjects were evaluated. A decreased serum level of vitamin E was consistently found to be associated with a memory deficit (Ricciarelli *et al.,* 2007).

2.5.3 Parkinson's disease

Parkinson's disease is the second most prevalent neurodegenerative disease that affects approximately 2% of individuals after the age of 65 years (De Rijk *et al.,* 1997). Parkinson's disease is a terminal progressive late-onset neurodegenerative disorder with patients experiencing a debilitating loss of movement that include muscle pain, muscle rigidity, bradykinesia (slowness of movement) and resting tremor (Alexi *et al.,* 2000). These symptoms grow progressively worse with the continuation of neurodegeneration until the patients are virtually unable to move (Alexi *et al.,* 2000).

Mitochondrial dysfunction in idiopathic Parkinson's disease has been demonstrated (Schapira *et al.,* 1989). There also exists evidence of the involvement of free radicals in Parkinson's disease. In the presence of iron, which is elevated in Parkinson's disease substantia nigra (Dexter *et al.,* 1989a), peroxide is catalyzed and oxygen radicals are formed (Szeto, 2006). Accelerated metabolism of dopamine by monoamine-oxidase-B may induce an excessive formation of hydrogen peroxide, superoxide anions, and hydroxyl radicals

(Dexter et al., 1989b; Fahn & Cohen, 1992; Alam *et al.,* 1997) that has been suggested to overwhelm endogenous antioxidant mechanisms.

One of the earliest events in Parkinson's disease is a drastic decrease in the antioxidant GSH (Perry & Young, 1986; Sofic *et al.,* 1992). This decrease in GSH levels makes these neurons especially vulnerable to 4-hydroxynonenal and other ROS and RNS (Blaylock, 1998). Therefore, compounds with antioxidant capacities and the ability to reduce mitochondrial dysfunction may prove to be efficacious in slowing the progression of Parkinson's disease. Previous studies assessed the therapeutic benefit of the antioxidant coenzyme Q_{10} in a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model of Parkinson's disease which has demonstrated a significant neuroprotective potential and subsequent clinical trials have demonstrated therapeutic efficacy. It was previously shown in a study that a combination of high doses of α-tocopherol and ascorbic acid may delay the progression of Parkinson's disease by an average of 2.5 years compared to the time required in the placebo group (Fahn & Cohen, 1992). Antioxidants are thus among the therapies that have been tested as a potential treatment for Parkinson's disease by promoting neuronal survival and function (Alexi *et al.,* 2000).

2.5.4 Dementia associated with the human immunodeficiency virus (HIV)

Acquired immunodeficiency syndrome (AIDS) is a serious condition that encompasses a deficiency of the immune system. AIDS is the final stage in a progression of diseases resulting from an infection with the human immunodeficiency virus (HIV) (Roederer *et al.,* 1990).

AIDS patients are constantly under powerful oxidative stress. Oxidative stress is the result of the excessive production of ROS by the white blood cells in response to the virus infection (Famularo *et al.,* 1997). Infection with the human immunodeficiency virus is associated with decreased levels of zinc and selenium (metals that are essential for the proper functioning of antioxidant enzymes) (table 2.3) and of the antioxidant glutathione (Buhl *et al.,* 1989; Eck *et al.,* 1989). As the disease progresses, there is an increase in the production of ROS and levels of fat oxidation as a result of the attack of the human immunodeficiency virus on the immune system (Famularo *et al.,* 1997). HIV-1-associated dementia is a common neurodegenerative disease in patients infected with the human immunodeficiency virus-1 (Price *et al.,* 1988; Davies *et al.,* 1998; Hofman *et al.,* 1999) that occurs in approximately 15 - 20% of patients with advanced HIV disease. HIV-1-associated dementia is currently the primary cause of dementia in people younger than 60 years of age. There exists a significant similarity between the neuropsychological performance by normal elderly patients and the much younger AIDS patients (Van Gorp *et al.,* 1989; Hardy *et al.,* 1999).

Several studies have observed that blood levels of vitamin A, β-carotene, vitamin E and the antioxidative status in HIV/AIDS patients was significantly decreased compared to a control group of healthy persons. This indicates a lack of antioxidants in AIDS patients which can most likely be altered by providing the patient with dietary antioxidants (Folkers *et al.,* 1991; Ireton-Jones & Stiller, 1998; Neves *et al.,* 2006).

2.6 Compounds used to induce oxidative stress

2.6.1 Cyanide

Cyanide is a toxic ion that induces neurotoxicity through a sequence of steps that results in the production of ROS. These ROS are responsible for cellular damage and exert their destructive effects on cellular components with neurodegenerative diseases as a consequence. Cyanide exerts it's neurodegenerative effect by elevating intracellular calcium levels (Johnson *et al.,* 1987) and also inhibits a number of antioxidant enzymes within the brain in addition to increasing the generation of ROS and RNS which leads to oxidative injury (Ardelt *et al.,* 1989; Gunasekar *et al.,* 1996). Cyanide causes inhibition of Complex IV of the ETC and thereby augments ROS and especially $O_2^{\cdot-}$ generation. The inhibition of Complex IV also leads to impaired oxygen utilization and inhibition of oxidative phosphorylation resulting in a disruption of homeostatic ATP-dependent Na^{2+}/K^{+} and Ca^{2+} pumps. The inhibition of oxidative phosphorylation leads to an energy deficit within the target tissues. The disruption in the Ca^{2+} pump causes an increase in intracellular calcium and thereby initiates a cascade of events, ending in free radical generation that can damage lipids, proteins and DNA (Johnson *et al.,* 1987; Southgate & Daya, 1999).

As a result of the dependence of the central nervous system on oxidative metabolism and limited anaerobic capacity, it is particularly vulnerable to cyanide toxicity (Way, 1984) and the brain is seen as a primary target organ in cyanide toxicity (Gunasekar *et al.,* 1996). Cyanide produces a Parkinson-like condition as a post toxicity effect (Utti *et al.,* 1985).

2.6.2 Hydrogen peroxide

Hydrogen peroxide has been shown to induce oxidative stress in animal models (Mahanom *et al.,* 2007; Linden *et al.,* 2008). Ferrous salts react with H_2O_2 to form $^{\cdot}OH$ via the Fenton reaction. $^{\cdot}OH$ can initiate lipid peroxidation. Ferric salts are also a product of the Fenton reaction. Ferric salts react much slower with H_2O_2 than ferrous salts. The inclusion of reducing agents (such as ascorbic acid) stimulates the Fenton reaction. Mixtures of iron salts, ascorbic acid and H_2O_2 are good sources of $^{\cdot}OH$ radicals (Halliwell & Chirico, 1993).

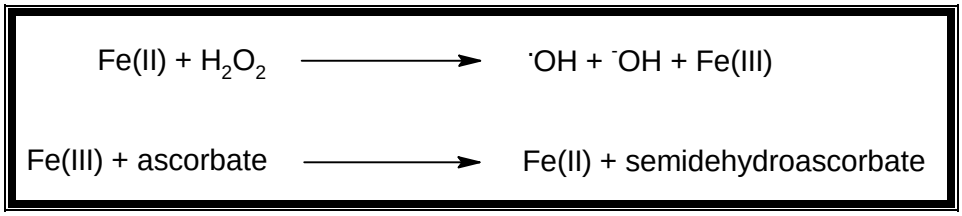

Figure 2.16 The Fenton reaction initiated with the TBARS assay employing a toxin-solution that consists of iron salts, ascorbic acid and H_2O_2 (Halliwell & Chirico, 1993).

2.7 Plants and medicine

Finding healing powers in plants is an ancient idea. For centuries, people have used plants as medicine in the ancient civilizations of China, India and North Africa. These civilizations used plants against a variety of diseases (Phillipson, 2001). There exists evidence of Neanderthals that lived 60 000 years ago in present-day Iraq, using hollyhock (*Alcea rosea* L.), which is still used in ethnomedicine around the world today (Cowan, 1999). Plants are recorded to have a prolonged history of use on the African continent for the treatment of various diseases and complaints (Hostettmann *et al.,* 2000). Developing countries continue to rely on ethnobotanical remedies as their main source of medicine since Western medicine is costly and often inaccessible as some rural communities are extremely isolated. It is reported that up to 80% of South Africans make use of traditional medicines (Van Wyk *et al.,* 1997). Higher plants are known to be the main source of drug therapy in traditional medicine (Aguilar, 2001) and approximately 75% of about 120 biologically active plant-derived substances have been discovered by following up on leads from traditional medicine throughout the world (Kinghorn, 2001).

There are approximately 119 drugs of known structure still extracted from about 90 species of commercially grown plants and there are at least 250 000 species of higher plants on earth. South Africa is blessed with being one of the richest centres of plant diversity in the world, consisting of approximately 30 000 indigenous plants (Van Wyk *et al.,* 1997). It therefore seems reasonable to presume that South Africa's enormous biodiversity has the potential to yield pharmacologically active compounds.

Compounds derived from higher plants contribute to the search for new drugs in a number of ways. The isolated pure compounds may act directly as new drugs to be used in an unmodified state. Examples include digoxin, digitoxin, morphine, reserpine, taxol, vinblastine and vincristine. The produced compounds may provide "building blocks" to be used in the synthesis of more complex compounds and the compounds may also serve as prototypes for the synthesis of drugs possessing physiological activities similar to the original compounds (Cox, 1990). The entire plant or part of it can also be used as a herbal remedy. Examples

include cranberry, Echinacea, feverfew, garlic, Ginkgo biloba, St. John's wort and Saw palmetto (Fabricant & Farnsworth, 2001).

The first chemical substance to be isolated from plants was benzoic acid in 1560. The search for useful drugs of known structure began in 1804 when morphine was isolated from *Papaver somniferum* L. (Opium). Salicylic acid is another compound that was isolated from willow bark that later led to the synthesis of aspirin (Raskin *et al.,* 2002).

Table 2.4 Drugs and chemicals derived from plants (Fabricant & Farnsworth, 2001; Kong *et al.,* 2003).

Plant source	Drug	Action/ Clinical use
Atropa belladonna	Atropine	Anticholinergic
Camellia sinensis (L.) Kuntze	Caffeine	CNS stimulant
Erythroxylum coca Lamk.	Cocaine	Local anaesthetic
Papaver somniferum L.	Codeine	Analgesic, antitussive
Mucuna spp.	*L*-Dopa	Anti-parkinsonism
Ephedra sinica Stapf.	Ephedrine	Sympathomimetic
Papaver somniferum L.	Morphine	Analgesic
Physostigma venenosum Balf.	Physostigmine	Cholinesterase inhibitor
Ephedra sinica Stapf.	Pseudoephedrine	Sympathomimetic
Rauvolfia serpentina (L.) Benth ex. Kurz	Reserpine	Antihypertensive, tranquilizer
Strychnos nux-vomica L.	Strychnine	CNS stimulant
Cannabis sativa	α-Tetrahydrocannabinol (THC)	Anti-emetic, decrease ocular tension

It has been documented that some naturally occurring substances in higher plants possess antioxidant activity. These naturally occurring substances include a wide variety of free radical scavenging molecules, such as flavonoids, anthocyanins, carotenoids, dietary glutathionine, vitamins and endogenous metabolites (Cao *et al.,* 1996).

2.8 Plant species with antioxidant properties

Several plants, (*Emblica officinalis*, *Curcuma longa, Mangifera indica, Santhum album* and *Withania somnifera*) have been highlighted for their antioxidant activity. Active principles have been isolated from plants, e.g. mangiferin, from *M. indica* L.; emblicanin A & B (two tannins) from *Phyllanthus emblica* L. and curcumin isolated from *C. longa* L. Fennel (*Foeniculum vulgare* Mill.) of the family Apiaceae has also exhibited antioxidant activity (Choi & Hwang, 2004). Galvez and co-workers (2005) have also observed promising antioxidant activity of *Plantago* species. Sage (*Salvia viridis*) possesses an inhibitory effect on H_2O_2. The antioxidant activity of Sideritis (*Sideritis montana* L.) of the Labiatae family is contributed to by the presence of flavonoids and phenylpropanoid glycosides (Abdel-Satter *et al.,* 1993).

Triterpenoids isolated from the leaves of wild African olive demonstrated antioxidant potential (Somova *et al.,* 2003). *M. officinalis* was also tested and exhibited promising free radical scavenging activity (Pourmorad *et al.,* 2006).

Table 2.5 Some South African medicinal plants with antioxidant activity (Atawodi, 2005).

Name of plant	Plant part studied	Type of assay (*in vitro/in vivo*)	Antioxidant potential (Good/Poor)
Sutherlandia frutescens	Not indicated	*In vitro*	Good
Pelargonium reniforme	Not indicated	*In vitro*	Good
Olea europa (Africana)	Leaves	*In vivo*	Good
Myrothamnus flabellifolia	Not indicated	*In vivo*	Good
Rhoicissus digitata	Leaves, stem, root	*In vitro*	Poor
Rhoicissus rhomboidea	Leaves, stem, root	*In vitro*	Good
Rhoicissus tomentosa	Leaves, stem, root	*In vitro*	Poor
Rhoicissus tridentate	Leaves, stem, root	*In vitro*	Good

2.9 Different antioxidant compounds found in plants

For the purpose of this study, a brief description of the following chemical constituents that occur in plants with antioxidant properties will be given:

- flavonoids;
- phenylpropanoid glycosides;
- and triterpenoids (Harborne, 1984).

2.9.1 Flavonoids

Flavonoids are widely found in plants as water soluble glycosylated derivatives and are responsible for the different brilliant shades such as blue, scarlet, and orange. Flavonoids can be found plant parts such as the leaves, flowers, fruits, seeds, nuts, grains, different medicinal plants and also beverages such as wine, tea and beer (Weisburger, 1997; Pietta, 2000; Gale, 2001).

These plant-derived antioxidants have been shown to function as singlet and triplet oxygen quenchers, peroxide decomposers, enzyme inhibitors and synergists. Some flavonoids were found to be a great deal more effective in preventing the damaging effects of aging by ROS than vitamin C and vitamin E (Cantuti-Castelvetri *et al.,* 2000). Flavonoids are essential for processing vitamin C and maintaining capillary walls. All flavonoids are phenolic substances that are structurally derived from the parent substance, flavone. There are about ten classes of flavonoids namely: anthocyanins, leucoanthocyanidins, flavonols, flavones, glycoflavones,

biflavonyls, chalcones and aurones, flavanones, isoflavones and betalains (see figure 2.17) (Harborne, 1984).

Flavonoids demonstrate several biological actions such as anti-tumoural, anti-ischaemic, anti-allergic, anti-hepatotoxic, anti-ulcerative, anti-inflammatory and antioxidant activities. Numerous biological activities of flavonoids can be attributed to their antioxidant properties and free radical scavenging capabilities. There exists an enormous variation in the antioxidant activities of flavonoids as a result of the differences of the backbone structure and functional groups of the different flavonoids. Some flavonoids can efficiently chelate metal ions (Shu, 1998). Polyphenols can chelate Fe^{2+} and thereby significantly changes its redox potential. The result is that polyphenols strongly interfere with the generation of free radicals (Halliwell & Gutteridge, 1990a; Halliwell & Gutteridge, 1992; Halliwell & Gutteridge, 1999).

2.9.2 Phenylpropanoids

Phenylpropanoids are naturally occurring phenolic compounds that consist of a three-carbon side chain attached to an aromatic ring. Phenylpropanoids include hydroxycinnamic acids, hydroxycoumarins, phenylpropenes and lignans (see figure 2.18). Hydroxycinnamic acids are usually found as esters in plants. Coumarin is the most widespread coumarin in plants. The phenylpropenes are typically isolated from the essential oil fraction of plant tissues. The phenylpropenes are lipid-soluble in comparison to most other phenolic compounds (Harborne, 1984).

2.9.3 Triterpenoids

The triterpenoids have relatively complex cyclic structures and are tipically found as alcohols, aldehydes or carboxylic acids. Triterpenoids are colourless and crystalline optically active substances with high melting points that exhibit a lack of chemical reactivity. Triterpenes can be divided into true triterpenes; steroids; saponins and cardiac glycosides (see figure 2.19). Saponins and cardiac glycosides are essentially triterpenes or steroids that occur as glycosides. Pentacyclic triterpenes, ursolic and oleanolic acids are widespread throughout the plant kingdom. These compounds usually occur in the waxy coatings of plant parts and may therefore serve a protective function. The three phytosterols, sitosterol (also known as β-sitosterol); stigmasterol and campesterol are generally found to be widespread in higher plants. Cholesterol is also found as a trace constituent in several higher plants. The cardiac glycosides are also known as cardenolides. The cardiac glycoside, oleandrin, is a toxin found in oleander (*Nerium oleander,* Apocynaceae). Most of the cardiac glycosides are toxic and exert their toxic effect on the heart, as the name implies (Harborne, 1984).

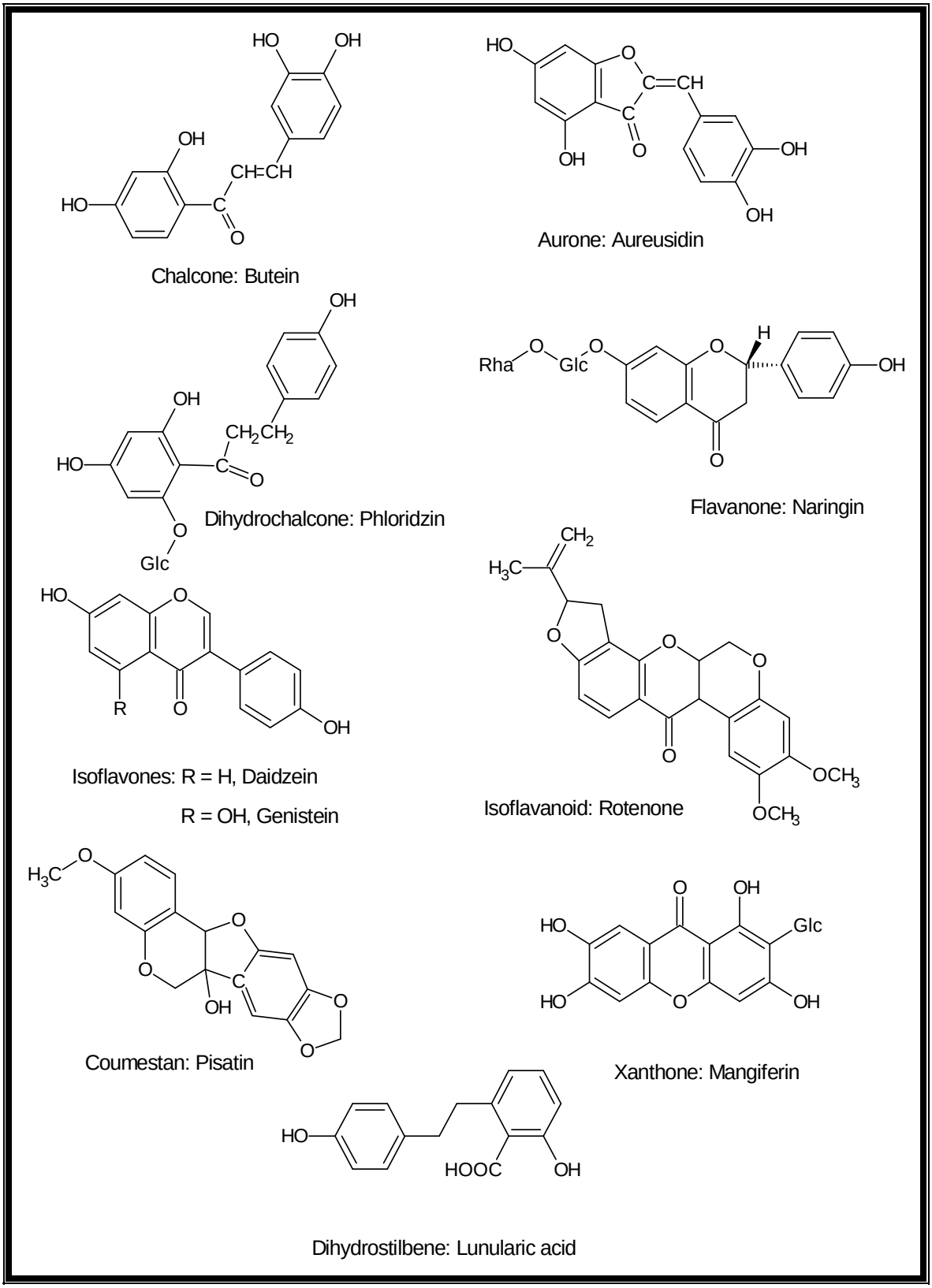

Figure 2.17 Structures of some flavonoids (Harborne, 1984).

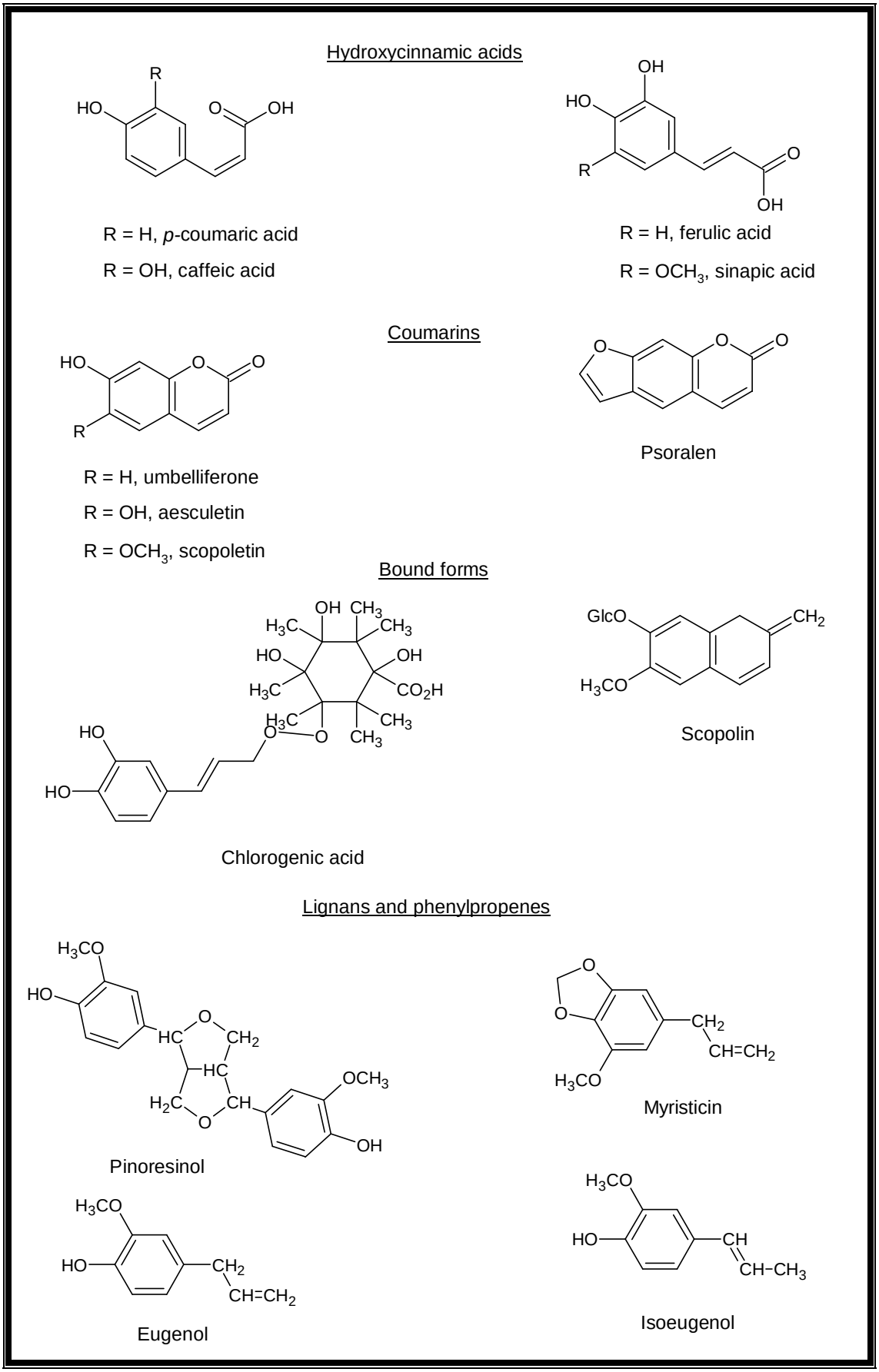

Figure 2.18 Structures of some phenylpropanoids (Harborne, 1984).

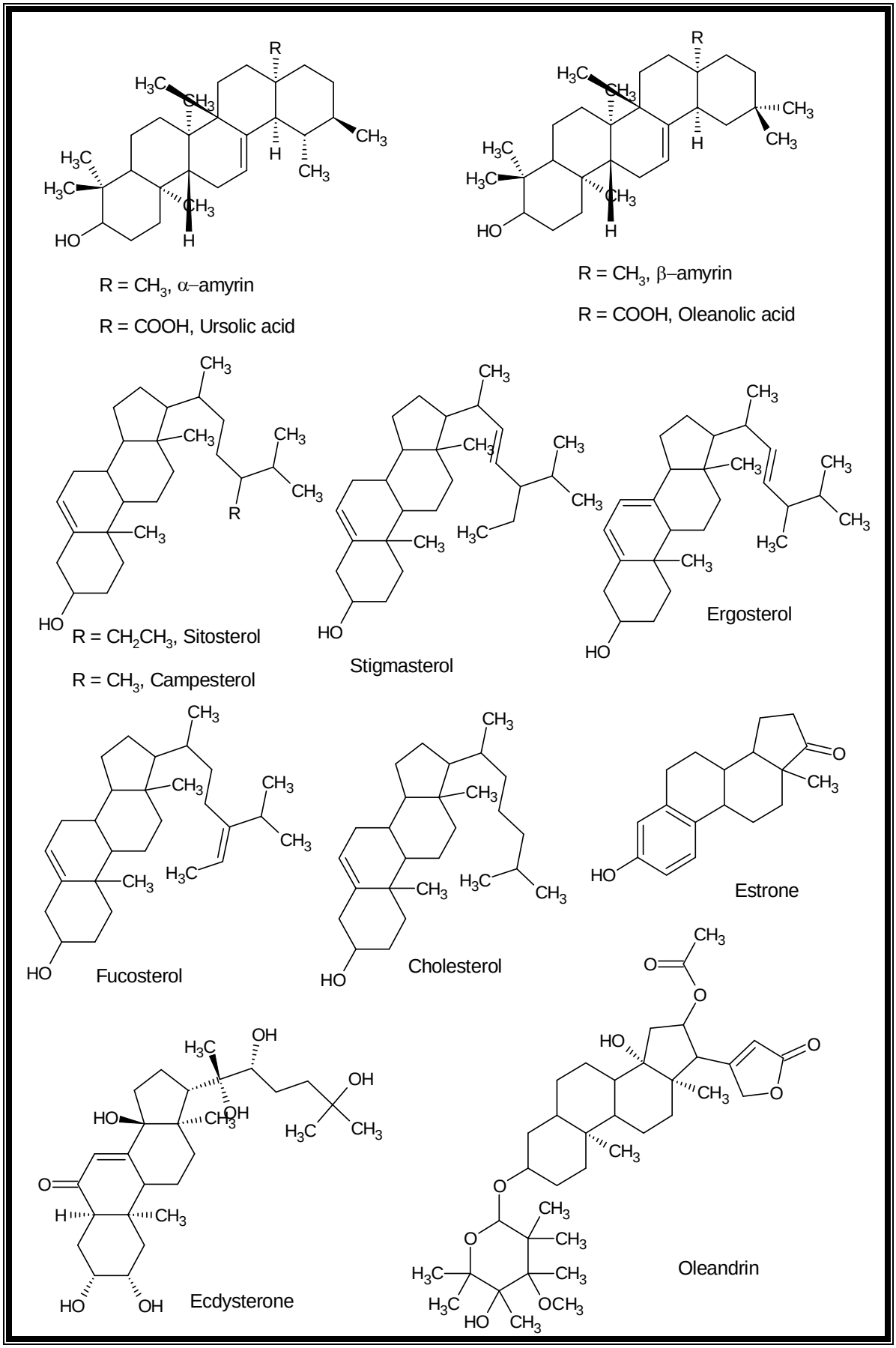

Figure 2.19 Structures of some triterpenoids (Harborne, 1984).

CHAPTER 3

Plants selected for screening

3.1 Selection of plants for screening for antioxidant activity

The first steps in a phytochemical investigation include the selection, collection and identification of plant material to be studied (Silva *et al.,* 1998). It is known that plants from the same family may contain the same classes of chemical compounds (Cox, 1990; Christensen & Kharazmi, 2001). Selecting plants on this basis is referred to as the chemotaxonomic approach. This chemotaxonomic approach was used in selecting the plants for this study. A thorough literature survey was conducted on plants that possess antioxidant activity. During the literature survey, care was taken that no previous research results regarding antioxidant activity had been published on the specific plant. Ten genuses were identified within those families. The final selection of twenty one plants was made taking the availability of adequate plant material into consideration. Table 3.1 lists the plants selected for this study.

Table 3.1 Plant families and species selected for this study

FAMILY	PLANT SPECIES
Apiaceae	*Berula erecta* *Heteromorpha arborescens*
Asteraceae	*Tarchonanthus camphorates* *Vernonia oligocephala*
Celastraceae	*Gymnosporia buxifolia*
Fabaceae	*Acacia karroo* *Elephantorrhiza elephantine* *Erythrina zeyheri*
Lamiaceae	*Leonotis leonurus* *Plectranthus ecklonii* *Plectranthus rehmanii* *Plectranthus venteri* *Salvia auretia* *Salvia runcinata* *Solenostemon latifolius* *Solenostemon rotundifolius*
Plumbaginaceae	*Plumbago auriculata*
Ranunculaceae	*Clematis brachiata*
Rubiaceae	*Vangueria infausta*
Solanaceae	*Physalis peruviana*
Verbenaceae	*Lippia javanica*

The plants selected for screening will be discussed briefly but *Lippia javanica* will be discussed in more detail as it was the plant selected from the initial screening. Voucher specimens are kept at the A.P. Goosens Herbarium (PUC), North-West University, Potchefstroom Campus, Potchefstroom.

3.1.1 Apiaceae

3.1.1.1 *Berula erecta* (Huds.) Coville

Figure 3.1 *Berula erecta* (Miles, 2007)

Herbarium: PUC 6804 (Schoeman, L.)

3.1.1.1.1 Botanical description of *B. erecta*

B. erecta is a perennial herb that can grow up to 1 m high. The leaves are elongated with the edges usually serrated or toothed. The flowers are white and borne in compound umbels. The fruits are ovate to oblong and somewhat ridged and rough (Leistner, 2000).

3.1.1.1.2 Chemical compounds isolated from *Berula* species

(Z)-falcarinol, β-sesquiphellandrene, β-caryophyllene and γ-terpinene were isolated from the essential oil fraction of *B. erecta.* Terpenoids, monoterpene hydrocarbons and sesquiterpene hydrocarbons were also isolated from *B. erecta* (Lazarevic *et al.,* 2010).

3.1.1.1.3 Traditional uses of *B. erecta*

The Zulu people take *B. erecta* in hot milk against fever and the South African people use the roots for toothache (Hutchings *et al*., 1996).

3.1.1.2 *Heteromorpha arborescens* (Spreng.) Cham. & Schltdl.

Figure 3.2 *Heteromorpha arborescens*

Herbarium: Van Wyk, A.E. 1433

3.1.1.2.1 Botanical description of *H. arborescens*

H. arborescens is a woody shrub or small tree reaching 7 – 15 m in height. The bark is reddish to purplish brown, smooth and somewhat waxy in appearance that peels off in characteristic horizontal papery flakes. It has thick, fleshy roots. The leaves vary in shape and are glossy, light or grey green, becoming yellow to red in autumn. The leaves are aromatic and when it is crushed it smells of parsley, hence the common name of Parsley tree (Fabian & Germishuizen, 1997; Van Wyk & Gericke, 2000; Coates-Palgrave, 2002; Van Wyk *et al*., 2002).

3.1.1.2.2 Chemical compounds isolated from *Heteromorpha* species

The antifungal compounds falcarindiol and asaricin, and α-pinene, germacrene C and sabinene were isolated from *H. arborescens* (Van Wyk *et al*., 2002).

3.1.1.2.3 Traditional uses of *H. arborescens*

Smoke from burning wood is inhaled to relieve headaches (Coates-Palgrave, 2002). The Sotho people administer leaf decoctions for mental and nervous diseases. Zimbabwean people use the roots for headaches, asthma, coughs, chest and back pain, fever and infertility (Hutchings *et al.,* 1996).

3.1.2 Asteraceae

3.1.2.1 *Tarchonanthus camphoratus* L.

Figure 3.3 *Tarchonanthus camphoratus* (Letsela *et al.,* 2002)

Herbarium: PUC 8761 (Van Heerden, M.)

3.1.2.1.1 Botanical description of *T. camphoratus*

T. camphoratus is a shrub or a small tree with a grey-green appearance, giving it the Afrikaans vernacular name "Vaalbos". It can grow up to 6 – 9 m high. The tree has a strong scent of camphor. The leaves are oblong in shape, with the upper surface dull-green to grey-green and strongly net-veined (Van Wyk et al., 2002). The lower surface is pale grey and velvety. The flowers are small, creamy white, followed by small nutlets covered with woolly hairs. The fruit resembles balls of cotton wool (Coates-Palgrave, 2002).

3.1.2.1.2 Chemical compounds isolated from *Tarchonanthus* species

Campor, α-fenchyl alcohol, 1,8-cineole, α-terpineol and the flavanone pinocembrin were isolated from *T. camphoratus* (Van Wyk *et al.,* 2002).

3.1.2.1.3 Traditional uses of *T. camphoratus*

Historic records show that early inhabitants of the Cape smoked the whole plant for its narcotic effect and dried leaves for its sedative effect (Van Wyk & Gericke, 2000). *T. camphoratus* is also used medicinally for headaches and respiratory complaints. The Sotho people smoke the green branches for headaches. People in the Western Cape smoke, chew or apply leaves as poultices for inflammation (Hutchings *et al.,* 1996).

3.1.2.2 *Vernonia oligocephala* (D.C) Sch.Bip. Ex Walp.

Figure 3.4 *Vernonia oligocephala* (Mankazana, 2009)

Herbarium: Bredenkamp, C. 175

3.1.2.2.1 Botanical description of *V. oligocephala*

V. oligocephala is an erect, leafy, perennial herb with the flowering branches growing from a woody rootstock. The leaves are elliptic, dark green, pointed sharply, smooth above and hairy and silvery below. The violet flowers are borne in large groups at the branch tips (Fabian & Germishuizen, 1997).

3.1.2.2.2 Chemical compounds isolated from *Vernonia* species

Sesquiterpenoid lactones, germacranolides and glaucolides were isolated from *Vernonia* species. Sesquiterpenoid lactones, 8α-(2-hydroxymethylacryloyloxy)-hirsutenolide-13-O-acetate and glaucolides were isolated from *V. oligocephala* (Hutchings *et al.,* 1996; Van Wyk *et al.,* 2002).

3.1.2.2.3 Traditional uses of *V. oligocephala*

The leaves and flowering stems are used in a health tonic and as an appetite stimulant, to treat malaise, abdominal pain, diarrhoea, dysentery, rheumatism, constipation and cilitis (Van Wyk & Gericke, 2000) and in Zambia, *V. oligocephala* is used for rheumatism (Hutchings *et al.,* 1996).

3.1.3 Celastraceae

3.1.3.1 *Gymnosporia buxifolia* (L.) Szyszyl. / *Maytenus heterophylla* (Eckl. & Zeyh.) N. Robson

Figure 3.5 *Gymnosporia buxifolia*

Herbarium: PUC 8766 (Killian, C.)

3.1.3.1.1 Botanical description of *G. buxifolia*

G. buxifolia is an evergreen shrub or small tree that can reach 9 m at times. The bark is light brown when young and becomes dark, flaking and rough with age. The spines are slender to robust, sharp and can be up to 10 cm long. The leaves are dull green and are either arranged in clusters or spirally on the branchlets. The lateral veins are apparent on both surfaces. The flowers are white and have an unpleasant smell (Fabian & Germishuizen, 1997). The fruit is a 3-lobed capsule that is smooth and nearly spherical. It has a green-yellow colour that becomes white to grey-brown when dry. There are 1 – 4 seeds per capsule and the seeds are reddish brown and glossy (Coates-Palgrave, 2002).

3.1.3.1.2 Chemical compounds isolated from *Gymnosporia* species

Dulcitol, celacinnine and three triterpenoids epifriedelanol, friedelin and epifriedelinol and maytansine were isolated from *M. heterophylla* (Hutchings *et al*., 1996).

3.1.3.1.3 Traditional uses of *G. buxifolia*

The Tswana, Koba and Subiya people take root and thorn decoctions for chest colds and coughs. In East Africa, root decoctions are taken for epilepsy. In West Africa, the leaves and roots are used as anti-inflammatory agents and for viral infections (Hutchings *et al.,* 1996).

3.1.4 Fabaceae

3.1.4.1 *Acacia karroo* Hayne

Figure 3.6 *Acacia karroo*

Herbarium: PUC 8763 (Van Heerden, M.)

3.1.4.1.1 Botanical description of *A. karroo*

A. karroo gets its common name (Sweet thorn) from the clear gum that exudes from the damaged trunk. The Sweet thorn varies from a multi-stemmed shrub to a tree with a dense, spreading and rounded crown, sometimes reaching a height of 12 – 15 m. The leaves are light-green and fern-like. The bark is dark brown to black, rough and flaky, revealing a reddish under bark. The thorns are white with dark tips, prominent, well developed and present as pairs on the twigs and branches. The flowers are borne in golden-yellow, ball-shaped heads and are sweetly scented. The fruit is long, narrow, spirally twisted pods (Fabian & Germishuizen, 1982; Van Wyk *et al.,* 2002).

3.1.4.1.2 Chemical compounds isolated from *Acacia* species

Tannins and gallotannins were isolated from *A. karroo*. 3,7,8,3',4'-pentahydroxyflavone, 7,8,3',4'-tetrahydroxy-3-methoxyflavone, 3,4,2',3',4'-pentahydroxy-*trans*-chalcone and 3,7,8,3'-tetrahydroxy-4'-methoxyflavone were isolated from *A. confusa* (Van Wyk *et al.,* 2002; Wu *et al.,* 2005).

3.1.4.1.3 Traditional uses of *A. karroo*

The pleasant tasting gum can be eaten, taken for oral thrush and applied to oral ulcers. It is also used in the pharmaceutical industry. The gum, bark and leaves have been used as an emollient and as a tonic for colds. An infusion made from the bark is given to cattle as an antidote to tulp (Moraea) poisoning (Coates-Palgrave, 2002; Van Wyk *et al.,* 2002).

3.1.4.2 *Elephantorrhiza elephantina* (Burch.) Skeels

Figure 3.7 *Elephantorrhiza elephantina*

Herbarium: PUC 7720 (Davoren, E.)

3.1.4.2.1 Botanical description of *E. elephantina*

E. elephantina has an enormous underground rhizome of up to 8 m long from which annual, unbranched stems grow. This shrub grows up to 1 m high. The leaves are finely divided and have numerous small, narrow leaflets, giving it a fern-like appearance. The small, cream-coloured flowers are sweetly scented and clustered along the bottom of aerial stems. The pods following the flowers are characteristically woody, dark to reddish brown, straight or slightly curved and strongly margined (Leistner, 2000; Van Wyk *et al.,* 2002).

3.1.4.2.2 Chemical compounds isolated from *Elephantorrhiza* species

Tannin (5.8 – 22.3%) and sugar (16.8%) were isolated from *E. elephantina* roots and tannin (25 – 30%) was isolated from *E. elephantina* root bark (Hutchings *et al.,* 1996).

3.1.4.2.3 Traditional uses of *E. elephantina*

Root decoctions can be used to treat fevers and chest complaints. *E. elephantina* may also be used to treat rheumatic heart conditions. In Zimbabwe, the roots are used for abdominal pain. The roots are also used in Botswana to clean the womb after an abortion and after a mother have just given birth (Hutchings *et al.,* 1996).

3.1.4.3 *Erythrina zeyheri* (Harv.)

Figure 3.8 *Erythrina zeyheri*

Herbarium: PUC 8767 (Killian, C.)

3.1.4.3.1 Botanical description of *E. zeyheri*

E. zeyheri is a small herb with the shoots arising from a large and tuberous underground rootstock. The rootstock is capable of causing great damage to a plough, giving rise to the common name, Plough-breaker. The leaves are large with sharp prickles. The flowers have a bright scarlet colour (Fabian & Germishuizen, 1997).

3.1.4.3.2 Chemical compounds isolated from *Erythrina* species

The alkaloids erysitrine, erythraline, 11-methoxyerythraline, erysodine, erysovine, erythrinine and erysopine have been isolated from *E. caffra*. The alkaloids erythraline, erysodine, erysovine, erysopine, erythratidine and erysosalvine have been isolated from *E. latissima* (Hutchings *et al.,* 1996).

3.1.4.3.3 Traditional uses of *E. zeyheri*

The bark fibres are used to treat asthma (Guillarmod, 1971).

3.1.5 Lamiaceae

3.1.5.1 *Leonotis leonurus* (L.) R. Br.

Figure 3.9 *Leonotis leonurus* (Reis, 2002)

Herbarium: Thompson, M. 68

3.1.5.1.1 Botanical description of *L. leonurus*

L. leonurus is classified as a shrub and can reach a height of 2 - 5 m. It has a thick woody base and the colour of the branches is pale brown. The leaves are simple, narrow, long, toothed, noticeably hairy and opposite each other on the stems. The flowers are bright orange, tubular and borne in definite rounded groups. These are distributed along the ends of the branches. The hairy flowers bear a resemblance to a lion's ears, hence the name "*leonurus*", which means lion's ears. All parts of the plant are aromatic (Van Wyk *et al*., 2002).

3.1.5.1.2 Chemical compounds isolated from *Leonotis* species

The diterpenoids marrubiin and resin were isolated from *L. leonurus* (Hutchings *et al.*, 1996; Van Wyk *et al.,* 2002).

3.1.5.1.3 Traditional uses of *L. leonurus*

There are early reports of people smoking *L. leonurus* as a substitute for dagga, but it is only mildly narcotic. It has also been smoked to relieve epilepsy. Decoctions of flowers, stems and leaves have been used externally to treat eczema, boils, skin diseases and itching (Van Wyk *et al.,* 2002). It is also used to treat colds, influenza, muscular cramps, skin diseases, high blood pressure and headaches. The Nama people apply ointments containing powdered leaves for pain above the eye (Hutchings *et al.,* 1996).

3.1.5.2 *Plectranthus ecklonii* (Benth.)

Figure 3.10 *Plectranthus ecklonii* (Van Jaarsveld, 2001)

3.1.5.2.1 Botanical description of *P. ecklonii*

P. ecklonii is an erect, soft and aromatic shrub that can grow up to 2.5 m high. The stems are hairy and much-branched growing from a woody base. The leaves are opposite, the margins toothed and dotted with reddish brown gland-dots on the undersurface. The flowers are tubular and ranges from pale blue or mauve to bluish purple, rarely white or pink and are borne in 6-flowered whorls (Fabian & Germishuizen, 1997; Manning *et al.,* 2001).

3.1.5.2.2 Chemical compounds isolated from *Plectranthus* species

A sesquiterpenoid, 15-hydroxyspathulenol and apigenin-7,4'-dimethyl ether, genkwanin, salvigenin, cirsimaritin, eupatorin, ursolic acid and oleanolic acid, 10(14)-aromadendrene-4*β*,15-diol, *ent*-3*β*-acetoxylabda-8(17),12*Z*,14-trien-2α-ol, *ent*-12*β*-acetoxy-15*β*-hydroxykaur-16-en-19-oic acid, *ent*-12*β*-acetoxy-7*β*-hydroxykaur-16-en-19-oic acid, *ent*-7*β*-hydroxykaur-15-en-19-oic acid, *ent*-12*β*-acetoxy-17-oxokaur-15-en-19-oic acid, *ent*-7*β*-hydroxy-15*β*,16*β*-epoxykauran-19-oic acid and *ent*-labda-8(17),12*Z*,14-triene-2α,3*β*-diol have been isolated from the polar fractions of the acetone extract of *P. fruticosus* (Gaspar-Marques *et al.,* 2004).

3.1.5.2.3 Traditional uses of *P. ecklonii*

P. ecklonii has been used to treat headaches and hayfever (Manning *et al.,* 2001). *P. ecklonii* is traditionally used to treat stomach aches, nausea, vomiting, meningitis, diarrhoea and skin diseases in South Africa (Nyila *et al.,* 2009).

3.1.5.3 *Plectranthus rehmanii* Guerke

Figure 3.11 *Plectranthus rehmanii*

3.1.5.3.1 Botanical description of *P. rehmanii*

P. rehmanii is an erect, branched herb or shrub that can reach a height of 1.2 m with the stems ascending. The leaves are ovate to oblong-ovate and the under-surface has orange-brown to dark glad-dots. The flowers of *P. rehmanii* are sigmoidal and predominantly white (Potgieter *et al.,* 2009)

3.1.5.3.2 Chemical compounds isolated from *Plectranthus* species

See section 3.1.5.2.2

3.1.5.3.3 Traditional uses of *P. rehmanii*

The tubers of *P. rehmanii* are a popular food in South Africa (Lukhoba *et al.,* 2006).

3.1.5.4 *Plectranthus venteri* Van Jaarsv. & Hankey

Figure 3.12 *Plectranthus venteri*

3.1.5.4.1 Botanical description of *P. venteri*

P. venteri is an erect herb that can grow up to 30 cm high with small leaves that usually have 2 pairs of lateral teeth. The leaves are aromatic with the flowers mauve to purple (Edwards *et al.,* 2000).

3.1.5.4.2 Chemical compounds isolated from *Plectranthus* species

See section 3.1.5.2.2

3.1.5.5 *Salvia auretia* Thunb.

Figure 3.13 *Salvia auretia*

3.1.5.5.1 Botanical description of *S. auretia*

S. auretia is a straggling, hairy perennial that can grow up to 1 m high. The leaves are usually lyrate-pinnatifid. The flowers are white, pinkish or mauve and borne in verticals (Goldblatt & Manning, 2000).

3.1.5.5.2 Chemical compounds isolated from *Salvia* species

Rosmanol is a major constituent of many *Salvia* species that possess strong antioxidant activities. Other major phenolic compounds include rosmarinic acid, carnosic acid, salvianolic acid as well as its derivatives carnosol, rosmanol, epirosmanol, rosmadial and methyl carnosate. Other compounds isolated from *S. sclarea* include ferruginol, salvipisone, microstegiol, candidissol, 2,3-dehydrosalvipisone, aethiopinone, 1-oxoaethiopinone, salvinolone, crytojaponol, acetylsalvipisone and sclareapinone. 6β-hydroxyisopimaric acid and 3-acetylvergatic acid have been isolated from *S. caespitosa* and 11β—hydroxymanoyl oxide, 8,13-diepimanoyl oxide, spathulenol, salvigenin, crysoeriol, diosmetin and *o,p*-dimethoxybenzoic acid have been isolated from *S. candidissima* (Tepe *et al.,* 2006).

3.1.5.6 *Salvia runcinata* L.f.

Figure 3.14 *Salvia runcinata* (Mountain Herb Estate, 2008)

3.1.5.6.1 Botanical description of *S. runcinata*

S. runcinata is a hairy, erect, perennial herb that grows up to 70 cm from a woody, creeping rootstock. The leaves are lyrate and hairy. The flowers are tubular, white or pale blue to mauve or purplish, 7 - 14 mm long and borne in verticals (Fabian & Germishuizen, 1997).

3.1.5.6.2 Chemical compounds isolated from *Salvia* species

See section 3.1.5.5.2

3.1.5.6.3 Traditional uses of *S. runcinata*

S. runcinata is used as a medicine for coughs. The Xhosa people administer medicine made from leaves and mothers' milk to new-born infants. The Sotho people make decoctions of parts of *S. runcinata* and *Graderia scabra* to prevent miscarriages and to relieve menstrual pain (Hutchings *et al.,* 1996).

3.1.5.7 *Solenostemon latifolius* (Hochst. Ex Benth.) J.K. Morton

Figure 3.15 *Solenostemon latifolius*

3.1.5.7.1 Botanical description of *S. latifolius*

S. latifolius is a perennial herb with semi-erect hairy stems. The leaves are teethed and hairy with a dark V-shaped blotch on the upper surface and red gland-dotted on the lower surface. The flowers are tubular and violet to purple (Fabian & Germishuizen, 1997).

3.1.5.7.2 Traditional uses of *S. latifolius*

S. latifolius leaves are taken orally in Uganda to induce labour (Kamatenesi-Mugisha & Oryem-Origa, 2007). Infusions and decoctions are also made from the leaves of *S. latifolius* in Uganda to treat intestinal worms (Ssegawa & Kasenene, 2007).

3.1.5.8 *Solenostemon rotundifolius* (Poir.) J.K. Morton

Figure 3.16 *Solenostemon rotundifolius* (Nkansah, 2004)

3.1.5.8.1 Botanical description of *S. rotundifolius*

S. rotundifolius is a small herbaceous perennial reaching 300 mm in height with aromatic leaves. The flowers are violet or blue and borne in elongated clusters. The tubers are formed on the lower parts of the stems and are rounded, dark brown and edible (Van Wyk & Gericke, 2000).

3.1.5.8.2 Traditional uses of *S. rotundifolius*

S. rotundifolius is used medicinally for treatment of eye diseases, dysentery as a result of indigestion, foot itching and ulcers (Kishorekumar *et al.,* 2008).

3.1.6 Plumbaginaceae

3.1.6.1 *Plumbago auriculata* Lam.

Figure 3.17 *Plumbago auriculata*

Herbarium: PUC 9764 (Manyakara, B.)

3.1.6.1.1 Botanical description of *P. auriculata*

P. auriculata is a shrub or scrambler reaching heights of up to 2 m or more. The stem is well-branched and climbing. The leaves are oblong, 20 – 25 mm long and the glands often dotted. The leaf stalk is characteristically winged at the base and partly clasps the stem. The flowers are sticky, glandular, pale blue or rarely white and borne in clusters on the tips of the branches (Fabian & Germishuizen, 1982; Van Wyk *et al.,* 2002).

3.1.6.1.2 Chemical compounds isolated from *Plumbago* species

The naphthoquinones plumbagin and *epi*-isoshinanolole and the steroids sitosterol and 3-O-glycosylsitosterol and plumbagic and palmitic acids were isolated from *P. auriculata* (Van Wyk *et al.,* 2002; De Paiva *et al.,* 2005).

3.1.6.1.3 Traditional uses of *P. auriculata*

The Zulu people snuff powdered roots or dried leaves to relieve headaches. The Xhosa people roasts and powder the roots and rub it onto fractures and stitches. Nigerian people use the roots for skin lesions (Hutchings *et al.,* 1996; Van Wyk & Gericke, 2000; Van Wyk *et al.,* 2002).

3.1.7 Ranunculaceae

3.1.7.1 *Clematis brachiata* Thunb.

Figure 3.18 *Clematis brachiata* (Immelman, 2008)

Herbarium: PUC 8765 (Manyakara, B.)

3.1.7.1.1 Botanical description of *C. brachiata*

C. brachiata is a vigorous climber that can reach heights of 3.5 – 4.5 m. It has spreading and hairy stems and bears masses of small (1 – 2 cm in diameter) white flowers. From time to time, the abundance of flowers causes the plant to bear a resemblance to a white mantle or robe, giving it the common name of Old Man's Beard. The leaves are dark green, thin and toothed. The fruit are brown, hairy and flattened (Fabian & Germishuizen, 1997; Fabian & Germishuizen, 1982).

3.1.7.1.2 Chemical compounds isolated from *Clematis* species

Oleanolic acid-3,28-biglucoside and phenylalanine-phenyl-alaninol-dipeptide were isolated form *C. montana* and aesculetin, chlorogenic acid, ferulic acid, gentisin and terniflorin were isolated from *C. terniflora* (Hegnauer, 1990).

3.1.7.1.3 Traditional uses of *C. brachiata*

The Zulu people apply a mixture of pounded leaves and red earth to children's rashes. To clear bad colds, the Xhosa people sniff bruised stems. Fresh leaves are used for colds and headaches by the Vhavenda people. The Ronga people take hot decoctions for steaming, malaria and colds (Hutchings *et al.,* 1996).

3.1.8 Rubiaceae

3.1.8.1 *Vangueria infausta* Burch.

Figure 3.19 *Vangueria infausta* (Hyde & Wursten, 2007)

Herbarium: PUC 8762 (Van Heerden, M.)

3.1.8.1.1 Botanical description of *V. infausta*

V. infausta is a shrub or small deciduous tree. The leaves are ovate, opposite and hairy with prominent veins below. The flowers are small, greenish-white to yellow and borne in clusters along the branchlets. The fruit is globular, yellow to brown, characteristically scarred around the tip and sweet-sour tasting (Joffe & Low, 1996; Fabian & Germishuizen, 1982; Van Wyk & Gericke, 2000).

3.1.8.1.2 Chemical compounds isolated from *Vangueria* species

Sterols were isolated from *V. infausta* (Hutchings *et al.,* 1996).

3.1.8.1.3 Traditional uses of *V. infausta*

Zimbabwean people use the roots for coughs, pneumonia and to wash the naval cords of newborn babies to prevent inflammation. In Mozambique, leaf infusions are taken for dental pain (Hutchings *et al.,* 1996).

3.1.9 Solanaceae

3.1.9.1 *Physalis peruviana* L.

Figure 3.20 *Physalis peruviana* (Forest & Starr, 2006)

3.1.9.1.1 Botanical description of *P. peruviana*

P. peruviana is a perennial herb that can grow up to 1.5 m high. The stems are stout, covered with hairs and woody at the base. The leaves are ovate and 8 – 10 cm long. The flowers are yellow with a purple centre (Adamson, 1950).

3.1.9.1.2 Chemical compounds isolated from *Physalis* species

Oleic acid, linoleic acid, palmitic acid, saponins, glucose, ascorbic acid, tigloidine, 3α-tigloyloxytropane, physalin A, withanolide E, physalolactone B, physoperuvine,

(+)-N,N-dimethylphysoperuvinium and β-sitosterol were isolated from *P. peruviana* (Hutchings *et al.,* 1996).

3.1.9.1.3 Traditional uses of *P. peruviana*

South African people use warm leaves as poultices for inflammation. In East Africa, root decoctions are used for skin rashes, excess bile, colds, labour pain, gonorrhoea and general ill-health. In East Africa, warm leaves are applied to the body to relieve pain (Hutchings *et al.,* 1996).

3.1.10 Verbenaceae

3.1.10.1 *Lippia javanica* (Burm.f.) Spreng.

Figure 3.21 *Lippia javanica* (Le Roux, 2004)

Herbarium: De Feijter, C. 33

3.1.10.1.1 History of *L. javanica*

L. javanica was first classified as part of the Lamiaceae family (Van Wyk & Gericke, 2000) but is now included in the Verbenaceae family. The genus *Lippia* includes approximately 200 species that varies form herbs and shrubs to small trees (Leistner, 2000). There are only 6 species that are indigenous to South Africa (Pascual *et al.,* 2001). *Lippia* was named after Augustin Lippi (1678 – 1701), an Italian traveller and natural historian who was killed in modern day Ethiopia. This plant also occurs in Java, hence the epiphet "*javanica*" (Latin) (Fabian & Germishuizen, 1982; Fabian & Germishuizen, 1997).

3.1.10.1.2 Botanical description of *L. javanica*

L. javanica is a woody shrub that can reach a height of 1 – 2 m. It stands erect and is multi-stemmed. The leaves are hairy with noticeable veins and are highly aromatic, giving off a

strong lemon-like smell when crushed. The leaves are simple, opposite each other or in whorls of 3 along the stems. The flowers are small and yellowish-white and are arranged in dense, rounded flower heads (Fabian & Germishuizen, 1997; Van Wyk & Gericke, 2000; Van Wyk *et al.,* 2002).

3.1.10.1.3 Distribution of *L. javanica*

L. javanica is spread throughout large parts of South Africa, with the exception of the Western Cape. *L. javanica* grows from the Eastern Cape northwards extending into tropical Africa including Swaziland and Botswana (Fabian & Germishuizen, 1982; Van Wyk *et al.,* 2002). This plant occurs in woodland, bushveld and grassland, often in disturbed areas (Coates-Palgrave, 2002).

3.1.10.1.4 Chemical compounds isolated from *Lippia* species

The monoterpenoids: myrcene, caryophyllene, linalool, p-cymene and ipsdienone and the organic acids: stearic-, palmitic-, myristic-, oleic-, arachidic-, behenic-, and lignoceric acids and alcohols were isolated from *L. javanica.* (+)-hernandulcin was isolated form *L. dulcis.* Iridoid glycosides, triterpenoids, camphor, carbone, cineole, citral, citronellol, geraniol, isovaleric acid, lactose, limonene, maltose, saponin, terpineol, thujone and verbenone were isolated from *Lippia* species (Hutchings *et al.,* 1996; Pascual *et al.,* 2001; Van Wyk *et al.,* 2002).

3.1.10.1.5 Traditional uses of *L. javanica*

Teas are made as infusions to treat coughs, colds, bronchitis and fever. It is also used for a variety of chest ailments, measles, influenza, malaria, rashes, headaches and stomach problems. A general health tea is made from weak infusions (Van Wyk *et al.,* 2002). Zulu people use cold infusions of the leaves for the condition gangrenous rectitis. The Xhosa people take weak leaf and stem infusions for coughs, colds and bronchial ailments. *Artemesia afra* is added to infusions to treat fevers and measles. *L. javanica* is also used to disinfect meat suspected of being infected with anthrax. In Zimbabwe, the leaves are used for asthma, headaches, fever, respiratory ailments and convulsions. Leaf infusions are used for respiratory and febrile ailments and prophylactic against dysentery, diarrhoea and malaria by the Vhavenda people. In Botswana, the roots are used for bronchitis, sore eyes and as an antidote for food poisoning. The leaves and roots are used for fevers, headaches and skin diseases in West Africa (Hutchings *et al.,* 1996).

CHAPTER 4

Selection of plants, collection and storage of plant material and preparation of extracts

4.1 Selection of plants

Following an extensive literature survey, ten plant families with described antioxidant activity were identified. The selected families are as follows: Apiaceae, Asteraceae, Celastraceae, Fabaceae, Lamiaceae, Plumbaginaceae, Ranunculaceae, Rubiaceae, Solanaceae and Verbenaceae. Species from each family were selected keeping the following factors in mind:

- Reported antioxidant activity of plants from the same family,
- Availability in the Potchefstroom area and
- No previous reports of antioxidant testing on the chosen species.

4.2 Collection and storage of plant material

Plant material was harvested from the Potchefstroom Botanical Garden and Potchefstroom area. Plant material was collected during March 2005. Plant specimens were authenticated by Mr. Peter Mortimer, the then Curator of the Botanical Garden, North-West University (Potchefstroom Campus). The green plants were separated into different morphological parts and dried. Separating plants into different morphological parts produces less complex extracts, whilst eliminating possible contamination from other plant parts (Cannel, 1998). The leaves were chosen for the experiment in order to enhance conservation by ensuring sustainable harvesting of medicinal plants. Also, flowers occur only seasonally and Angioni and co-workers (2006) found a decrease in essential oil yields as the flowering stage progressed, while yields from the leaves remained constant throughout the year. Before drying the leaves, care was taken to remove old, damaged or infected leaves in order to eliminate infection as infection can cause changes in metabolites produced by the plant (Silva *et al.,* 1998). The healthy leaves were spread out and dried in our laboratory at room temperature for approximately 3 weeks or until the leaves broke easily by hand. The dried leaves were stored in brown paper bags until time of use.

4.3 Preparation of extracts

4.3.1 Soxhlet extraction

Samples were ground using a mortar and pestle. Extraction was performed with 1 - 53 grams of plant leaf material. In this study conventional Soxhlet extraction was used

since it is a very convenient way of preparing plant extracts. A range of pure solvents was used successively in order of increasing polarity starting with petroleum ether (PE) (to remove fats and waxes) (Silva *et al.,* 1998), then dichloromethane (DCM) (to separate lipids and terpenoids) (Harborne, 1984), followed by ethyl acetate (EtOAc) and ethanol (EtOH) for more polar compounds. This range of solvents was selected to reduce the complexity of extracts by selective extraction based on the polarity of phytochemicals (Eloff, 1998). By using a wide range of solvents, it was ensured that all possible plant constituents with different polarities were present in the screened extracts.

The important advantages of Soxhlet extraction are that the plant material is separated from the extract and the sample is repeatedly brought into contact with fresh portions of the solvent. Furthermore, the temperature of the system is close to the boiling point of the solvent, providing energy in the form of heat that helps to increase the extraction kinetics of the system (Ganzler & Salgó, 1987). Soxhlet extraction is relatively inexpensive and selectivity of the extraction can easily be manipulated by altering the polarity of the solvent.

The disadvantages of Soxhlet extraction are that it requires several hours or days of extraction and losses of compounds occur due to thermal degradation and volatilization because of the heat supplied (Ganzler & Salgó, 1987).

The plant material was exhaustively extracted until the solvent appeared clear. After the extraction was complete, the solvent was removed by rotary evaporation and the extract was dried completely *in vacuo*. For quantitative determination, the extracts were placed in pre-weighed polytops before drying. Technical grade acetone [Merck] was used as solvent to subsequently re-dissolve the extracts for further analysis. All operations were performed under light protection.

4.3.2 Extracts obtained

Table 4.1 Description of extracts obtained

Plant	Mass of plant material (grams)	Extract (Solvent)	Mass of extract (grams)	Yield (%)	Description
Berula erecta	3.45	PE	0.017	0.49	Yellow substance
		DCM	0.065	1.88	Brown yellowish substance
		EtOAc	0.055	1.59	Green brownish substance
		EtOH	0.094	2.74	Green substance
Heteromorpha arborescens	8.75	PE	0.151	1.72	Yellowish white deposit
		DCM	0.049	0.56	Dark green substance
		EtOAc	0.133	1.52	Dark green substance
		EtOH	3.551	40.58	Dark green substance

Plant	Mass of plant material (grams)	Extract (Solvent)	Mass of extract (grams)	Yield (%)	Description
Tarchonanthus camphoratus	5.55	PE	0.206	3.71	Light yellow substance
		DCM	0.176	3.17	Brown-green substance
		EtOAc	0.042	0.76	Green substance
		EtOH	0.320	5.77	Dark green substance
Vernonia oligocephala	10.12	PE	0.191	1.89	Yellow powder
		DCM	0.763	7.54	Green substance
		EtOAc	0.267	2.64	Dark green substance
		EtOH	0.468	4.62	Brown-green substance
Gymnosporia buxifolia	28.70	PE	0.886	3.09	Yellow-green substance
		DCM	0.375	1.31	Dark green substance
		EtOAc	0.203	0.71	Dark green substance
		EtOH	0.821	2.86	Yellow-brown substance
Acacia karroo	36.30	PE	0.453	1.25	Bright milky yellow deposit
		DCM	0.386	1.06	Dark green deposit
		EtOAc	0.519	1.43	Dark green deposit
		EtOH	2.061	5.68	Brown green substance
Elephantorrhiza elephantina	24.20	PE	1.064	4.40	Brownish yellow substance
		DCM	0.590	2.44	Yellow-green substance
		EtOAc	0.618	2.55	Dark green deposit
		EtOH	0.732	3.00	Brownish green substance
Erythrina zeyheri	25.19	PE	0.350	1.39	Bright yellow substance
		DCM	0.348	1.38	Dark green substance
		EtOAc	0.373	1.48	Dark green substance
		EtOH	0.407	1.62	Bright green substance
Leonotis leonurus	11.91	PE	0.331	2.78	Orange powder
		DCM	0.588	4.94	Green substance
		EtOAc	0.101	0.85	Dark green substance
		EtOH	0.675	5.67	Greenish brown substance
Plectranthus ecklonii	8.66	PE	0.169	1.95	Brownish orange substance
		DCM	0.274	3.17	Brown substance
		EtOAc	0.107	1.23	Dark green substance
		EtOH	0.173	2.00	Dark green substance
Plectranthus rehmanii	4.83	PE	0.182	3.76	Brownish yellow substance
		DCM	0.118	2.45	Green substance
		EtOAc	0.083	1.72	Dark green substance
		EtOH	0.387	8.01	Brownish green substance
Plectranthus venteri	2.76	PE	0.078	2.83	White-green substance
		DCM	0.043	1.58	Green substance
		EtOAc	0.028	1.03	Dark green substance
		EtOH	0.124	4.47	Dark green substance

Plant	Mass of plant material (grams)	Extract (Solvent)	Mass of extract (grams)	Yield (%)	Description
Salvia auretia	2.08	PE	0.087	4.19	Light orange substance
		DCM	0.043	2.06	Green powder
		EtOAc	0.023	1.09	Dark green substance
		EtOH	0.180	8.65	Dark green substance
Salvia runcinata	7.43	PE	0.136	1.83	Bright yellow substance
		DCM	0.346	4.66	Green substance
		EtOAc	0.098	1.31	Bright green substance
		EtOH	0.064	0.86	Green substance
Solenostemon latifolius	1.16	PE	0.017	1.47	Light green powder
		DCM	0.031	2.71	Orange-brown substance
		EtOAc	0.020	1.72	Green substance
		EtOH	0.026	2.24	Dark green deposit
Solenostemon rotundifolius	2.64	PE	0.072	2.73	Bright yellow deposit
		DCM	0.056	2.11	Brown-green substance
		EtOAc	0.060	2.25	Green substance
		EtOH	0.298	11.27	Green substance
Plumbago auriculata	6.16	PE	0.045	0.73	Light green powder
		DCM	0.030	0.47	Dark green substance
		EtOAc	0.081	1.31	Dark green substance
		EtOH	0.547	8.88	Brown-green substance
Clematis brachiata	8.40	PE	0.079	0.94	Greenish brown deposit
		DCM	0.105	1.25	Dark brownish green substance
		EtOAc	0.137	1.63	Greenish brown deposit
		EtOH	0.836	9.96	Dark green deposit
Vangueria infausta	53.80	PE	0.307	0.57	Greenish yellow powder
		DCM	0.682	1.27	Black-green deposit
		EtOAc	0.419	0.78	Black-green deposit
		EtOH	0.884	1.64	Green substance
Physalis peruviana	6.69	PE	0.130	1.95	Yellow-green deposit
		DCM	0.096	1.43	Brown-green deposit
		EtOAc	0.094	1.41	Green substance
		EtOH	0.265	3.96	Brownish green substance
Lippia javanica	4.17	PE	0.043	1.02	Orange-yellow substance
		DCM	0.083	1.99	Brown-green substance
		EtOAc	0.107	2.56	Dark green substance
		EtOH	0.388	9.30	Green-yellow substance

CHAPTER 5

Primary screening

5.1 Primary screening of plant extracts

Several assays for measuring antioxidant activity have been developed during the last several decades. The most common assays include the ferric reducing antioxidant power (FRAP) assay and the oxygen radical absorbance capacity (ORAC) assay among others. These methods differ in terms of their assay principles and experimental conditions, as a relatively different aspect of antioxidant activity is measured by each test. None of these tests however measure the antioxidative actions of a compound in a living organism, since all of these tests are done in a laboratory using artificial environments (Cao & Prior, 1999).

Antioxidant screening methods may thus provide the required preliminary observations to select among crude leaf extracts those with potentially useful properties for further chemical and pharmacological investigations.

5.1.1 Oxygen radical absorbance capacity assay

5.1.1.1 Introduction and general principles of the ORAC assay

The ORAC assay was originally developed by Cao and co-workers (1993) and was significantly improved by Ou and co-workers (2001). The ORAC assay is based on the ability of antioxidants to protect a fluorescent probe from damage by free radicals initiated by the thermal decomposition of azo-compounds (Glazer, 1990). For this assay, 2,2'-azobis-(2-amidinopropane) hydrochloride (AAPH) was used to produce the peroxyl radical ($ROO^{\cdot}$) at a constant rate through thermal decomposition (Cao & Prior, 1998).

The ORAC assay utilizes a biologically relevant free radical, the reaction is run to completion and the change in fluorescence of the probe over time is calculated using the area under the fluorescence decay curve (AUC) method (figures 5.4 & 5.5) (Huang, *et al.,* 2002b; Awika *et al.,* 2003). The ORAC assay is thus ideally suited to measure the antioxidant capacity of a sample (Ghiselli *et al.,* 2000).

The peroxyl radical ($ROO^{\cdot}$) initiates oxidation of fluorescein and in so doing causes a decrease in fluorescein fluorescence intensity. The decrease in fluorescein fluorescence intensity is the index of the extent of damage to fluorescein (Ou *et al.,* 2001). In the presence of an antioxidant, free radical damage is inhibited and a more stable fluorescence signal is produced (Cao & Prior, 2001).

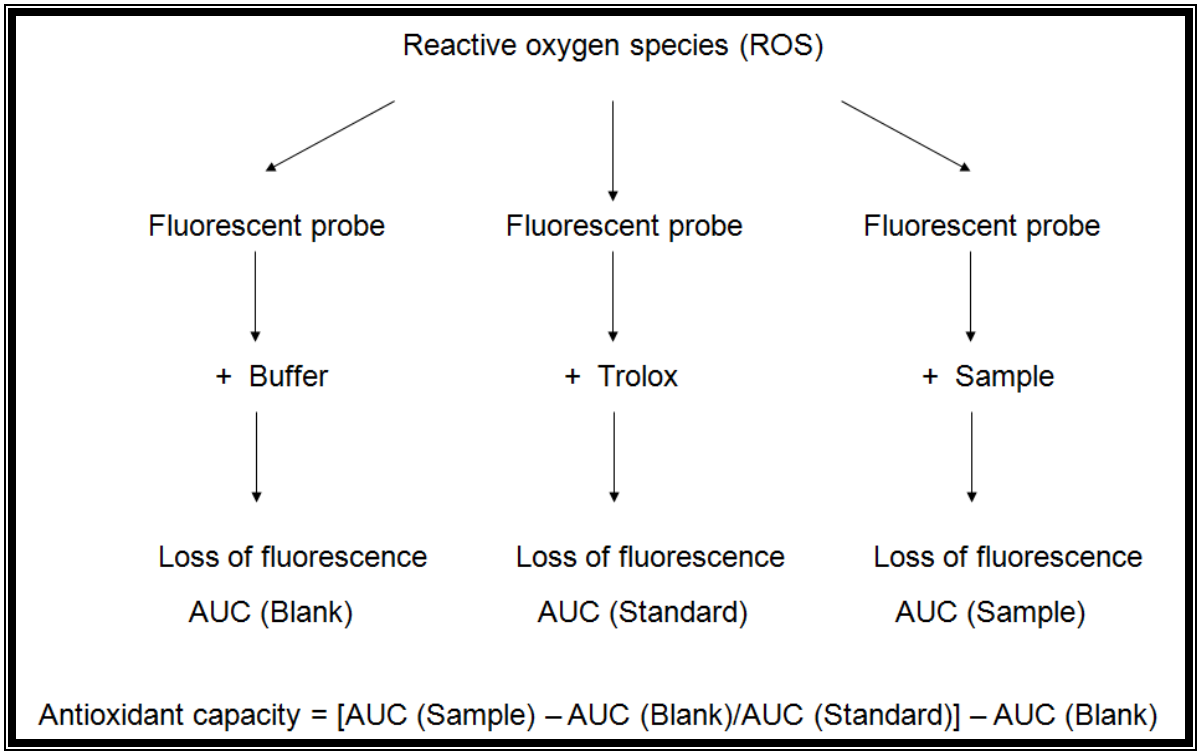

Figure 5.1 Schematic depiction of the ORAC assay. ROS generators are added to parallel reactions that contain equal amounts of a fluorescent probe. Reactions contain either a buffer blank or antioxidant samples and standards. The antioxidant capacity of a sample is the net difference between the area under the curve (AUC) of the sample and that of the blank. Figure adapted from Huang and co-workers (2002b).

The reference standard to which all the other antioxidant compounds were compared to is Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (a water-soluble analogue of vitamin E). Trolox is a potent antioxidant but unlike vitamin E, it does not occur naturally in the body. Being the standard, all ORAC results are reported in Trolox Equivalents (TE).

There exists a very complex reaction among the free radical, the substrate and the antioxidant. It is impossible to use a fixed equation to express the kinetic order because of this complexity. One has to take both the inhibition degree and inhibition time into account to accurately measure antioxidant capacity (Cao & Prior, 1999).

Most methods for determining antioxidant capacity are carried out in an aqueous environment. The antioxidant capacity of lipophilic antioxidants have therefore been overlooked in the past. A validated ORAC assay for lipophilic antioxidants was developed by introducing RMCD (methylated β-cyclodextrin) as the solubility enhancer (Ou *et al.,* 2001).

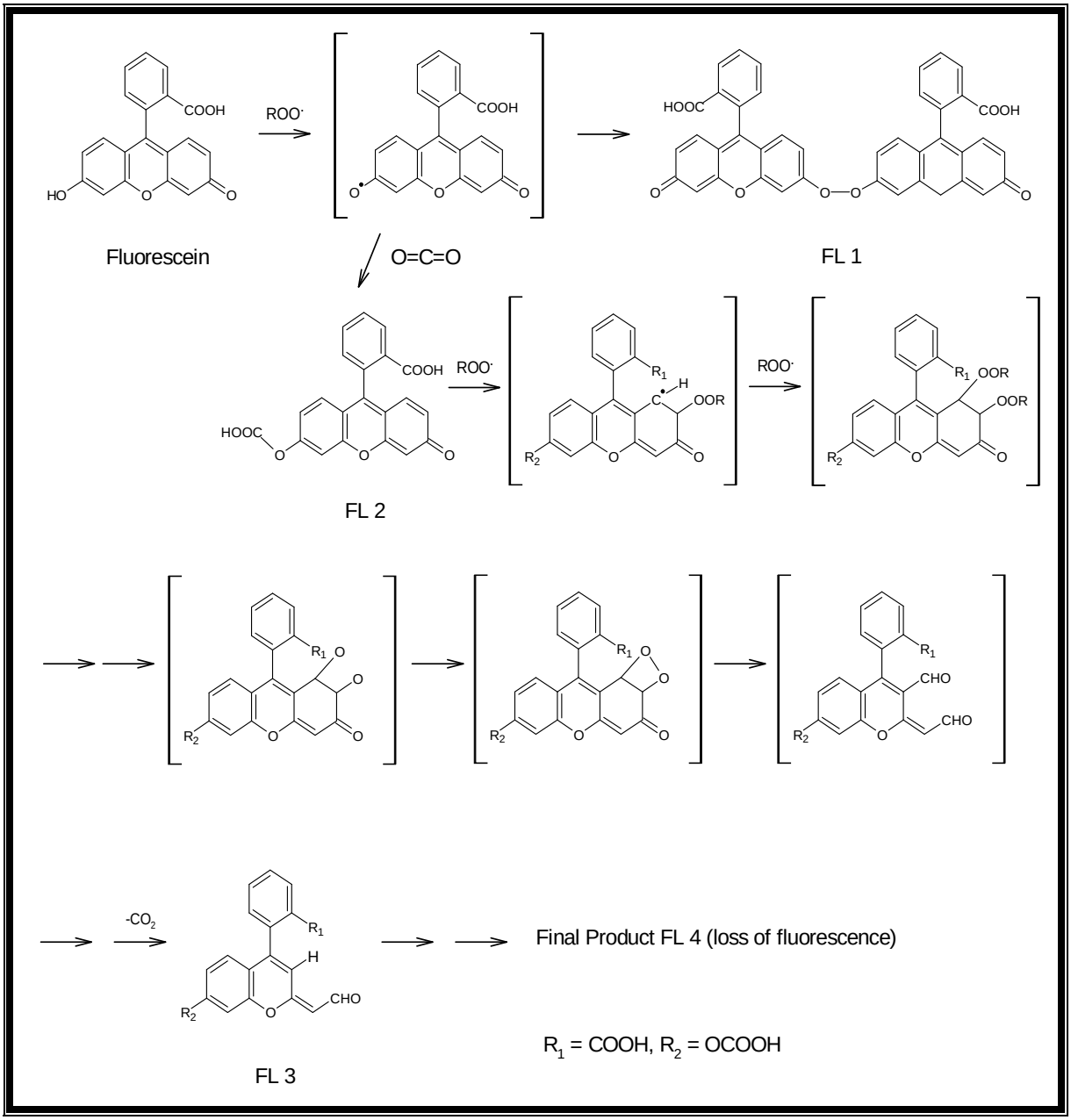

Figure 5.2 The proposed fluorescein pathway in the presence of AAPH (Ou *et al.,* 2001).

A high ORAC score does not necessarily mean a high antioxidant activity in the body. The current antioxidant measure is merely approximations of how an antioxidant might behave in the body.

5.1.1.2 Experimental procedure

5.1.1.2.1 Chemicals

Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), 2,2'-azobis(amidino-propane) dihydrochloride (AAPH) and fluorescein (3',6'-dihydroxy-spiro[isobenzofuran-1[3*H*],9'[9*H*]-xanthen]-3-one) were purchased from Sigma Chemical Co., St. Louis, MO. Randomly methylated cyclodextrin (RMCD) was a generous gift from Prof. F.H. van der

Westhuizen (Department of Biochemistry, NWU, Potchefstroom campus). All other chemicals were of analytical grade.

5.1.1.2.2 Reagents

Phosphate buffer (75 mM, pH 7.4): 1M K_2HPO_4 and NaH_2PO_4, (61.6:38.9, v/v) were diluted with deionised water (1:9, v/v) to make up 75 mM phosphate buffer. The pH was ascertained to be 7.4.

A main stock solution of fluorescein, 10% (w/v) in water [265 mM] was prepared. Before each assay, 1 µl of the stock solution [265 mM] was diluted with 999 µl phosphate buffer (solution #1) and 8 µl of solution #1 was further diluted with 1996 µl phosphate buffer to give the effective solution #2 [112 nM].

A 72 mM solution of 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) in phosphate buffer was freshly prepared for each run and kept on ice until it was loaded onto the microtitre plate. This was done as AAPH is temperature sensitive.

Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), 250 µM, in phosphate buffer was aliquoted in vials and kept at -70 °C for 4 months. A new set of stock Trolox vials were removed from the freezer daily for use. A solution of 20 µM was prepared by diluting the stock solution with buffer (1:4, v/v). All solutions were prepared fresh daily.

5.1.1.2.3 Preparation of samples (Extraction)

The samples were extracted in two stages to obtain lipophilic and hydrophilic antioxidant constituents. 10 mg of each extract was accurately weighed and dissolved in the same solvent used for the Soxhlet extraction (PE, DCM, EtOAc and EtOH). This was done since the crude extracts showed poor solubility in hexane.

The leaf extracts were then extracted with 1 ml of hexane followed by centrifugation for 5 minutes at 4300 x g. The hexane layer (supernatant) was removed and transferred to another set of tubes. The extraction and centrifugation steps were repeated. The supernatant was removed again and combined with the previously transferred hexane layer. The hexane of the lipophilic fraction tubes was evaporated under nitrogen after which it was dissolved in 125 µl of acetone and diluted with 375 µl of a 7% solution of randomly methylated cyclodextrin (RMCD). This was the lipophilic fraction of the plant extract.

The hexane residues of the initial tubes were evaporated under nitrogen gas and were then extracted with 1 ml of an acetone/water/glacial acetic acid (70:29.5:0.5, v/v/v) solution. The tubes were vortexed for 30 seconds and then sonicated for 5 minutes at 37 °C. The tubes were inverted once during the sonication step. The tubes were kept at room temperature for

10 minutes while shaking them regularly, followed by centrifugation for 15 minutes at 3500 x g. The supernatant was removed and transferred to another set of tubes. This was the hydrophilic fraction of the plant extract.

5.1.1.2.4 Instrumentation

All ORAC analyses were performed on a BioTek® FL600 microplate fluorescence reader using an excitation wavelength of 485 nm and an emission filter of 520 nm.

5.1.1.2.5 ORAC-assay

The ORAC assay was performed as detailed by Ou and co-workers (2001). Analyses were conducted at ambient conditions of pH 7.4 and temperature of 25 °C. A black 96-well Costar opaque microtitre plate was used for the ORAC assay and every sample was pipetted in triplicate. The first row of the opaque plate was set aside for the Trolox reference standards. Different dilutions of Trolox® (0 µM – 20 µM) were prepared in phosphate buffer (pH 7.4, 75 mM). Sample wells received 20 µl of sample. 80 µl of fluorescein was added before adjusting the sensitivity to ± 146. To determine the background signal, the initial 485/520 reading was determined. The initial 485/520 reading should be in the region of 65000 relative fluorescence units (RFU). The sensitivity should be adjusted to attain a RFU of 65000.

After detecting the background signal, the reaction was initiated by adding 100 µl of AAPH. Once AAPH is added, the plate is mixed briefly and fluorescence is measured instantly. This had to be done as quickly as possible since the ROS-generator displays immediate activity after addition. The test was then resumed and fluorescence intensity was monitored kinetically in static mode and fluorescence readings were taken every 5 minutes for up to 3 hours. The fluorescence of the last reading should be less than 5% of the initial reading. If this was not achieved, the dilution of the analyzed sample was adjusted accordingly and the sample was run again.

All fluorescent measurements were expressed relative to the initial reading. The raw RFU data, recorded by KC4 software, was then exported to a disk.

5.1.1.3 Data collection

The raw data was imported to a Microsoft Excel worksheet.

Increasing concentrations of Trolox were used as a standard for measuring the peroxyl radical scavenging activity by means of the ORAC assay. The standards, containing 0 - 20 µM Trolox at increments of 5 µM were used to generate a standard curve using a second order polynomial equation (Eq. 5.1).

$y = ax^2 + bx + c$ Equation 5.1

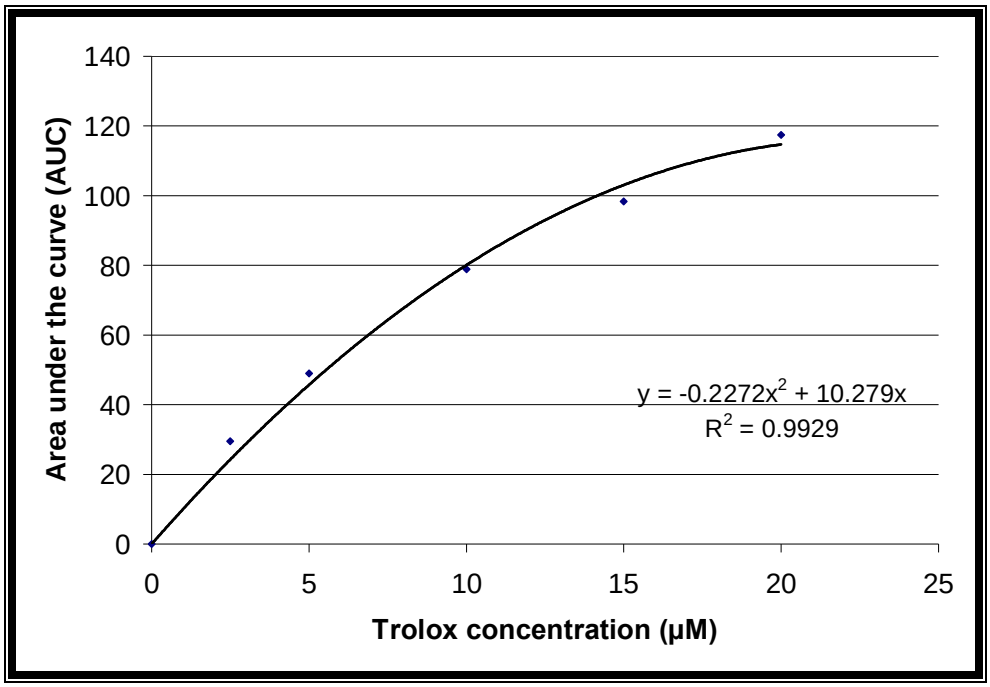

Figure 5.3 The ORAC assay standard curve generated from Trolox. Data is expressed as concentration Trolox plotted against the absorbance values measured at 485 nm and results in a regression of 0.9929. n = 2

The ORAC value is attained by determining the area under the quenching curve of the sample and subtracting the area under the blank quenching curve. The same calculation is done with the Trolox standard curve and corresponding blanks. The resulting net protection area of the sample is then divided by the Trolox net protection area. One ORAC unit is equivalent to the net protection area provided by 1 µM of Trolox. The greater the extent of fluorescent decay, the smaller the expected AUC value would be.

The net area under the fluorescence curve was calculated as follows:

$AUC = (0.5 + f_5/f_0 + f_{10}/f_0 + f_{15}/f_0 + \ldots\ldots\ldots + f_{175}/f_0 + f_{180}/f_0) \times T$ Equation 5.2

where f_0 is the initial fluorescence at 0 minutes, f_{180} is the fluorescence measurement at 180 minutes and T is the cycle time (Cao & Prior, 1999).

Final ORAC values were calculated using the regression equation between Trolox concentration and the net AUC. The final ORAC values were calculated as follows:

$$\text{ORAC value} = -b + \sqrt{\frac{b^2 - 4a(c-y)}{2a}} \times \text{dilution factor}$$ Equation 5.3

Equation 5.3 was mathematically derived from equation 5.1 to calculate the final ORAC value. The final ORAC values were expressed as micromole Trolox equivalents (TE) per 10 milligram of crude plant extract (µmole of TE/10 mg of plant extract).

The basis for the ORAC value calculation is presented schematically in Figure 5.4 (Cao & Prior, 1999):

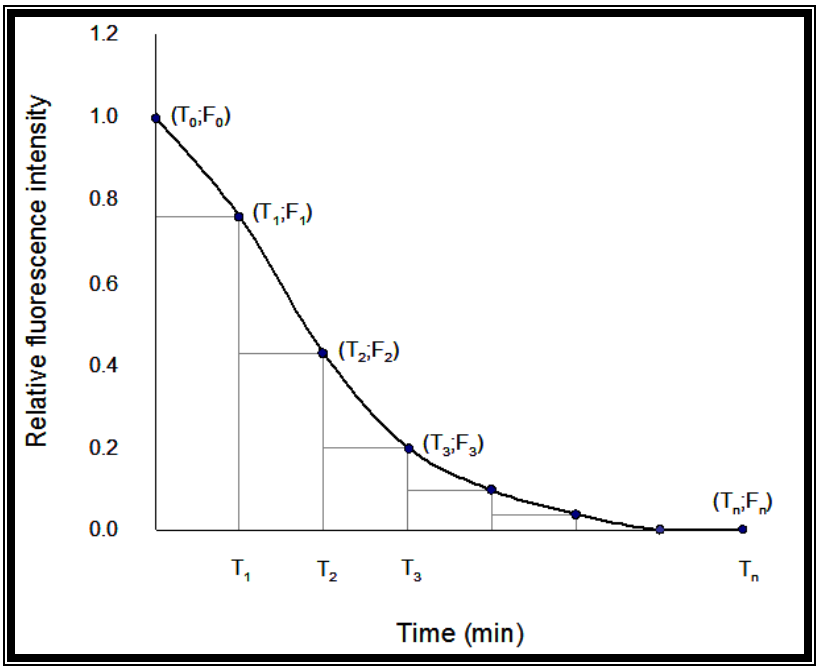

Figure 5.4 Calculation of ORAC value. AUC = 0.5 + f_1/f_0 + f_2/f_0 + ……. + f_n/f_0.

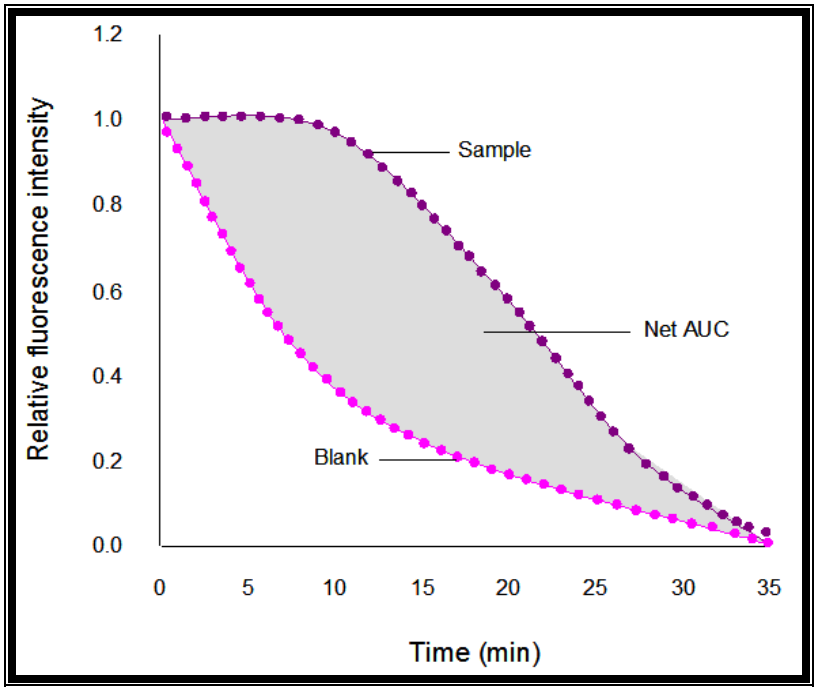

Figure 5.5 Illustration of calculation of the ORAC value expressed as the net area under the curve (AUC) (Huang *et al.,* 2002b).

After subtracting the AUC for the blank, the resultant difference would be the protection conferred by the antioxidant compound (figure 5.5).

The advantage of using the AUC approach to calculate the antioxidant capacity of a sample is that it applies equally well for antioxidants that exhibit a distinctive lag phase as well as those samples that exhibit no lag phase. The AUC approach combines the lag phase method with the initial rate method and is therefore for the most part useful for plant extracts, which often contain numerous compounds that possess complex reaction kinetics (Huang *et al.,* 2005).

5.1.1.4 Statistical analysis

The results obtained were statistically analyzed with GraphPad InStat 3 software. The data from the ORAC assay is presented as the mean ± standard error of the mean (SEM) from 3 determinations.

5.1.1.5 Results and discussion

The lipophilic ORAC values were not available since the lipophilic assay could not be standardized. The hydrophilic values were therefore used to determine the plants with the highest ORAC value.

The experiments were done in triplicate. The results are expressed as Trolox equivalents per 10 mg of plant extract and are presented as mean ± standard error of the mean (SEM).

The hydrophilic ORAC values of the twenty one plants are presented in table 5.1 and figure 5.6. Four different extracts from each plant were examined for their antioxidant activity.

Table 5.1 Relative ORAC values of the 21 plants

Plant	Extract	ORAC value (mean) µM TE/10 mg of plant extract (n = 3)	Standard error of the mean (SEM)
Berula erecta	PE	3 604.00	± 791.57
	DCM	6 804.33	± 479.74
	EtOAc	8 405.00	± 968.12
	EtOH	20 318.33	± 479.74
Heteromorpha arborescens	PE	3 340.67	± 1 445.50
	DCM	6 434.00	± 527.60
	EtOAc	13 247.00	± 562.93
	EtOH	10 413.33	± 1 341.90

Plant	Extract	ORAC value (mean) µM TE/10 mg of plant extract (n = 3)	Standard error of the mean (SEM)
Tarchonanthus camphoratus	PE	4 689.67	± 1 648.70
	DCM	25 822.67	± 1 473.70
	EtOAc	18 347.67	± 1 636.00
	EtOH	30 422.67	± 1 849.70
Vernonia oligocephala	PE	5 302.00	± 1 310.30
	DCM	19 838.67	± 1 631.50
	EtOAc	24 993.67	± 711.51
	EtOH	18 302.67	± 1 368.50
Gymnosporia buxifolia	PE	11 380.00	± 848.94
	DCM	21 091.67	± 559.68
	EtOAc	26 974.67	± 518.36
	EtOH	72 262.33	± 7 944.7
Acacia karroo	PE	4 465.33	± 282.97
	DCM	3 838.00	± 218.28
	EtOAc	4 754.00	± 359.88
	EtOH	22 406.00	± 980.34
Elephantorrhiza elephantina	PE	8 020.33	± 3 551.30
	DCM	9 923.33	± 837.96
	EtOAc	10 413.33	± 329.43
	EtOH	9 219.67	± 1 899.00
Erythrina zeyheri	PE	5 250.67	± 538.17
	DCM	26 486.67	± 425.44
	EtOAc	11 144.67	± 1 372.90
	EtOH	64 516.33	± 4 627.50
Leonotis leonurus	PE	3 153.00	± 1 327.00
	DCM	9 504.33	± 1 780.90
	EtOAc	13 743.00	± 1 287.00
	EtOH	12 559.67	± 1 193.40
Plectranthus ecklonii	PE	6 529.00	± 1 527.60
	DCM	7 080.00	± 414.10
	EtOAc	9 484.66	± 741.01
	EtOH	10 548.67	± 1 392.20
Plectranthus rehmanii	PE	14 783.00	± 754.14
	DCM	7 302.00	± 889.43
	EtOAc	8 293.33	± 481.73
	EtOH	13 987.67	± 1 448.20
Plectranthus venteri	PE	6 380.33	± 3 294.20
	DCM	13 539.33	± 2 497.80
	EtOAc	13 068.33	± 1 121.70
	EtOH	7 520.67	± 921.16
Salvia auretia	PE	-1 258.33	± 817.09
	DCM	8 834.67	± 707.65
	EtOAc	1 757.33	± 168.06
	EtOH	4 977.33	± 943.95

Plant	Extract	ORAC value (mean) µM TE/10 mg of plant extract (n = 3)	Standard error of the mean (SEM)
Salvia runcinata	PE	32 218.67	± 556.85
	DCM	14 915.00	± 7 274.00
	EtOAc	12 415.67	± 305.09
	EtOH	26 969.33	± 2 112.40
Solenostemon latifolius	PE	4 234.33	± 1 131.20
	DCM	9 853.67	± 669.17
	EtOAc	7 015.00	± 233.60
	EtOH	9 744.00	± 397.79
Solenostemon rotundifolius	PE	-1 217.67	± 289.65
	DCM	-444.67	± 41.83
	EtOAC	4 305.67	± 190.54
	EtOH	13 245.00	± 1 331.30
Plumbago auriculata	PE	2 198.67	± 1 207.20
	DCM	6 802.00	± 1 215.00
	EtOAc	5 406.67	± 870.81
	EtOH	31 505.67	± 4 673.60
Clematis brachiata	PE	6 426.00	± 1 447.20
	DCM	12 276.33	± 1 223.90
	EtOAc	27 462.33	± 598.63
	EtOH	19 058.33	± 708.60
Vangueria infausta	PE	5 203.00	± 1 545.30
	DCM	10 469.33	± 1 156.00
	EtOAc	11 581.33	± 403.90
	EtOH	12 443.00	± 1 450.60
Physalis peruviana	PE	2 524.67	± 541.51
	DCM	2 210.00	± 2 031.80
	EtOAc	13 271.00	± 92.09
	EtOH	15 468.67	± 1 046.40
Lippia javanica	PE	12 664.33	± 1 549.10
	DCM	49 147.67	± 8 355.10
	EtOAc	75 908.00	± 6 545.20
	EtOH	NA	± NA

NA = not available

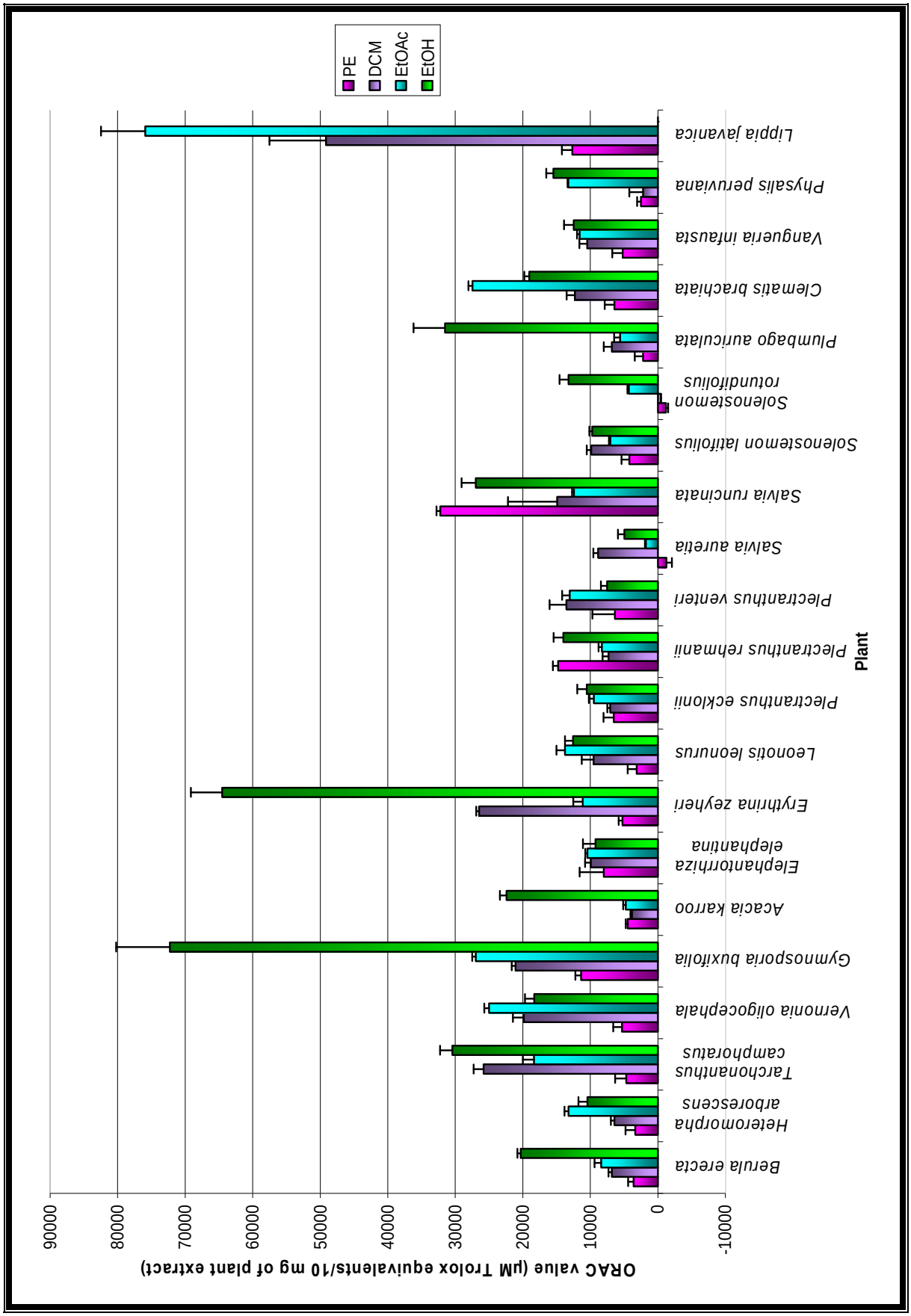

Figure 5.6 Hydrophilic ORAC values of the leaf extracts of the 21 plants (expressed as μmoles Trolox equivalents/10 mg of plant extract). Data is presented as mean ± SEM, n = 3.

Several plants among the 21 that were analyzed exhibited activity. The ORAC values varied from -1 258.33 ± 817.09 to 75 908.00 ± 6 545.20 µM TE/10 mg of plant extract.

The EtOAc extract of *L. javanica* exhibited the most promising antioxidant activity with a value of 75 908.00 ± 6 545.20 µM TE/10 mg of plant extract. The PE extract of *S. auretia* and *S. rotundifolius,* with values of -1 258.33 ± 817.09 and -1 217.67 ± 289.65 µM TE/10 mg of plant extract respectively, exhibited poor antioxidant capacity compared to the other extracts.

L. javanica, G. buxifolia and *E. zeyheri* exhibited high ORAC values. Some plants exhibited little to no activity with the ORAC assay. This could be due to low activity or unspecific interaction with other components of the plant extract. The fact that some plants did not exhibit any activity in the ORAC assay does not mean they do not have any antioxidant activity at all. The compounds in these plant extracts may have a total different mechanism of action.

Based on the results obtained from data in table 5.1, it can be seen that as a general rule the highest radical scavenging capacity was found in the EtOH extracts. It was clear that the polarity of the extractants markedly influences the antioxidant activity. This may suggest that polyphenols, flavanones and flavonoids which are known to exhibit antioxidant activity (Cao *et al.,* 1997; Lien *et al.,* 1999) may be responsible (Tepe *et al.,* 2005). One should keep in mind that only the hydrophilic fractions were analyzed as the method for the lipophilic fractions could not be standardized.

We can conclude from our studies that, when compared to the standard dietary antioxidant, vitamin E, *L. javanica* was effective in scavenging $ROO^{\cdot}$ radicals, being superior to the other plants in this regard.

5.1.2 Ferric reducing antioxidant power assay

5.1.2.1 Introduction and general principles of the FRAP assay

A biological antioxidant is able to significantly prevent or delay the oxidation of a specific substrate. When an antioxidant is present, it will react with the oxidizing species preventing the oxidizing species to react with the substrate. Therefore, the antioxidant reduces the oxidant. In basic terms, electron-donating antioxidants can be referred to as reductants and the inactivation of oxidants by these reductants is described as redox reactions in which one reactive species is reduced while the other is oxidized. The “total antioxidant power” may therefore be referred to as total reducing power (Benzie & Strain, 1999).

The FRAP assay employs an easily reduced oxidant (present in stoichiometric excess) and antioxidants act as reductants in a redox-linked colorimetric method (Benzie & Strain, 1999).

Total antioxidant potential were determined using the FRAP assay of Benzie and Strain (1996). The FRAP assay does not involve a pro-oxidant nor an oxidizable substrate and is therefore an indirect measure of total antioxidant power (Prior & Cao, 1999). The FRAP assay is therefore completely different from the ORAC assay as there are no free radicals present in the assay (Cao & Prior, 1999).

The FRAP assay involves two components in the reaction mixture, antioxidants and an oxidant, which is also the probe. The principle of the FRAP assay is based on the reduction of a ferric tripyridyltriazine (Fe^{3+}-TPTZ) complex (the oxidant) to ferrous tripyridyltriazine (Fe^{2+}-TPTZ) (TPTZ = 2,4,6-tripyridyl-*s*-triazine) by a reductant at a low pH (figure 5.7). Fe^{3+}-TPTZ has an intense blue colour that can spectrophotometrically be measured at 593 nm (Benzie & Strain, 1996; Benzie & Strain, 1999).

The FRAP assay follows the single electron transfer (SET) mechanism (figure 2.11) (Huang *et al.,* 2005) and as a result, does not measure chain-breaking antioxidant activity nor preventative antioxidant activity.

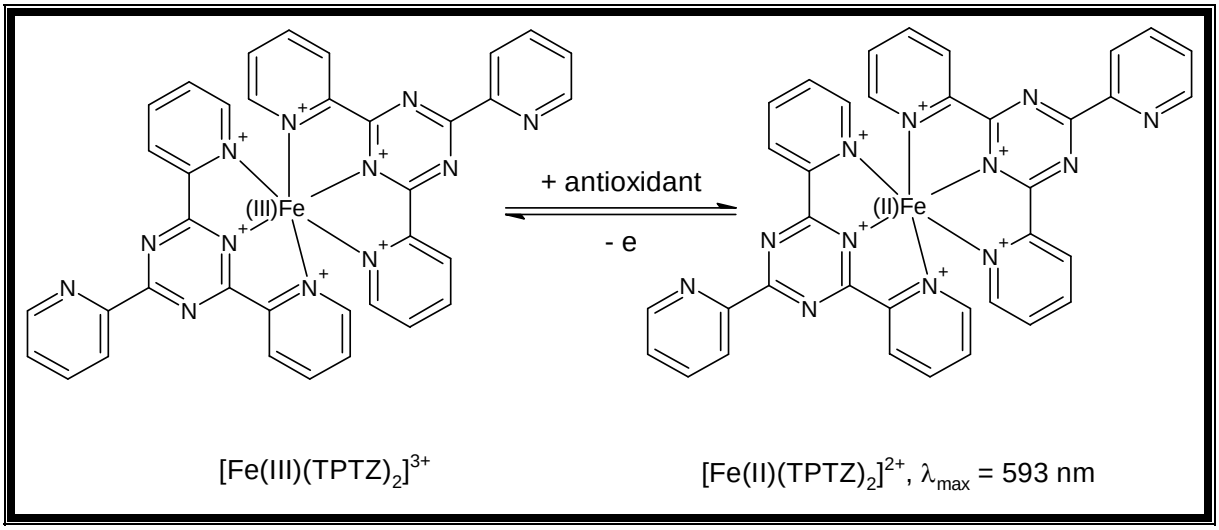

Figure 5.7 Reaction mechanism by which $[Fe(III)(TPTZ)_2]^{3+}$ is reduced to $[Fe(II)(TPTZ)_2]^{2+}$ by a reductant (Huang *et al.,* 2005).

The FRAP assay is based on the following electron-transfer reaction:

Probe (oxidant) + e^- (from antioxidant) → reduced probe + oxidized antioxidant

$M(n) + e^-$ (from AH) → $AH^{\cdot+}$ + M(n-1) Equation 5.4

In equation 5.4, M represents the iron metal ion, e^- represents the electron, AH represents the antioxidant and $AH^{\cdot+}$ represents the oxidized antioxidant.

The FRAP assay basically measures the ability of a sample to reduce Fe^{3+} to produce Fe^{2+}. Fe^{3+} is not essentially a pro-oxidant but Fe^{2+}, produced from the reduction of Fe^{3+} in the FRAP assay, can act as a pro-oxidant and is capable of reacting with H_2O_2 to produce $^{\cdot}OH$ (Benzie & Strain, 1996; Prior & Cao, 1999; Ou *et al.,* 2001). The use of Fe^{2+} as the final indicator in the FRAP assay may be problematic since some antioxidants, such as ascorbic acid, may not only reduce Fe^{3+} to Fe^{2+}, but may also generate further free radicals by reacting with Fe^{2+} (Cao & Prior, 1999).

Some of the advantages of the FRAP assay is that it is simple, speedy, sensitive, inexpensive and robust. No sample pre-treatment is required and reproducibility is excellent. The stoichiometric factors are constant; linearity is maintained over a wide range and the assay does not need highly specialized equipment or skills (Benzie & Strain, 1999). One of the drawbacks of the FRAP assay is that it does not measure the SH-group containing antioxidants (Prior & Cao, 1999). As a result, FRAP results do not necessarily reflect antioxidant activities. In addition, the FRAP assay seemingly relies on the hypothesis that the redox reactions proceed so fast that all reactions are complete within a short period of time. However, this is not always the case (Ou *et al.,* 2001).

5.1.2.2 Experimental procedures

5.1.2.2.1 Chemicals

All chemicals, including glacial acetic acid, 2,4,6-tripyridyl-*s*-triazine (TPTZ), ascorbic acid and $FeCl_3.6H_2O$ were obtained from Sigma Chemical Co., St Louis, MO. All other reagents were of analytical grade.

5.1.2.2.2 Reagents

The FRAP reagent was prepared by combining 40 mM HCl; 10 mM TPTZ in 40 mM HCl and 20 mM $FeCl_3.6H_2O$ in 300 mM acetate buffer in a ratio of 10:1:1, v/v/v.

300 mM acetate buffer was prepared by dissolving sodium acetate trihydrate in 8 ml of glacial acetic acid and made up to a volume of 500 ml with distilled water.

For the FRAP calibration standard, ascorbic acid in concentrations of 0 – 1000 µM was used.

5.1.2.2.3 Sample preparation

The hydrophilic fractions, used in the ORAC assay were used in the FRAP assay. For detailed sample preparation, see section 5.1.1.2.3.

5.1.2.2.4 Instrumentation

The FRAP assay was performed on a BioTek® FL600 microplate fluorescence reader and the absorbance was measured at 593 nm.

5.1.2.2.5 The FRAP assay

The FRAP assay was performed as detailed by Benzie & Strain (1999). Analyses were conducted at a pH 3.6 and temperature of 37 °C. A transparent 96-well microtitre plate was used for the FRAP assay and sample was pipetted in triplicate. The first row of the microtitre plate was set aside for the ascorbic acid reference standards. Sample wells received 10 µl of sample. 200 µl of FRAP reagent was added to each well. Colouration was observed within approximately 1 minute. The microtitre plate was then placed in the reader and the absorbance recorded at 593 nm.

5.1.2.3 Data collection

Data was analyzed with Microsoft Excel. The FRAP value was obtained by converting the absorbance readings to µM ascorbic acid equivalents (AE)/10 mg of plant extract. This was done by inserting the absorbance value of the sample (plant leaf extract) into the straight line equation of the ascorbic acid standard curve (figure 5.8). Increasing concentrations of ascorbic acid were used as a standard for measuring the ferric reducing ability by means of the FRAP assay. The standards, containing 0 – 1000 µM ascorbic acid prepared in water at increments of 100 µM, were used to generate a standard curve using a straight line equation (equation 5.5).

$$y = mx + c \qquad \text{Equation 5.5}$$

Equation 5.6 is mathematically derived from equation 5.5:

$$x = \frac{y - c}{m} \qquad \text{Equation 5.6}$$

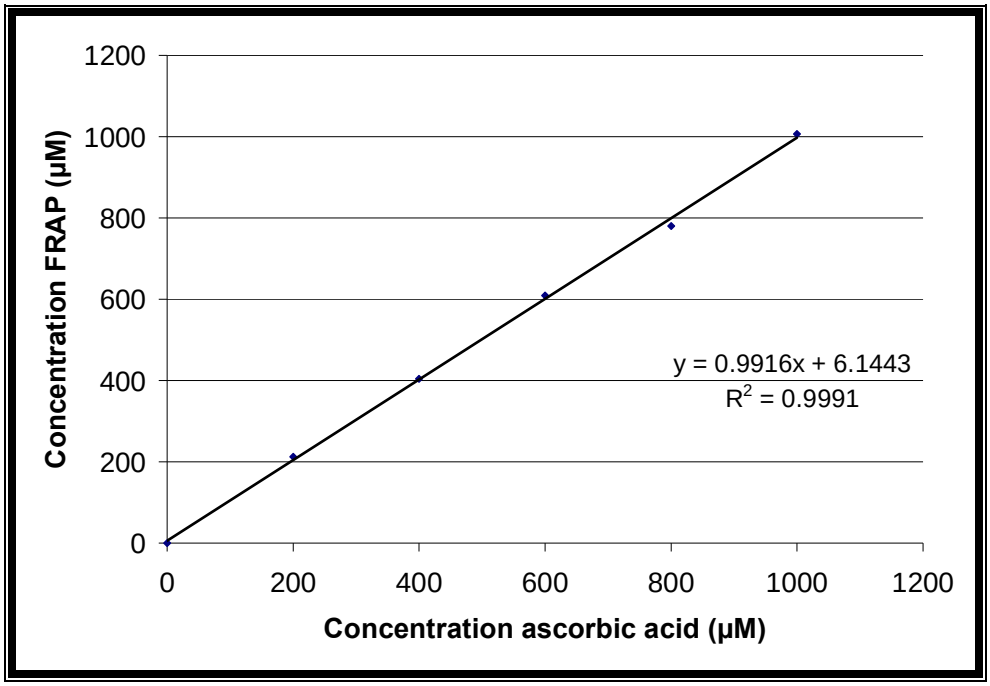

Figure 5.8 The FRAP assay standard curve generated from ascorbic acid. Data is expressed as concentration ascorbic acid (µM) plotted against the absorbance values measured at 593 nm and results in a regression of 0.9991. n = 2

5.1.2.4 Statistical analysis

The results obtained were statistically analyzed with GraphPad InStat 3 software. The data from the FRAP assay is presented as the mean ± standard error of the mean (SEM) from 3 determinations.

5.1.2.5 Results and discussion

The experiments were done in triplicate. The results are expressed as µM ascorbic acid equivalents per 10 mg of plant leaf extract and results are presented as mean ± standard error of the mean (SEM).

The twenty one plants were evaluated for their ferric reducing ability at a non physiological pH of 3.6. The FRAP values of the twenty one plants are presented in table 5.2 and figure 5.9. Four different extracts from each plant were examined for their antioxidant activity.

Table 5.2 FRAP values of the 21 plants

Plant	Extract	FRAP value (mean) µM AE/10 mg of plant extract (n = 3)	Standard error of the mean (SEM)
Berula erecta	PE	33.28	± 7.41
	DCM	76.51	± 8.58
	EtOAc	126.78	± 9.59
	EtOH	1 460.94	± 56.62
Heteromorpha arborescens	PE	30.93	± 19.90
	DCM	48.02	± 10.85
	EtOAc	719.71	± 29.84
	EtOH	618.72	± 48.66
Tarchonanthus camphoratus	PE	101.08	± 23.22
	DCM	1 292.90	± 26.53
	EtOAc	846.13	± 52.35
	EtOH	4 445.60	± 62.36
Vernonia oligocephala	PE	53.13	± 11.83
	DCM	372.35	± 17.57
	EtOAc	1 416.08	± 33.90
	EtOH	1 536.43	± 85.54
Gymnosporia buxifolia	PE	105.80	± 57.71
	DCM	432.50	± 49.38
	EtOAc	433.51	± 33.59
	EtOH	3 332.04	± 61.34
Acacia karroo	PE	52.65	± 52.65
	DCM	0.00	± 0.00
	EtOAc	15.19	± 8.28
	EtOH	4 641.33	± 73.73
Elephantorrhiza elephantina	PE	18.34	± 4.70
	DCM	15.51	± 7.39
	EtOAc	51.25	± 11.87
	EtOH	324.40	± 18.32
Erythrina zeyheri	PE	5.40	± 5.40
	DCM	490.13	± 13.61
	EtOAc	65.96	± 6.10
	EtOH	1 060.04	± 75.90
Leonotis leonurus	PE	320.94	± 15.33
	DCM	212.18	± 9.49
	EtOAc	712.98	± 42.70
	EtOH	893.72	± 60.47
Plectranthus ecklonii	PE	86.98	± 6.96
	DCM	162.20	± 10.25
	EtOAc	438.17	± 37.00
	EtOH	920.08	± 32.78

Plant	Extract	FRAP value (mean) µM AE/10 mg of plant extract (n = 3)	Standard error of the mean (SEM)
Plectranthus rehmanii	PE	2 957.87	± 74.08
	DCM	174.90	± 7.97
	EtOAc	274.33	± 17.65
	EtOH	3 239.53	± 182.03
Plectranthus venteri	PE	127.25	± 3.74
	DCM	221.51	± 2.69
	EtOAc	1 044.44	± 50.70
	EtOH	1 067.20	± 8.12
Salvia auretia	PE	123.18	± 2.05
	DCM	1 535.97	± 40.73
	EtOAc	180.07	± 4.31
	EtOH	81.57	± 14.64
Salvia runcinata	PE	407.09	± 203.73
	DCM	0.00	± 0.00
	EtOAc	651.55	± 116.26
	EtOH	2 400.53	± 42.82
Solenostemon latifolius	PE	2.89	± 2.89
	DCM	678.28	± 58.73
	EtOAc	276.92	± 10.51
	EtOH	801.02	± 36.40
Solenostemon rotundifolius	PE	130.88	± 19.90
	DCM	1 092.07	± 85.48
	EtOAC	110.16	± 5.84
	EtOH	1 542.21	± 61.18
Plumbago auriculata	PE	28.04	± 20.39
	DCM	85.30	± 8.85
	EtOAc	42.84	± 5.60
	EtOH	1 983.95	± 45.71
Clematis brachiata	PE	128.35	± 26.21
	DCM	129.76	± 11.64
	EtOAc	1 976.46	± 47.94
	EtOH	1 334.27	± 24.50
Vangueria infausta	PE	59.71	± 8.36
	DCM	71.46	± 5.34
	EtOAc	291.02	± 22.42
	EtOH	581.10	± 21.70
Physalis peruviana	PE	116.72	± 10.39
	DCM	107.67	± 3.52
	EtOAc	744.54	± 45.32
	EtOH	1 062.83	± 4.03
Lippia javanica	PE	234.45	± 50.37
	DCM	698.13	± 39.25
	EtOAc	6 742.13	± 24.33
	EtOH	9 502.13	± 85.47

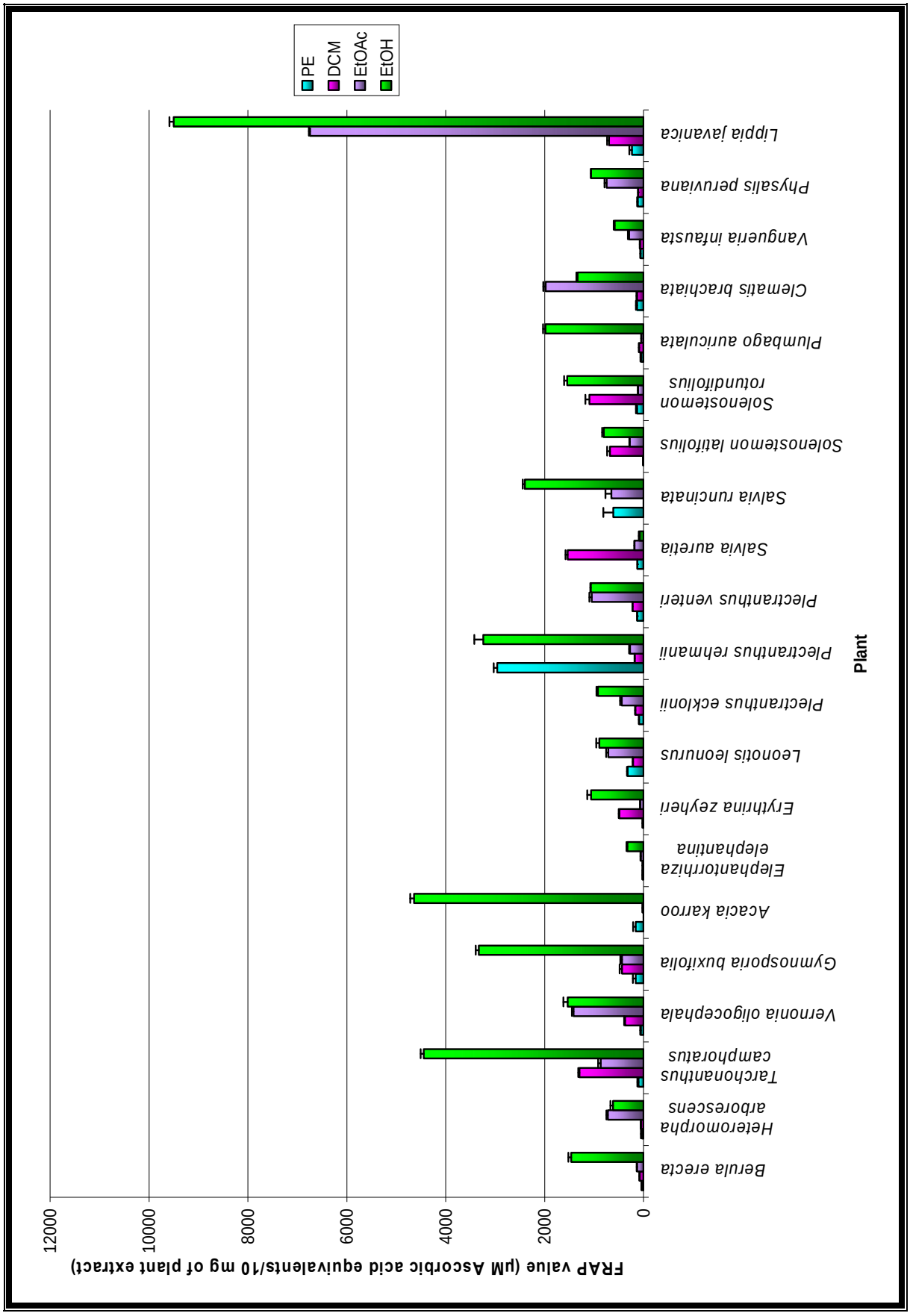

Figure 5.9 FRAP values of the leaf extracts of the 21 plants (expressed as µM ascorbic acid equivalents/10 mg of plant extract). Data are presented as mean ± SEM. n = 3.

Several plants among the 21 that were analyzed exhibited activity. The FRAP values varied from 0.00 ± 0.00 to 9 502.13 ± 85.47 µM AE/10 mg of plant extract.

The EtOH extract of *L. javanica* exhibited the most promising reducing ability with a value of 9 502.13 ± 85.47 µM AE/10 mg of extract. The DCM extract of *S. runcinata* and *A. karroo* exhibited poor reducing capacity compared to the other plant extracts, both with a value of 0.00 ± 0.00 µM AE/10 mg of plant extract.

The EtOH extracts of *L. javanica, A. karroo* and *T. camphoratus* exhibited high FRAP values. Some plants exhibited little to no activity with the FRAP assay. This could be due to the fact that the FRAP assay is carried out in an acidic environment (pH 3.6). pH values greatly influences the reducing capacity of antioxidants. Reducing capacity may be suppressed at acidic conditions due to protonation of antioxidant compounds, whereas a sample's reducing capacity is enhanced in basic conditions due to proton dissociation of phenolic compounds (Huang *et al.,* 2005).

Based on the results obtained from data in table 5.2 and figure 5.9, one can see that as a general rule the highest reducing capacity was found in the EtOH extracts. It is clear that the polarity of the extractants markedly influences the reducing activity. This may indicate that the active compounds are of a more polar nature. One should keep in mind that only the hydrophilic fractions analyzed in the ORAC assay were analyzed in the FRAP assay as the method with the lipophilic fractions could not be standardized during the ORAC assay. It is therefore obvious that the polar extracts did exhibit higher activity than the non-polar extracts.

The reducing capacity of a sample reflects a single aspect of its antioxidant properties. To refer to the result as the total antioxidant capacity, is oversimplified. The total antioxidant capacity encompasses much broader aspects including metal chelating capacity, reactive oxygen species scavenging capacity and oxidative enzyme inhibition capacity (Huang *et al.,* 2005).

5.1.2.6 Conclusion

The results of the ORAC and FRAP assays were used to identify a plant with promising antioxidant and reducing capacity. The ORAC and FRAP methods provided quantitative estimations of the total antioxidant activity, which was expressed in terms of an equivalent concentration of a standard antioxidant. There wasn't a perfect correlation between the ORAC and FRAP values. This was expected since they use different technologies and mechanisms of action (Cao & Prior, 1999). The two assays can be seen as two significant components of the antioxidant system.

Among the 21 plants analyzed, several exhibited antioxidant activity. *L. javanica, G. buxifolia* and *T. camporatus* exhibited high values for both the ORAC and FRAP assays (see table 8.1).

The EtOAc leaf extract of *L. javanica* exhibited the most promising activity with a value of 75 908.00 ± 6 545.20 µM TE/10 mg of extract for the ORAC assay and the EtOH leaf extract of *L. javanica* exhibited the most promising activity with a value of 9 502.13 ± 85.47 µM AE/10 mg of extract for the FRAP assay.

The ORAC and FRAP assays are performed at a pH of 7.4 and 3.6, respectively. The pH at which the ORAC assay is performed is close to that in human physiology and may therefore be a better indicator of the antioxidant activity (Awika *et al.,* 2003).

The most promising antioxidant properties when measured with the FRAP and ORAC assays was that of the leaves of *L. javanica.* This study demonstrated that *L. javanica* extracts possess significant reducing power and free radical scavenging ability. The *in vitro* antioxidative properties of *L. javanica* were the next step in discovering an extract exhibiting promising antioxidant activity.

Based on these results, the evaluation of the *in vitro* superoxide scavenging ability and the *in vitro* attenuation of lipid peroxidation of the extracts of *L. javanica* was undertaken.

CHAPTER 6

Antioxidant screening of *L. javanica*

6.1 *In vitro* antioxidant activity of *L. javanica* leaf extracts and selection of a promising extract using different *in vitro* methods

During the primary screening of the 21 plants, *L. javanica* leaf extracts exhibited the highest ORAC and FRAP values (see table 8.1). This was the basis for selecting *L. javanica* for further examination in the determination of the *in vitro* antioxidant activity. The aim of the present chapter was to investigate the ability of the neurotoxin, cyanide, to produce superoxide generation in rat brain homogenate and whether the extracts of the leaves of *L. javanica* were able to reduce the cyanide-induced $O_2^{\cdot -}$ generation, comparing the results with Trolox. Also, the ability of a toxin-solution consisting of H_2O_2, $FeCl_3$ and ascorbic acid to induce lipid peroxidation through the formation of the hydroxyl radical ($^{\cdot}OH$) was investigated as well as the ability of the extracts of the leaves of *L. javanica* to attenuate the toxin-induced lipid peroxidation. The results were also compared to that of Trolox.

6.1.1 Nitro-blue tetrazolium assay

During the *in vitro* antioxidant screening of *L. javanica*, the ability of the extracts of *L. javanica* to scavenge the superoxide anion was determined using a well established nitro-blue tetrazolium (NBT) assay as described by Ottino and Duncan (1997). The rational for determining the superoxide anion scavenging activity is that superoxide is the first product formed from the univalent reduction of oxygen and is capable of producing much more powerful and damaging radicals (Fridovich, 1975; Halliwell & Gutteridge, 1984; Fridovich, 1989).

6.1.1.1 Introduction and general principles of the Nitro-blue tetrazolium (NBT) assay

The NBT assay was first described by Das and co-workers (1990) and Sagar and co-workers (1992). This method was modified by Ottino and Duncan in 1997. The NBT assay was used to spectrophotometrically determine the level of superoxide anions induced by the neurotoxin potassium cyanide (KCN) in rat brain homogenate as well as the ability of the petroleum ether (PE), dichloromethane (DCM), ethyl acetate (EtOAc) and ethanol (EtOH) leaf extracts of *L. javanica* to scavenge the induced radicals *in vitro.* The results were compared to that of Trolox (a water-soluble vitamin E analogue), since vitamin E is known to be a chain breaking antioxidant that reacts directly with the superoxide anion (Machlin & Bendich, 1987).

The basic principle of the NBT assay is based on the reduction of yellow nitro-blue tetrazolium chloride (NBT^{2+}) to an insoluble purplish blue nitro-blue diformazan (NBD) (figure 6.1) during incubation at a pH of 7.4 (Das *et al.,* 1990; Sagar *et al.,* 1992; Wang *et al.,* 1998; Grimm *et al.,* 2004). The diformazan complex is then extracted with glacial acetic acid and measured spectrophotometrically at 560 nm (Parejo *et al.,* 2003). The presence of radical scavenging compounds in the solution decreases the formation of diformazan and thus decreases the absorption at 560 nm (Grimm *et al.,* 2004).

The absorbance of the extracts could potentially influence the bioassay results due to the presence of chlorophyll that absorb at the same wavelengths measured in the different absorbance-based assays (NBT assay, 560 nm; TBARS assay, 532 nm). The extracts were therefore dissolved in acetone and photobleached using a photochemical reactor to avoid any interference from chlorophyll.

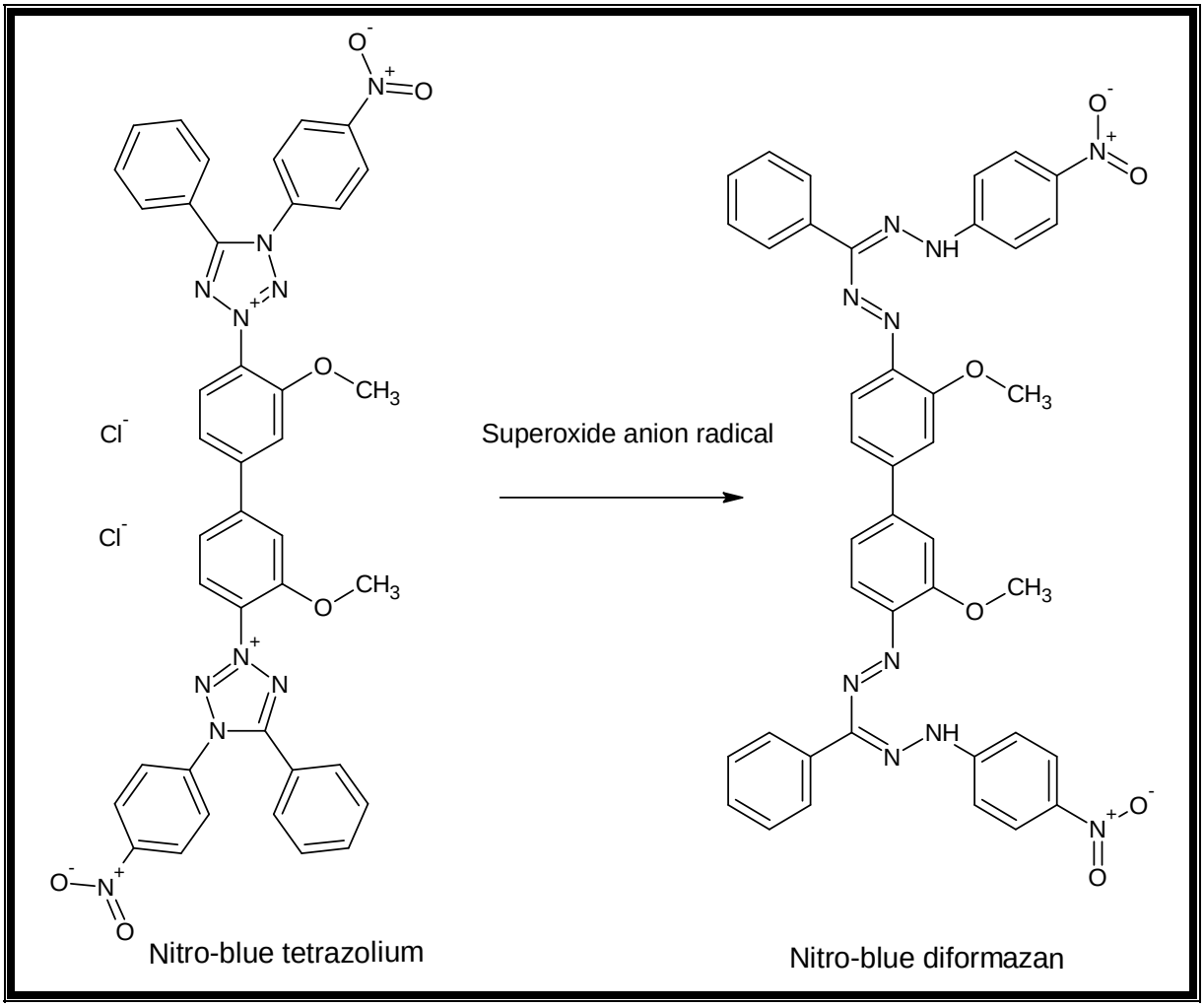

Figure 6.1 The reaction of the reduction of nitro-blue tetrazolium (NBT) to nitro-blue diformazan (NBD) in the presence of the superoxide anion radical (Kaur & Geetha, 2006).

6.1.1.2 Experimental

6.1.1.2.1 Chemicals

Potassium cyanide (KCN), nitro-blue tetrazolium (NBT), nitro-blue diformazan (NBD), Folin & Ciocalteu's reagent, dimethyl sulfoxide (DMSO), potassium chloride (KCl), glacial acetic acid, di-sodium hydrogen orthophosphate anhydrous (Na_2HPO_4), potassium dihydrogen orthophosphate (KH_2PO_4) and hydrochloric acid (HCl) were purchased from Saarchem (PTY) Ltd., 259 Davidson Road, Wadeville, Gauteng, South Africa. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), Bradford reagent and bovine serum albumin (BSA) were purchased from Sigma-Aldrich Chemical Corporation, Reidstreet 2, D-89555 Steinheim, Germany). All other chemicals were obtained locally and were of the highest available purity.

6.1.1.2.2 Reagents

Phosphate buffer solution (PBS) consisted of 8 g NaCl (137 mM), 0.2 g KCl (2.7 mM), 1.44 g Na_2HPO_4 (10 mM) and 0.24 g KH_2PO_4 (2 mM) dissolved in 1 L double distilled water and the pH was ascertained to be 7.4 and stored in the refrigerator until time of use.

Stock solutions of cyanide were prepared by dissolving KCN in double distilled water, so that on addition of 250 µl of the toxin, the solution would be diluted to the correct incubation concentration. KCN was tested at the following concentrations 0; 0.25; 0.5 and 1 mM.

Copper reagent solution was prepared by mixing 1 ml of a 1% aqueous copper sulphate solution, ($CuSO_4.5H_2O$), 1 ml of a 2% aqueous sodium tartrate solution and 98 ml of a 2% disodiumcarbonate solution (Na_2CO_3) in 0.1M sodium hydroxide (NaOH).

A 0.1% NBT solution was prepared by dissolving NBT in ethanol before diluting it to the required volume with double distilled water. Fresh solutions were prepared daily and the solution was protected from light by covering the container with aluminium foil.

Trolox was dissolved in EtOH before diluting it to the required volume with double distilled water. Trolox was tested at 1 mM (the same concentration as the toxin) to compare the superoxide scavenging activity of the extracts of *L. javanica* with that of the known antioxidant, vitamin E (Di Mascio *et al.,* 1991; Halliwell, 1994; Ottino & Duncan, 1997; Agarwal *et al.,* 2004).

The PE, DCM, EtOAc and EtOH extracts of *L. javanica* were dissolved in DMSO before diluting it to the required volume with double distilled water. The extracts were tested at the following concentrations 1.25, 2.5 and 5 mg/ml. The final DMSO concentration in the incubation flasks was less than 10%. The extracts were protected from light by covering the containers with aluminium foil.

6.1.1.2.3 Animals and preparation of brain homogenate

Three month old, adult male albino rats of the Sprague Dawley strain, weighing between 250 and 300 g were used throughout this study and were obtained from the Experimental Animal Center at the North West University (Potchefstroom Campus). The rats were housed in separate opaque plastic cages with metal grid floors and covers. All of the rats were kept in the same room under a constant temperature (24 ± 3˚C) and at a lighting cycle of 12 hours light and 12 hours dark. The rats were given access to standard laboratory food and water *ad libitum.*

All experiments were carried out in accordance with the ethical principles in animal research adopted by the Ethics Committee, North-West University, Potchefstroom Campus [Application # 05D05].

Rats were sacrificed by rapid decapitation and whole brain was excised by removing the top of the skull. Decapitation was performed by a trained laboratory professional. The brain was immediately weighed and placed on ice. The whole brain was homogenized using a Teflon homogenizer in 0.1M phosphate buffered saline (PBS), pH 7.4 to give a final concentration of 10% (w/v). The significance of using the PBS is to prevent any lysosomal damage of the tissue. PBS has shown not to scavenge free radicals (Anoopkumar-Dukie *et al.,* 2001).

6.1.1.2.4 Preparation of standard curves

6.1.1.2.4.1 Bovine serum albumin standard curve

To express the scavenging of superoxide anions in terms of µmoles NBD/mg protein, one has to determine the protein content of each brain before conducting the NBT assay.

6.1.1.2.4.1.1 Lowry method

The Lowry method was chosen to determine the protein content of each brain in the initial experiments conducted on the crude leaf extracts of *L. javanica* (Chapter 6). The principle behind the Lowry method of determining protein concentration lies in the reactivity of the peptide nitrogen(s) with the copper [II] ions under alkaline conditions and the subsequent reduction of the Folin-Ciocaltea phosphomolybdic-phosphotungstic acid mixture to heteropolymolybdenum blue by the copper-catalyzed oxidation of aromatic acids. Most studies that involve the measurement of biological activity require the normalization of the activity to the protein content (Lowry *et al.,* 1951).

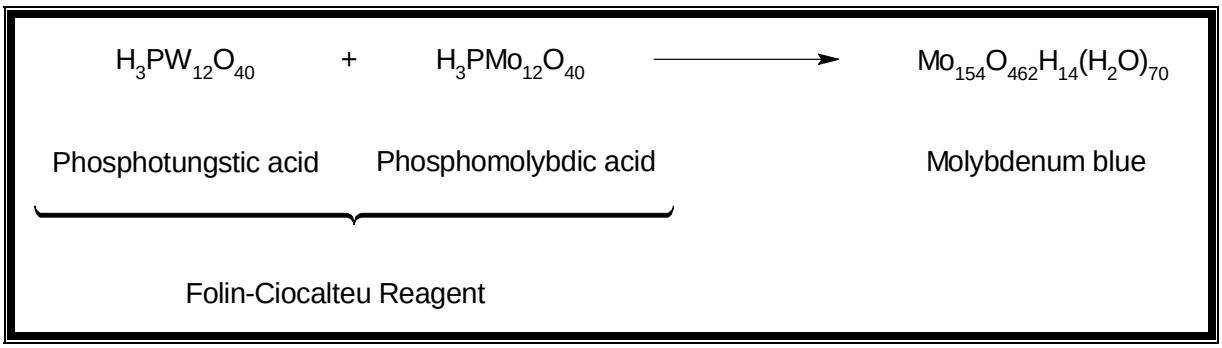

Figure 6.2 Principle of the Lowry method for determination of the protein concentration (Karimi *et al.,* 2009; Prado *et al.,* 2009; Zhang *et al.,* 2010).

Increasing concentrations of bovine serum albumin (BSA) were used as a standard for measuring the protein concentration by means of the Lowry protein assay. The standards, containing 0 – 300 µg/ml of BSA at increments of 60 µg/ml, were used to generate a standard curve (figure 6.3) using equation 5.5.

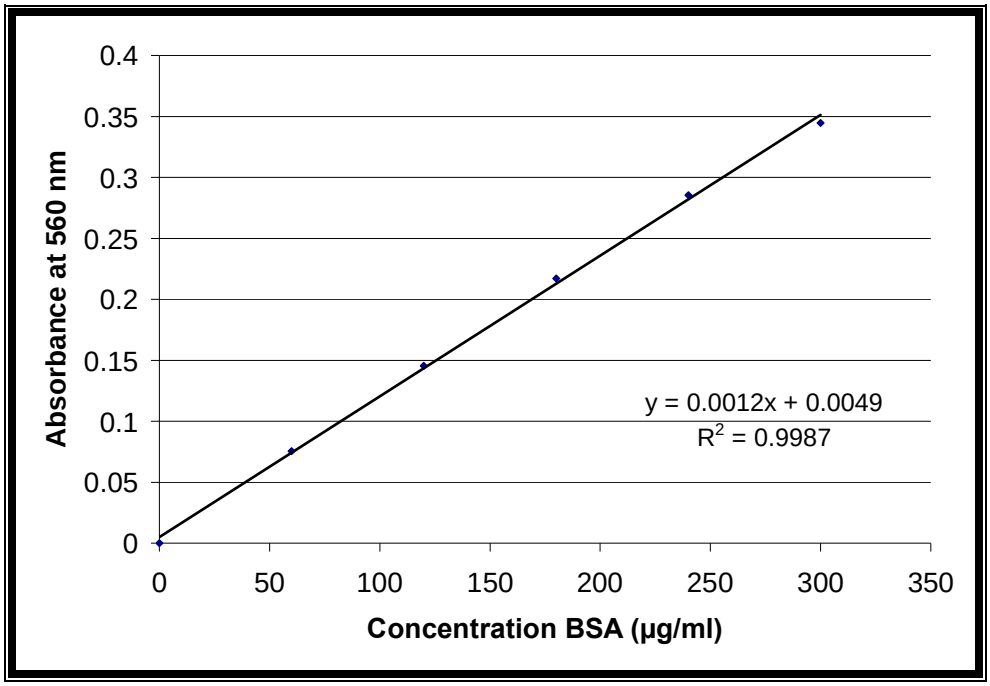

Figure 6.3 The Lowry protein assay standard curve generated from bovine serum albumin. Data is expressed as concentration bovine serum albumin (µg/ml) plotted against the absorbance values measured at 500 nm and results in a regression of 0.9987. n = 2.

6.1.1.2.4.1.2 Bradford method

The NBT assay experiments during bio-assay guided fractionation (Chapter 7) had been adapted for the multiplate reader and the Bradford assay replaced the Lowry method for the determination of the protein content. The Bradford protein assay was first described by Bradford in 1976. The Bradford assay is a colorimetric assay and the principle behind the

assay lies in the binding of the acidic Coomassie Brilliant Blue G-250 to arginyl and lysyl residues of proteins, producing a change in colour from brown to blue in proportion to the amount of protein present in the sample. The absorbance is spectrophotometrically measured at 560 nm using a microplate reader (Lü *et al.,* 2007). The technique is simpler, faster, and more sensitive than the Lowry method. Protein determinations are made by comparison to the colour response of protein assay standards, usually prepared as a series of known dilutions of bovine serum albumin (BSA). One adverse effect of the Bradford assay is that the Bradford reagent can form complexes with plant tannins and phenolics (Kilkowski & Gross, 1999).

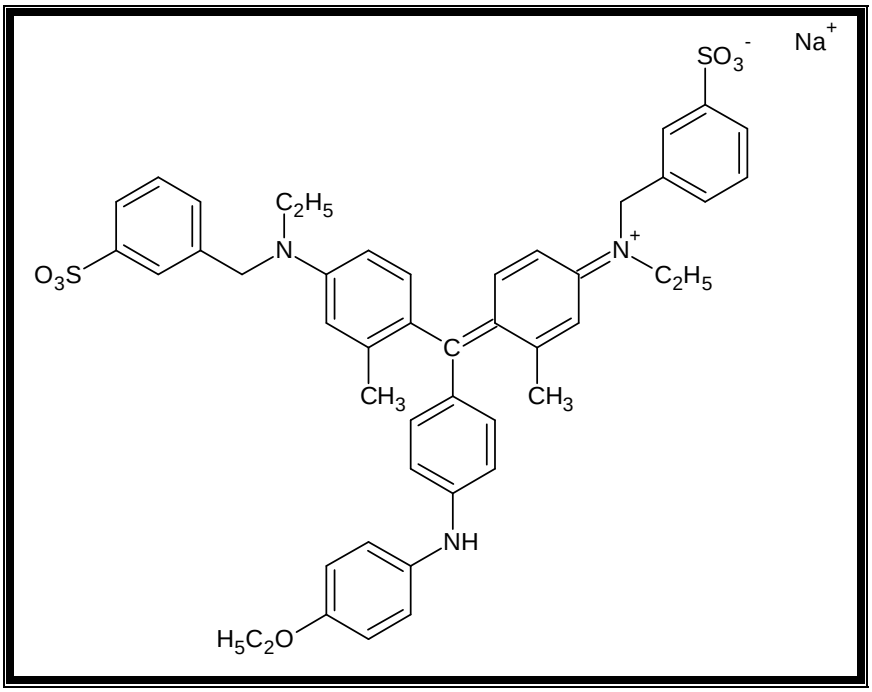

Figure 6.4 Structure of Coomassie Brilliant Blue (Bukallah *et al.,* 2007).

Increasing concentrations of BSA were used as a standard for measuring the protein concentration by means of the Bradford protein assay. The standards, containing 0 - 1.4 µg/ml of BSA at increments of 0.2 µg/ml, were used to generate a standard curve (figure 6.5) using equation 5.5.

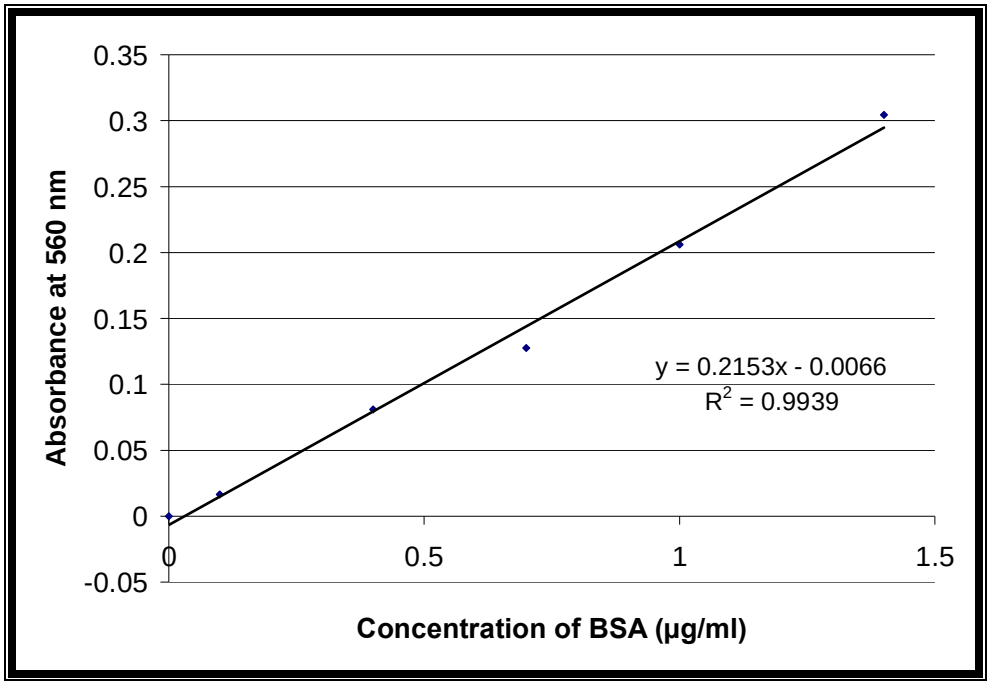

Figure 6.5 The Bradford protein assay standard curve generated from bovine serum albumin. Data is expressed as concentration bovine serum albumin (µg/ml) plotted against the absorbance values measured at 560 nm and results in a regression of 0.9939. n = 5.

6.1.1.2.4.2 Nitro-blue diformazan (NBD) standard curve

To express the scavenging of superoxide anions in terms of µmoles NBD/mg protein, one has to determine the µmoles nitro-blue diformazan formed from the reduction of NBT.

Increasing concentrations of NBD was used as a standard for measuring the concentration of NBD formed from NBT by means of the NBT assay. The standards, containing 0 - 400 µM/ml of NBD at increments of 100 µM, were used to generate a standard curve (figure 6.6) using equation 5.5.

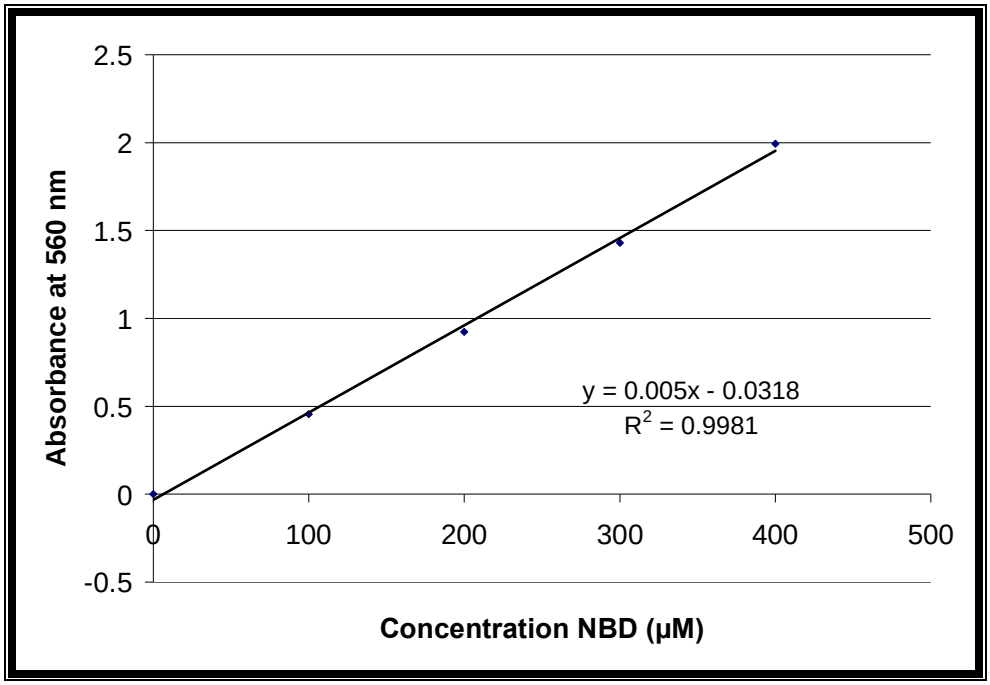

Figure 6.6 The nitro-blue diformazan standard curve generated from nitro-blue diformazan. Data is expressed as concentration of nitro-blue diformazan (µM) plotted against the absorbance values measured at 560 nm and results in a regression of 0.9981. n = 2.

6.1.1.2.5 Instrumentation

The absorbance values for the NBT assay of the initial experiments conducted on the crude extracts of *L. javanica* (Chapter 6) were analyzed at 560 nm, using a BOE COS-22 UV/visible recording spectrophotometer and the absorbance values for the NBT assay of the experiments conducted during bio-assay guided fractionation (Chapter 7) were also analyzed at 560 nm, using a LABSYSTEMS MULTISCAN RC multiplate reader and Genesis software.

6.1.1.2.6 The protein assay

6.1.1.2.6.1 Lowry method

The protein content estimation for each brain was performed preceding the NBT assay, using the method described by Lowry and co-workers (1951). In brief, an aliquot of 0.1 ml homogenate was added to 4.9 ml PBS, vortexed and 1 ml was added to 6 ml alkaline copper reagent solution. This mixture was vortexed and left to stand at room temperature for 10 minutes. To this 0.3 ml Folin & Ciocalteau's reagent was added and the tubes were vortexed. Afterwards, the tubes were left to stand in the dark, at room temperature for 30 minutes. After the 30 minute incubation, the absorbance was measured at 500 nm and

converted to mg protein using the standard curve generated from BSA (Figure 6.3). The protein content for each brain was measured in duplicate.

6.1.1.2.6.2 Bradford method

In brief, the dye reagent was removed from the refrigerator and warmed to room temperature. The dye reagent was inverted a few times before use. 5 µl of each standard and unknown sample solution was pipetted into separate microplate wells. 250 µl of the dye reagent was then added to each well and the samples were mixed using the microplate mixer. The microplate was then incubated at room temperature for 15 minutes. The unknown protein concentration was determined by making 10, 20 and 50 fold dilutions with PBS of the 10% (w/v) whole rat brain homogenate used in the assay using PBS as the blank. Dilutions were necessary since the assay gave unreliable absorbance measurements above 1.4 mg/ml. The absorbance values were inserted into the calibration curve (figure 6.5) and adjusted according to the dilution to yield the protein concentration (mg/ml) present in the homogenate.

6.1.1.2.7 NBT assay

During the initial experiments conducted on the crude leaf extracts of *L. javanica* (Chapter 6), 0.5 ml homogenate containing 0.25 ml of the toxin (KCN, 1 mM), in the absence and presence of varying concentrations of the PE, DCM, EtOAc and EtOH extracts of *L. javanica* (1.25, 2.5 and 5 mg/ml) and trolox (1 mM) was incubated with 0.4 ml of the 0.1% NBT solution for 1 hour at 37 ˚C in an oscillating water bath. The leaf extracts of *L. javanica* were added to the homogenate at the beginning of the incubation period in a volume of 0.25 ml. In the case of control experiments, volume adjustments were done with PBS. After incubation the suspensions were centrifuged at 2000 x g for 10 minutes. Following the centrifugation, the supernatant was decanted and the pellet resuspended with 2 ml glacial acetic acid. The reduced NBT is extracted into the glacial acetic acid. After centrifugation at 4000 x g for 5 minutes, 1.5 ml of the upper layer was transferred to cuvettes and the absorbance values were measured at a wavelength of 560 nm with glacial acetic acid as the blank. Absorbance values were converted to µmoles NBD using the standard curve (figure 6.6). All experiments were measured in 5 replicates and repeated once to ensure reproducibility.

The NBT assay experiments during bio-assay guided fractionation (Chapter 7) had been adapted for the multiplate reader. In brief, 100 µl homogenate containing 50 µl of the toxin (KCN, 1 mM), in the absence and presence of the specific column fractions (only at a concentration of 1.25 mg/ml) and trolox (1 mM) was incubated with 80 µl of the 0.1% NBT solution for 1 hour at 37 ˚C in an oscillating water bath. The column fractions were dissolved in DMSO and PBS and were added to the homogenate at the beginning of the incubation

period in a volume of 50 µl. In the case of control experiments, volume adjustments were done with PBS. After incubation the suspensions were centrifuged at 2000 x g for 10 minutes. Following the centrifugation, the supernatant was decanted and the pellet resuspended with 400 µl of glacial acetic acid. The reduced NBT is extracted into the glacial acetic acid. After centrifugation at 4000 x g for 5 minutes, 200 µl of the upper layer was transferred to a 96-well multiplate and the absorbance spectrophotometrically measured at a wavelength of 560 nm with glacial acetic acid as the blank. Absorbance values were converted to µmoles NBD using the standard curve (figure 6.6). All experiments were measured in 5 replicates and repeated once to ensure reproducibility.

6.1.1.2.7.1 KCN

For the first experiment, the effective concentration of KCN was determined. Varying concentrations of KCN (0, 0.25, 0.5 and 1 mM) were assayed to determine the concentration of cyanide that caused the most significant superoxide generation. The assay was further carried out as described in section 6.1.1.2.7.

6.1.1.2.7.2 PE, DCM, EtOAc and EtOH extracts of *L. javanica*

After determining the effective concentration of KCN, the superoxide scavenging ability of the PE, DCM, EtOAc and EtOH extracts of the leaves of *L. javanica* was determined. Homogenate contained 1 mM KCN in the presence of varying concentrations (1.25, 2.5 and 5 mg/ml) of the extracts of *L. javanica.* The assay was further carried out as described in section 6.1.1.2.7.

6.1.1.2.8 Data collection

The raw data was imported to a Microsoft Excel worksheet.

The absorbance values of the protein assay were converted to mg protein, using the calibration curves generated from increasing concentrations of bovine serum albumin (figures 6.3 & 6.5). These values were used in expressing the superoxide anion scavenging results when converting the results to µmoles/mg protein.

The absorbance values of the NBT assay were then converted to µmoles NBD detected, using the calibration curve generated from increasing concentrations of nitro-blue diformazan (figure 6.6). The final results were expressed as µmoles NBD/mg protein.

6.1.1.2.9 Statistical analysis

The results obtained were statistically analyzed with GraphPad InStat 3 software. The data from the NBT assay is presented as the mean ± SEM from 5 determinations in each run and the experiments were repeated once. Statistical significance was evaluated using one-way

analysis of variance (ANOVA), followed by the Student-Newman Keuls Multiple Range test for comparison of different means. One-way analysis of variance (ANOVA) was used since there was only one independent variable and the data was subjected to post-hoc comparisons. p-values indicate the significant difference between different means. A difference was considered statistically significant when $p \leq 0.05$ (Zar, 1974).

6.1.1.2.10 Results and discussion

For the first experiment (section 6.1.1.2.7.1), the increase in $O_2^{\cdot -}$ generation by different concentrations of potassium cyanide (KCN) was determined. This was done to select the concentration of KCN that produced the highest $O_2^{\cdot -}$ generation for the rest of the experiments.

The *in vitro* exposure of whole rat brain homogenate to increasing concentrations of KCN caused a significant increase in $O_2^{\cdot -}$ generation, in a concentration-dependent manner when compared to the control value. The 1mM KCN was therefore chosen for the subsequent experiments. The NBT values for KCN are presented in table 6.1 and figure 6.7.

Table 6.1 The *in vitro* effect of increasing concentrations of KCN on $O_2^{\cdot -}$ generation in rat brain homogenate.

Compound/Extract	Concentration	NBD (µM/mg protein) (n = 5)	Standard error of the mean (SEM)
Control		16.660	± 0.454
KCN	0.25 mM	22.380	± 0.705
	0.5 mM	23.439	± 0.659
	1 mM	26.032	± 0.835

The *in vitro* superoxide scavenging activity of the PE, DCM, EtOAc and EtOH extracts of *L. javanica* was then determined using the NBT assay. The results are expressed as µmoles NBD/mg protein and results are presented as mean ± standard error of the mean (SEM). The NBT values for the PE, DCM, EtOAc and EtOH extracts of the leaves of *L. javanica* are presented in table 6.2 and figure 6.8.

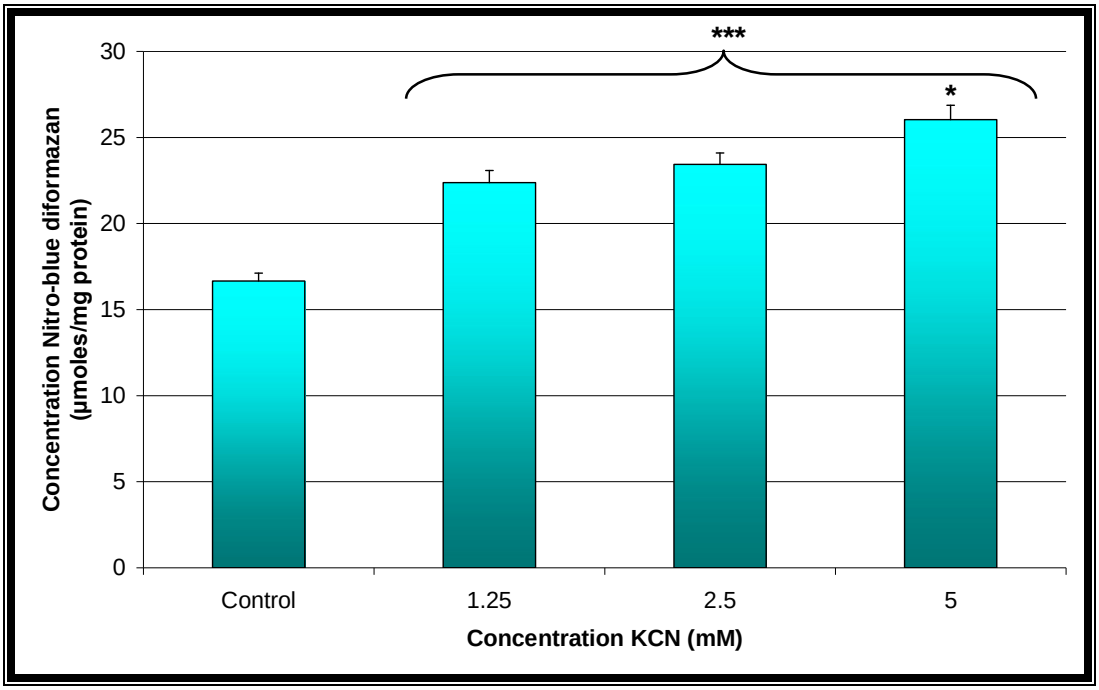

Figure 6.7 Concentration-dependent effect of KCN on $O_2^{\cdot -}$ generation in whole rat brain homogenate. Each bar represents the mean ± SEM; n = 5; *p<0.05 in comparison to 0.5 mM KCN; ***p<0.001 in comparison to control (Student-Newman-Keuls Multiple Range Test).

Table 6.2 The *in vitro* effect of the PE, DCM, EtOAc and EtOH extracts of the leaves of *L. javanica* on KCN-induced superoxide anion generation in rat brain homogenate.

Compound/Extract	Concentration	NBD (µM/mg protein) (n=5)	Standard error of the mean (SEM)
Control		20.474	± 1.248
KCN	1 mM	33.246	± 1.602
Trolox	1 mM	18.074	± 0.420
L. javanica$_{PE}$	1.25 mg/ml	14.305	± 0.947
	2.5 mg/ml	8.359	± 0.562
	5 mg/ml	10.493	± 0.557
L. javanica$_{DCM}$	1.25 mg/ml	22.336	± 0.484
	2.5 mg/ml	15.980	± 0.338
	5 mg/ml	11.690	± 0.223
L. javanica$_{EtOAc}$	1.25 mg/ml	18.433	± 0.484
	2.5 mg/ml	13.199	± 0.447
	5 mg/ml	7.755	± 0.274
L. javanica$_{EtOH}$	1.25 mg/ml	28.267	± 0.569
	2.5 mg/ml	26.033	± 0.632
	5 mg/ml	19.930	± 0.374

The extracts demonstrated varying degrees of activity with the NBT assay. Based on the results obtained from data in table 6.2 and figure 6.8, one can see that all the extracts of *L. javanica* exhibited activity with the NBT assay by lowering the superoxide anion concentration to lower than that of the toxin, KCN. The more effective the extract, the lower the concentration of NBD produced. The NBT values varied from 7.355 ± 0.274 to 33.245 ± 1.602 µmoles NBD/mg protein.

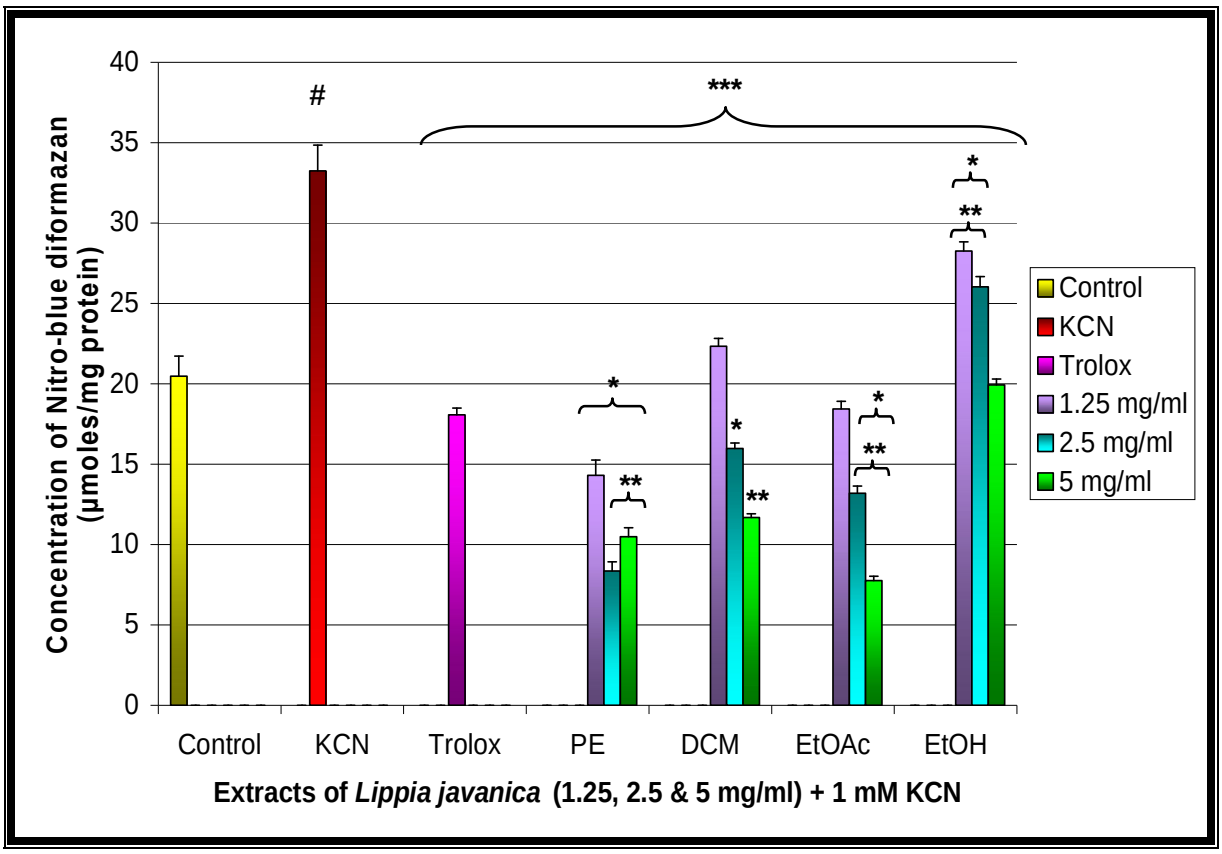

Figure 6.8 The superoxide scavenging effect of increasing concentrations of the PE, DCM, EtOAc and EtOH extracts of the leaves of *L. javanica* and Trolox (1 mM) on 1 mM KCN-induced superoxide anion generation on whole rat brain homogenate. Each bar represents the mean ± SEM. n = 5. #p<0.001 compared to the control, ***p<0.001 compared to 1 mM KCN alone, **p<0.001 compared to the control (1 mM Trolox, 1.25 mg/ml concentration of the DCM, EtOAc and EtOH extracts exhibited no significant difference compared to the control), *p<0.001 compared to 1 mM Trolox (2.5 mg/ml concentration of the DCM extract, 1.25 mg/ml concentration of the EtOAc extract and 5 mg/ml concentration of the EtOH extract exhibited no significant difference compared to 1 mM Trolox). There appeared to be no significant difference between the 2.5 mg/ml concentration of the PE extract when compared to the 5 mg/ml concentration of the EtOAc extract (Student-Newman-Keuls Multiple Range Test).

As shown in figure 6.7, treatment of the rat brain homogenate with 1 mM KCN caused a significant increase in $O_2^{\cdot-}$ generation in comparison to the control. However, treatment of the homogenate with increasing concentrations of the extracts resulted in an overall decline in cyanide-induced $O_2^{\cdot-}$ generation in comparison to the 1 mM KCN alone (figure 6.8). All the concentrations (1.25, 2.5 and 5 mg/ml) of the four extracts of *L. javanica* were significantly effective at reducing the neurotoxic effects of KCN in rat brain homogenate, $p<0.001$. Furthermore, all concentrations of the PE and EtOAc extracts and the 2.5 and 5 mg/ml concentration of the DCM extract of *L. javanica* were able to lower the $O_2^{\cdot-}$ levels below that measured in the control (homogenate with no drug or toxin present), indicating that these extracts offer complete protection against the neurotoxic effects of KCN at the specific concentrations.

The DCM, EtOAc and EtOH extracts of *L. javanica* caused a significantly lower concentration of NBD compared to the toxin in a dose dependent manner. The PE extract, at all concentrations used and the 2.5 mg/ml and 5 mg/ml concentrations of the DCM and EtOAc extracts exhibited values lower than that of Trolox (1 mM), indicating that these extracts, at the specific concentrations, were even more effective than Trolox in scavenging the superoxide anions induced by KCN. The 2.5 mg/ml concentration of the PE extract exhibited more promising superoxide anion scavenging activity than the 5 mg/ml concentration. It may therefore be concluded that the PE extract exhibits a pro-oxidant effect at a concentration of 5 mg/ml. The 5 mg/ml concentration of the EtOAc extract of *L. javanica* exhibited the highest superoxide anion scavenging activity with a value of 7.755 ± 0.274 µM NBD/mg protein followed by the 2.5 mg/ml concentration of the PE extract with a value of 8.359 ± 0.562 µM NBD/mg proteien. The PE and EtOAc extracts of *L. javanica* therefore exhibited the highest superoxide scavenging activity. There appeared to be no significant difference between the NBT values for the 5 mg/ml concentration of the EtOAc extract and the 2.5 mg/ml concentration of the PE extract. It can therefore be concluded that the PE extract exhibited similar activity to the EtOAc extract but at a lower concentration of 2.5 mg/ml compared to the 5 mg/ml concentration of the EtOAc extract.

The extracts of *L. javanica* were able to scavenge free radicals and to trap $O_2^{\cdot-}$, preventing its conversion into H_2O_2 and $^{\cdot}OH$.

6.1.2 Thiobarbituric acid reactive substances (TBARS) assay

The *in vitro* antioxidant screening of *L. javanica* also included the determination of the ability of the extracts of the leaves of *L. javanica* to attenuate lipid peroxidation using a modified thiobarbituric acid reactive substances (TBARS) method of Ottino and Duncan (1997). During lipid peroxidation various reactive aldehydes are generated. Malondialdehyde (MDA) is the most abundant reactive aldehyde (Esterbauer & Cheeseman, 1990; Guo *et al.,* 2009).

Determination of MDA by the TBARS assay is one of the most common assays used to assess lipid peroxidation. The rational for determining attenuation of lipid peroxidation is to determine whether the extracts of *L. javanica* were capable of inhibiting the chain reaction induced by the highly destructive $^{\cdot}OH$.

6.1.2.1 Introduction and general principles of the lipid peroxidation assay

The thiobarbituric acid reactive substances (TBARS) assay was used to spectrophotometrically determine the level of lipid peroxidation induced by a toxin-solution consisting of hydrogen peroxide (H_2O_2), iron(III)chloride ($FeCl_3$) and ascorbic acid (Aruoma *et al.,* 1989; Linden *et al.,* 2008) as well as the ability of the PE, DCM, EtOAc and EtOH extracts of *L. javanica* to attenuate the H_2O_2-induced lipid peroxidation *in vitro.* The results were compared to that of Trolox (1 mM), since Trolox is known to break the lipid peroxidation chain as a result of its reaction with lipid peroxyl radicals (Sahin *et al.,* 2001; Traber & Atkinson, 2007). The principle of the assay is based on the reaction of malondialdehyde (MDA) with thiobarbituric acid (TBA) that yields a pink chromagen that can be extracted with buthanol and spectrophotometrically measured at 532 nm (Ottino & Duncan, 1997; Nandita & Rajini, 2004; Dasgupta & De, 2006). The ability of compounds in the extracts of *L. javanica* to inhibit lipid peroxidation decreases the formation of the MDA-TBA complex and thus decreases the absorption measured at 532 nm. This assay is however not specific for MDA since other aldehydes can also react with TBA (Draper & Hadley, 1990).

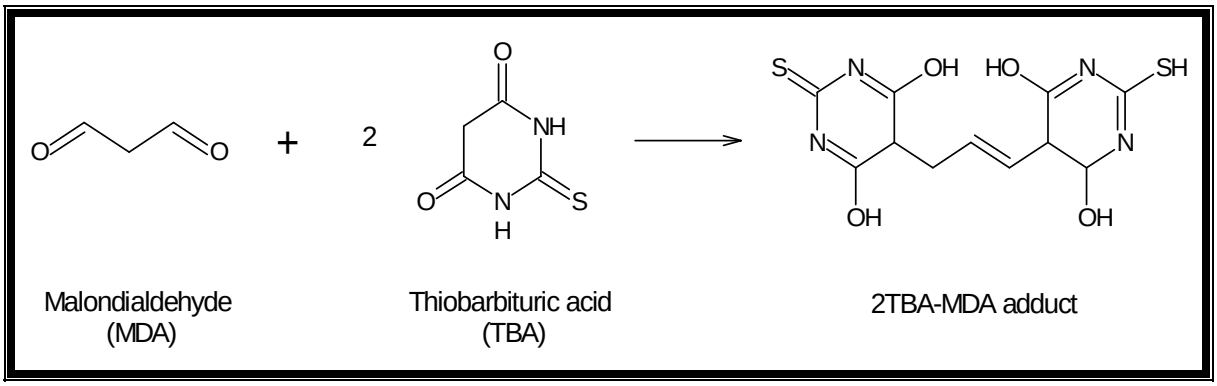

Figure 6.9 The reaction of malondialdehyde (MDA) with two molecules of thiobarbituric acid (TBA) that forms a MDA-TBA adduct (Kaur & Geetha, 2006).

6.1.2.2 Experimental

6.1.2.2.1 Chemicals

Ascorbic acid (Vitamin C), dimethyl sulfoxide (DMSO), potassium chloride (KCl), di-sodium hydrogen orthophosphate anhydrous (Na_2HPO_4), potassium dihydrogen orthophosphate (KH_2PO_4), hydrochloric acid (HCl), buthanol, methanol and iron(III)chloride hexahydrate

($FeCl_3.6H_2O$) were purchased from Merck-Chemicals (Saarchem (Pty) Ltd., 259 Davidson Road, Wadeville, Gauteng, South Africa). 1,1,3,3-tetramethoxypropane [malonaldehyde bis(dimethyl acetal)] (99%) (TMP), 2-thiobarbituric acid (98%) (TBA), butylated hydroxytoluene (2,6-di-tert-butyl-4-methylphenol) (BHT), trichloroacetic acid (TCA) and (±)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox) were purchased from Sigma-Aldrich Chemical Corporation (Reidstreet 2, D-89555 Steinheim, Germany). Sodium chloride (NaCl) was purchased from BHD, Midrand. Hydrogen peroxide was purchased at a local pharmay. All other chemicals used were obtained locally and of the highest chemical purity. Double distilled water was used throughout the experiment.

6.1.2.2.2 Reagents

Phosphate buffer solution (PBS): see section 6.1.1.2.2.

Butylated hydroxytoluene (BHT) solution (0.5 g/L) was prepared in methanol and stored in the refrigerator.

Trichloroacetic acid (TCA) solution (10%) was prepared in double distilled water and stored in the refrigerator.

Thiobarbituric acid (TBA) solution (0.33%) was prepared in double distilled water. Fresh solutions were prepared daily and the solution was protected from light by storing it in an amber bottle.

Tetramethoxypropane (TMP) stock solution (50 nmoles/L) was prepared in double distilled water. Fresh solutions were prepared daily.

Hydrogen peroxide (5 mM) was used as the toxin. The toxin was used to generate $^{\cdot}OH$ and initiated lipid peroxidation in rat brain homogenate. Ascorbic acid (1.4 mM) and $FeCl_3$ (4.8 mM) were added to increase the generation of $^{\cdot}OH$ according to the Fenton reaction (see figure 2.16) (Bhat *et al.,* 2001; Cui *et al.,* 2004; Zhu *et al.,* 2006).

Trolox (1 mM) was dissolved in EtOH before diluting it to the required volume with double distilled water.

The PE, DCM, EtOAc and EtOH extracts of *L. javanica*: see section 6.1.1.2.2.

6.1.2.2.3 Animals and preparation of brain homogenate

For detailed description see section 6.1.1.2.3.

6.1.2.2.4 Preparation of standard curves

6.1.2.2.4.1 MDA standard curve

To express the extent of lipid peroxidation, one has to determine the µmoles malondialdehyde (MDA) formed.

Increasing concentrations of 1,1,3,3-Tetramethoxypropane (TMP)/malondialdehyde (MDA) was used as a standard for measuring the level of lipid peroxidation by means of the TBARS assay. The standards, containing 0 – 25 nmoles/L of MDA at increments of 5 nmoles/L, were used to generate a standard curve (figure 6.10) using equation 5.5.

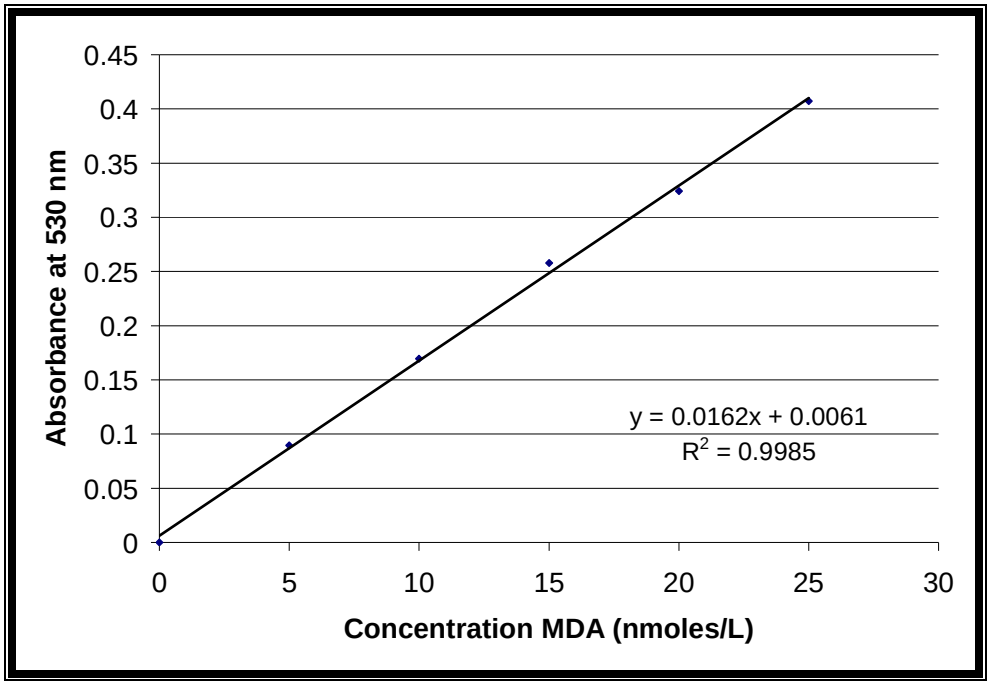

Figure 6.10 Lipid peroxidation standard curve generated from malondialdehyde. Data is expressed as concentration malondialdehyde (nmoles/L) plotted against the absorbance values measured at 530 nm and results in a regression of 0.9985. n = 5.

6.1.2.2.5 Instrumentation

Absorbance values for the lipid peroxidation assay were analyzed at 530 nm, using a LABSYSTEMS MULTISCAN RC multiplate reader and Genesis software.

6.1.2.2.6 TBARS assay

Lipid peroxidation was measured by the thiobarbituric acid reactive substances (TBARS) assay. In brief, 160 µl of brain homogenate containing 20 µl of the toxin (10 µl of H_2O_2; 5 µl of $FeCl_3$ and 5 µl of ascorbic acid) (to initiate lipid peroxidation), in the absence and presence of varying concentrations of the PE, DCM, EtOAc and EtOH extracts of *L. javanica*

(1.25, 2.5 and 5 mg/ml) and trolox (1 mM) was incubated at 37 °C in an oscillating waterbath. The PE, DCM, EtOAc and EtOH extracts of *L. javanica* (1.25, 2.5 and 5 mg/ml) were dissolved in DMSO and PBS and were added to the homogenate at the beginning of the incubation period in a volume of 20 µl (for the initial experiments conducted on the crude leaf extracts of *L. javanica*). During bio-assay guided fractionation (Chapter 7), the various column fractions were tested only at a concentration of 1.25 mg/ml. In the case of control experiments, volume adjustments were done with PBS. After incubation the suspensions were centrifuged at 2000 x g for 20 minutes. Following the centrifugation, the supernatant was transferred to a new set of tubes. 100 µl of a methanolic solution of BHT (to inhibit lipid peroxidation during the assay) (Aruoma *et al.,* 1989), 200 µl of an aqueous solution of TCA (to precipitate cellular proteins) and 100 µl of an aqueous solution of TBA were added and incubated at 90 °C for 1 hour in a waterbath. After cooling on ice, 400 µl of buthanol was added and tubes shaken vigorously on a vortex mixer. The MDA-TBA complex was extracted into buthanol. After centrifugation at 2000 x g for 10 minutes, 200 µl of the upper organic layer was transferred into a 96-well multiplate and the absorbance was measured at a wavelength of 530 nm with buthanol as the blank. Absorbance values were converted to nmoles MDA/mg tissue using the standard curve (figure 6.10). All experiments were measured in 5 replicates and repeated once to ensure reproducibility.

6.1.2.2.7 Data collection

The raw data was imported to a Microsoft Excel worksheet.

The absorbance values of the lipid peroxidation assay were converted to nmoles MDA detected, using the calibration curve generated from increasing concentrations of malondialdehyde (figure 6.10). The final results were expressed as nmoles malondialdehyde/mg tissue.

6.1.2.2.8 Statistical analysis

The results obtained were statistically analyzed with GraphPad InStat 3 software. The data from the TBARS assay is presented as the mean ± standard error fo the mean (SEM) from 5 determinations in each run and the experiments were repeated once. Also see section 6.1.1.2.9.

6.1.2.2.9 Results and discussion

The amount of TBARS was expressed as nmoles MDA equivalents formed per mg tissue and results are presented as mean ± standard error of the mean (SEM). The TBARS values for the PE, DCM, EtOAc and EtOH extracts of *L. javanica* are presented in table 6.3 and figure 6.11.

The extracts demonstrated varying degrees of activity with the TBARS assay. The *in vitro* exposure of whole rat brain homogenate to the toxin (H_2O_2, $FeCl_3$ and ascorbic acid in a ratio of 2:1:1) caused a significant increase in lipid peroxidation when compared to the control value, $p<0.001$. Based on the results obtained from data in table 6.3 and figure 6.11, one can see that all of the extracts of *L. javanica* exhibited activity with the TBARS assay by lowering the MDA formed from lipid peroxidation to values lower than that of the toxin as well as the control. The more effective the extract, the lower the concentration of MDA-TBA complex produced. The TBARS values varied from 0.212 ± 0.030 to 2.187 ± 0.543 nmoles MDA/mg tissue.

Table 6.3 The *in vitro* effect of the PE, DCM, EtOAc and EtOH extracts of the leaves of *L. javanica* on H_2O_2-induced lipid peroxidation in rat brain homogenate.

Compound/Extract	Concentration	MDA (nmoles/mg tissue) (n = 5)	Standard error of the mean (SEM)
Control		0.912	± 0.054
Toxin		2.188	± 0.192
Trolox	1 mM	0.213	± 0.010
L. javanica$_{PE}$	1.25 mg/ml	0.638	± 0.017
	2.5 mg/ml	0.520	± 0.042
	5 mg/ml	0.488	± 0.014
L. javanica$_{DCM}$	1.25 mg/ml	0.681	± 0.031
	2.5 mg/ml	0.387	± 0.044
	5 mg/ml	0.313	± 0.017
L. javanica$_{EtOAc}$	1.25 mg/ml	0.233	± 0.013
	2.5 mg/ml	0.235	± 0.009
	5 mg/ml	0.217	± 0.028
L. javanica$_{EtOH}$	1.25 mg/ml	0.293	± 0.010
	2.5 mg/ml	0.342	± 0.008
	5 mg/ml	0.430	± 0.010

As shown in figure 6.11, treatment of the rat brain homogenate with the toxin (H_2O_2, $FeCl_3$ and ascorbic acid) caused a significant increase in lipid peroxidation as determined by the TBARS assay in comparison to the control. However, treatment of rat brain homogenate with increasing concentrations of the extracts resulted in an overall decline in toxin (H_2O_2, $FeCl_3$ and ascorbic acid)-induced lipid peroxidation in comparison to the toxin alone. All the concentrations (1.25, 2.5 and 5 mg/ml) of the four extracts of *L. javanica* and 1 mM Trolox were significantly effective at reducing the lipid peroxidation products produced by the toxin solution in rat brain homogenate, $p<0.001$. Furthermore, all concentrations of all the extracts of *L. javanica* were able to lower the MDA-TBA complex levels below that measured

in the control (homogenate with no antioxidant or toxin present), p<0.001. The fact that at all of the concentrations of all the extracts of *L. javanica* were able to lower the MDA levels to below that of the control value indicates that these extracts offer complete protection against the neurotoxic effects of the toxin and may also indicate that these extracts inhibit lipid peroxidation that occurs during normal processes in the brain.

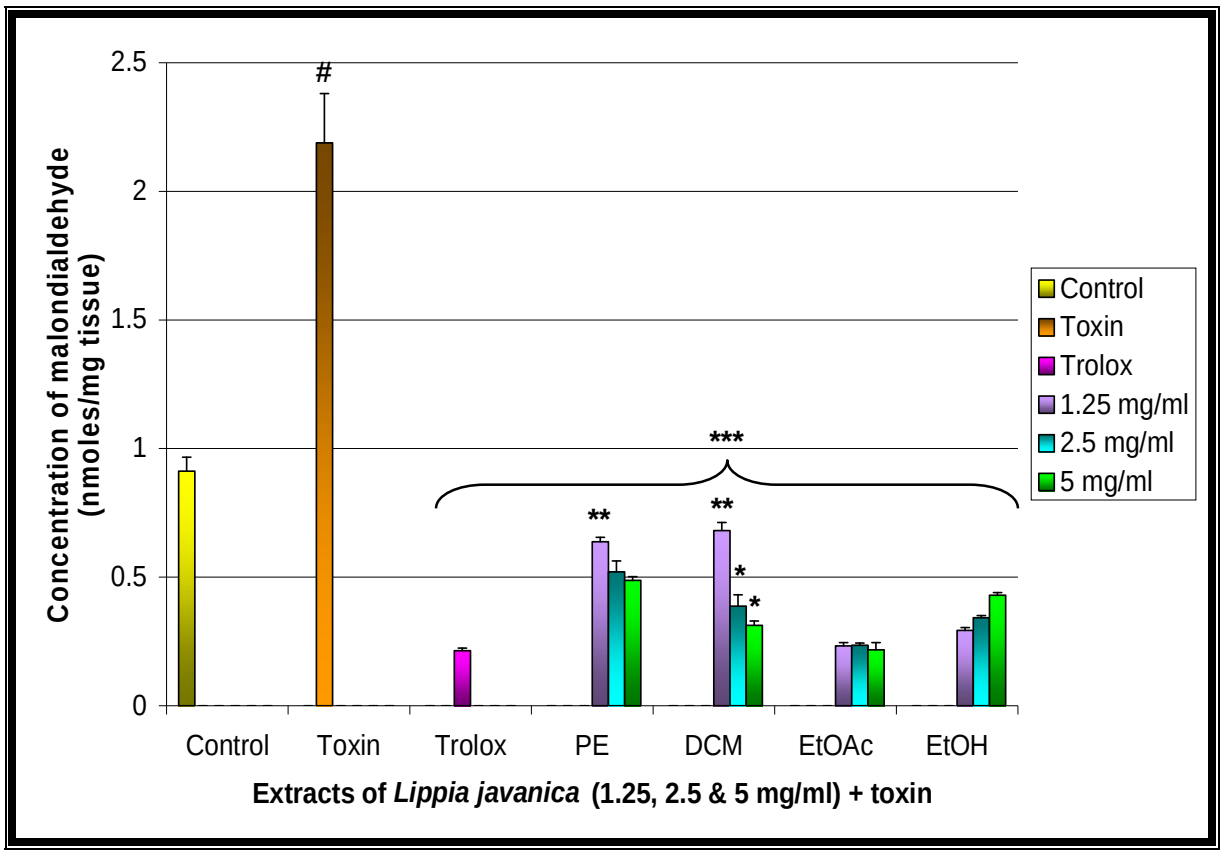

Figure 6.11 The attenuation of lipid peroxidation of increasing concentrations of the PE, DCM, EtOAc and EtOH extracts of the leaves of *L. javanica* and Trolox (1 mM) on toxin (H_2O_2, $FeCl_3$ & ascorbic acid)-induced lipid peroxidation on whole rat brain homogenate. Each bar represents the mean ± SEM. n = 5. #p<0.001 in comparison to the control, ***p<0.001 in comparison to the toxin as well as compared to the control, *p<0.001 in comparison to the 1.25 mg/ml concentration of the DCM extract and **p<0.01 in comparison to control (Student-Newman-Keuls Multiple Range Test).

The DCM extract of *L. javanica* caused a significantly lower concentration of MDA than the toxin in a dose dependent manner. There appeared to be no significant difference between the different concentrations of the PE, EtOAc and EtOH extracts. The activity of the 5 mg/ml and 2.5 mg/ml concentrations of the DCM extract as well as all concentrations of the EtOAc and EtOH extracts was comparable to that of Trolox. The 5 mg/ml concentration of the EtOAc extract of *L. javanica* exhibited the most pronounced attenuation of lipid peroxidation products with a value of 0.217 ± 0.081 nM MDA/mg tissue followed by the 2.5 mg/ml and

1.25 mg/ml concentrations of the EtOAc extract with values of 0.233 ± 0.037 and 0.235 ± 0.025 nM MDA/mg tissue, respectively. All the extracts of *L. javanica* exhibited excellent activity with the TBARS assay, lowering the MDA formed by the toxin to values below that of the toxin and the control (no drug or toxin present) and therefore the results of the NBT assay were used when selecting the most active extract of *L. javanica*. The extracts of *L. javanica* were able to scavenge free radicals and attenuate lipid peroxidation.

Based on these experiments, the evaluation of the PE extract of *L. javanica* by isolating and identifying a compound was undertaken.

CHAPTER 7

Bio-assay guided isolation and structure elucidation of a compound from *L. javanica*

7.1 Chromatographic techniques

The aim of this chapter was to simplify the petroleum ether extract of *L. javanica* leaves, thereby facilitating the isolation of a compound from a fraction with antioxidant activity by bioassay-guided fractionation. The large number of components present in a plant extract makes analysis of the bioactive compounds an extremely difficult task. The methodology for studying natural products therefore includes the fractionation of a complex mixture, the separation and isolation of components using various chromatographic methods and structure elucidation using various spectroscopic methods. Activity-guided isolation was used during the purification process. The phytochemical investigation of the PE extract of *L. javanica* leaves was initiated with the performance of thin-layer chromatography (TLC). Purification of the PE extract of the leaves *L. javanica* was achieved by using column chromatography. The separation was observed with the use of TLC and examination using visible and UV light (254 and 366 nm) and visualizing agents 5% H_2SO_4 (sulphuric acid) and/or anisaldehyde. The purified fraction was then analyzed by determining the nuclear magnetic resonance spectroscopy (NMR) and infrared spectroscopy (IR) to elucidate the chemical structure.

7.1.1 Thin-layer chromatography (TLC)

Analytical TLC was employed in the selection of suitable mobile phases for the isolation of the compound using precoated Merk silica gel aluminium backed plates, thickness 0,25 mm (Merk® 5554 DC – Alufolien 60 F_{254}). A variety of solvent systems were prepared until the optimal solvent system was obtained for the separation of the different constituents. Mobile phases were prepared by mixing solvents in a volume to volume ratio. The TLC plates were developed under saturated conditions with the chosen eluent system. Before dipping the TLC plates in 5% sulphuric acid and/or anisaldehyde, the plates were observed under UV and visible light, which identified fluorescent-quenching compounds in plant extracts. UV light usually identifies fluorescing compounds with many double bonds and the visible light only detects coloured compounds, usually with conjugated bonds. Compounds containing aromatic rings absorb UV light at 254 nm and therefore quench the fluorescence of the pigment included in the silica gel. The chromatograms were then dipped in 5% H_2SO_4 and/or anisaldehyde to aid with visualization of poor chromophores or non-chromogenic compounds. Once the plates were dipped they were heated in an oven at 100 ˚C to allow for colour development until maximal visualization of spots was obtained. After visualization, the

outline of the zones was marked with a soft pencil since some spots had the tendency to fade after a while. For documentation of the chromatogram, the plates were scanned.

7.1.2 Column chromatography (CC)

Vacuum liquid chromatography with a Versaflash was carried out using Merck silica gel 60 G columns. The PE extract or column fractions were dissolved in a small amount of mobile phase and adsorbed onto the dry silica column with a pasteur pipette. The mobile phases comprized of different ratios of different eluents as indicated for the individual columns. The flow rate was 5 ml/min and fractions of 2 ml each were collected. Compound elution was monitored by TLC.

7.2 Bioassay-guided fractionation

7.2.1 Fractionation of the petroleum ether extract of *L. javanica*

From serial extraction of *L. javanica* leaves, four extracts were obtained, PE, DCM, EtOAc and EtOH.

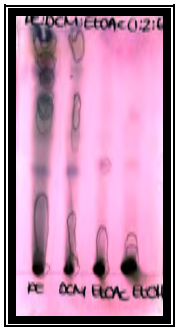

Figure 7.1 TLC chromatogram of *L. javanica* leaf extracts developed in PE:DCM:EtOAc (1:2:6). The solvents used for extraction are PE, DCM, EtOAc and EtOH (with polarity increasing from left to right. After the development of the TLC plate, the plate was dipped in anisaldehyde.

The TLC chromatogram (figure 7.1) revealed that the extracts contain various constituents.

1 g of crude *L. javanica* petroleum ether extract was loaded onto a vacuum liquid chromatography column (40 x 90 mm) packed with silica gel 60 particle size 0.063 – 0.2 mm (70 – 230 mesh) (Fluka Chemika). The column was developed with PE:DCM (1:1), EtOAc, EtOH and MeOH consecutively. Fractions from test tubes that exhibited a similar separation profile as indicated by TLC analysis upon dipping in 5% H_2SO_4 and visualized under UV, were pooled together and a total of 7 fractions were obtained and dried. This separation was repeated twice (fractions O, P & Q) (figure 7.3) and the fractions with a similar separation

profile were pooled together (fractions R) (see figure 7.3). None of the original column fractions were pure. See figure 7.10 for isolation flowchart.

After each purification stage the antioxidant activity of fractions of ample amount were tested using the NBT and TBARS assays. The NBT assay was carried out as described in section 6.1 and the TBARS assay was carried out as described in section 6.2. The results are given in figure 7.8 for the NBT assay and figure 7.9 for the TBARS assay.

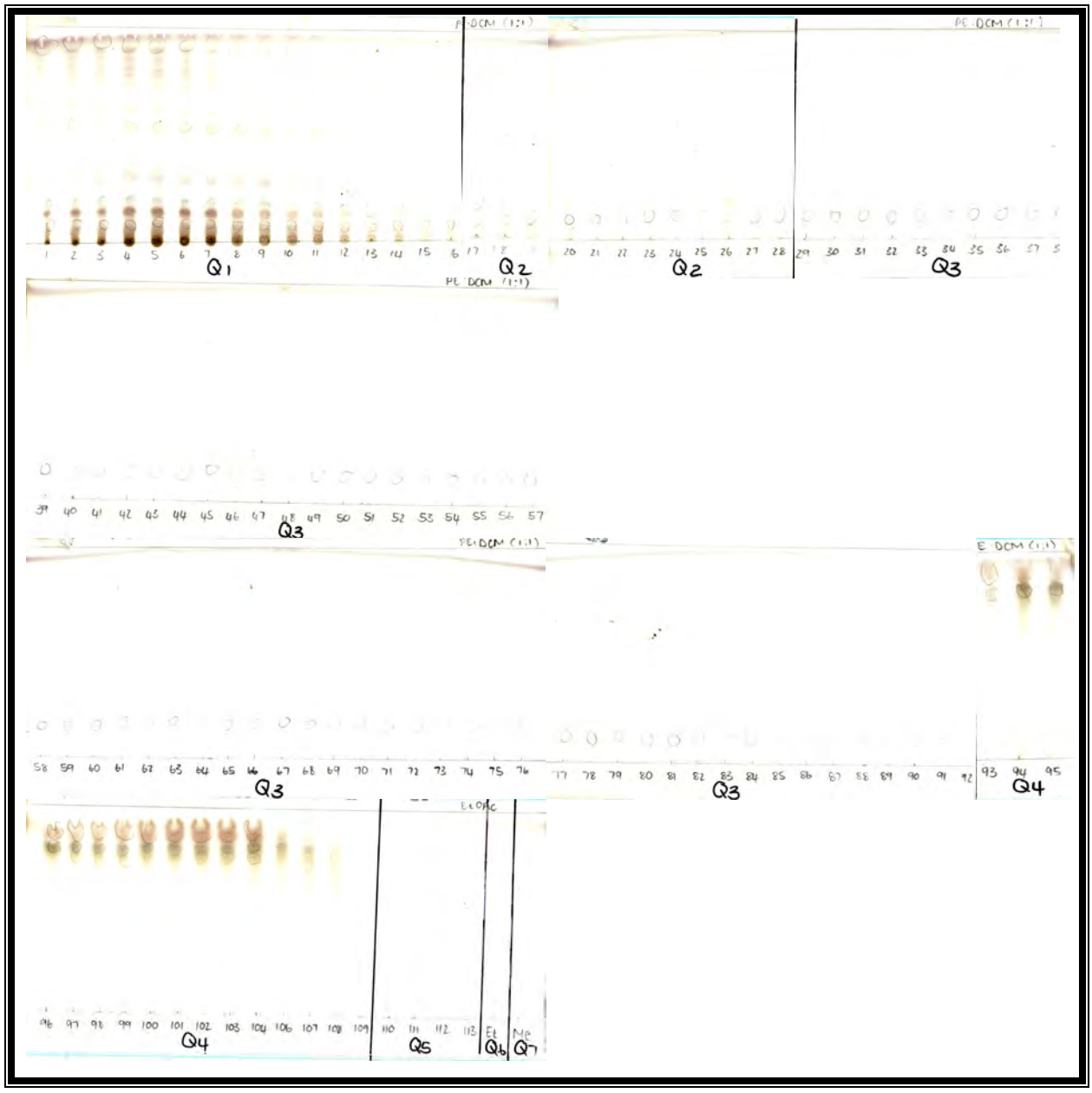

Figure 7.2 TLC chromatograms of pooled fractions from column chromatography of crude *L. javanica* PE extract with PE:DCM (1:1); EtOAc; EtOH and MeOH, consecutively. Fractions were collected from test tubes 1 – 16; 17 – 28; 29 – 92; 93 -109; 110 – 113; EtOH fraction and MeOH fraction. 5% H_2SO_4 was used as the visualizing agent.

The column in figure 7.2 was repeated twice and the fractions of columns O, P and Q were pooled together based on similarities in the separation profile.

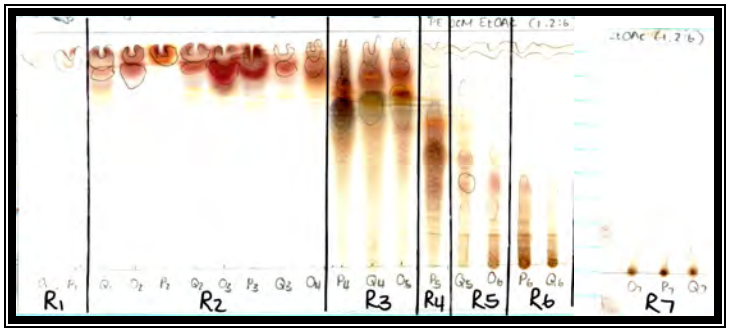

Figure 7.3 TLC chromatogram of the fractions from columns O, P and Q. Fractions were pooled together based on similarities in the separation profile and resulted in fractions R1 - R7. The TLC plates were developed in PE:DCM:EtOAc (1:2:6) and then dipped in 5% H_2SO_4.

Fraction R3 exhibited promising antioxidant activity (see figures 7.8 & 7.9) and was therefore subjected to subsequent vacuum liquid column chromatography over silica gel with PE:DCM:EtOAc (1:2:6); EtOAc:EtOH (1:1) and EtOH consecutively. 57 fractions of 2 ml each were collected. Each fraction was applied onto the TLC plates. The plates were developed in PE:DCM:EtOAc (1:2:6) and were visualized by dipping the plates in 5% H_2SO_4 and heating at 100 °C for approximately 5 minutes (see figure 7.4).

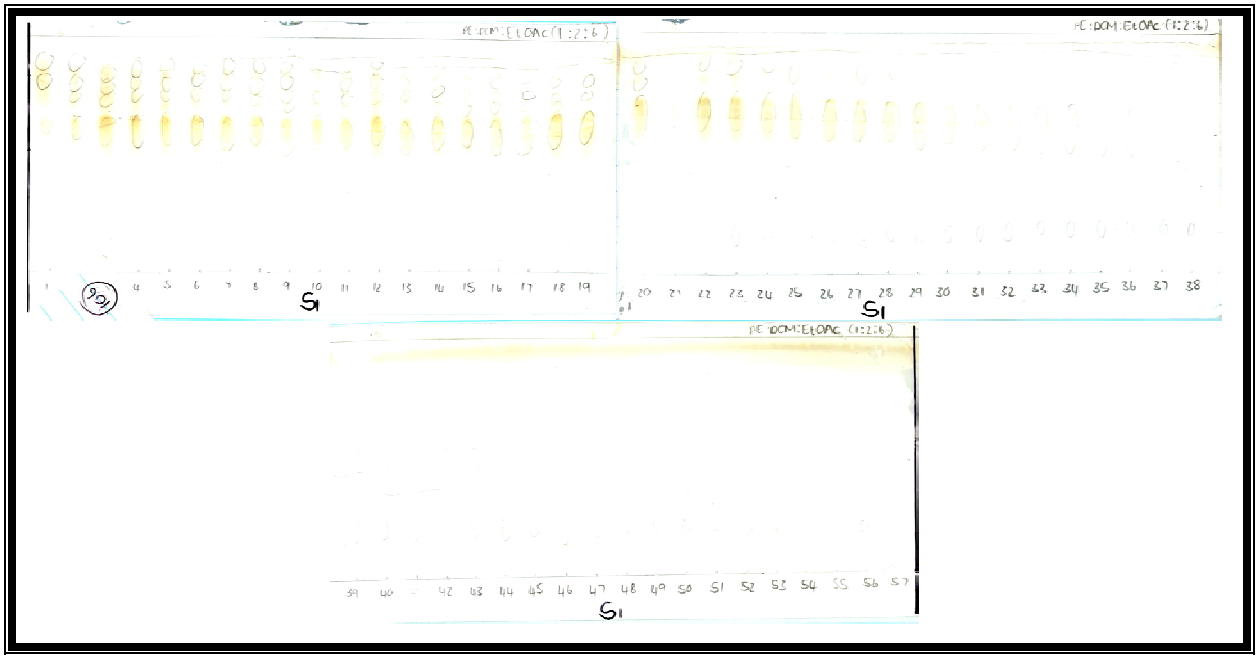

Figure 7.4 TLC chromatograms of fractions collected from column chromatography of fraction R3 with PE:DCM:EtOAc (1:2:6); EtOAc:EtOH (1:1); EtOAc and EtOH consecutively. Fractions were collected from test tubes 1 – 57. 5% H_2SO_4 was used as the visualizing agent.

Fraction S1 exhibited antioxidant activity (see figures 7.8 & 7.9) and was further purified using column chromatography and PE:DCM:EtOAc (1:1:1); PE:DCM:EtOAc (1:2:8); EtOAc and EtOH consecutively. 160 fractions of 2 ml each were collected and applied onto the TLC plates. The plates were developed in PE:DCM:EtOAc (1:2:8), dipped in 5% H_2SO_4 and heated at 100 ˚C (see figure 7.5).

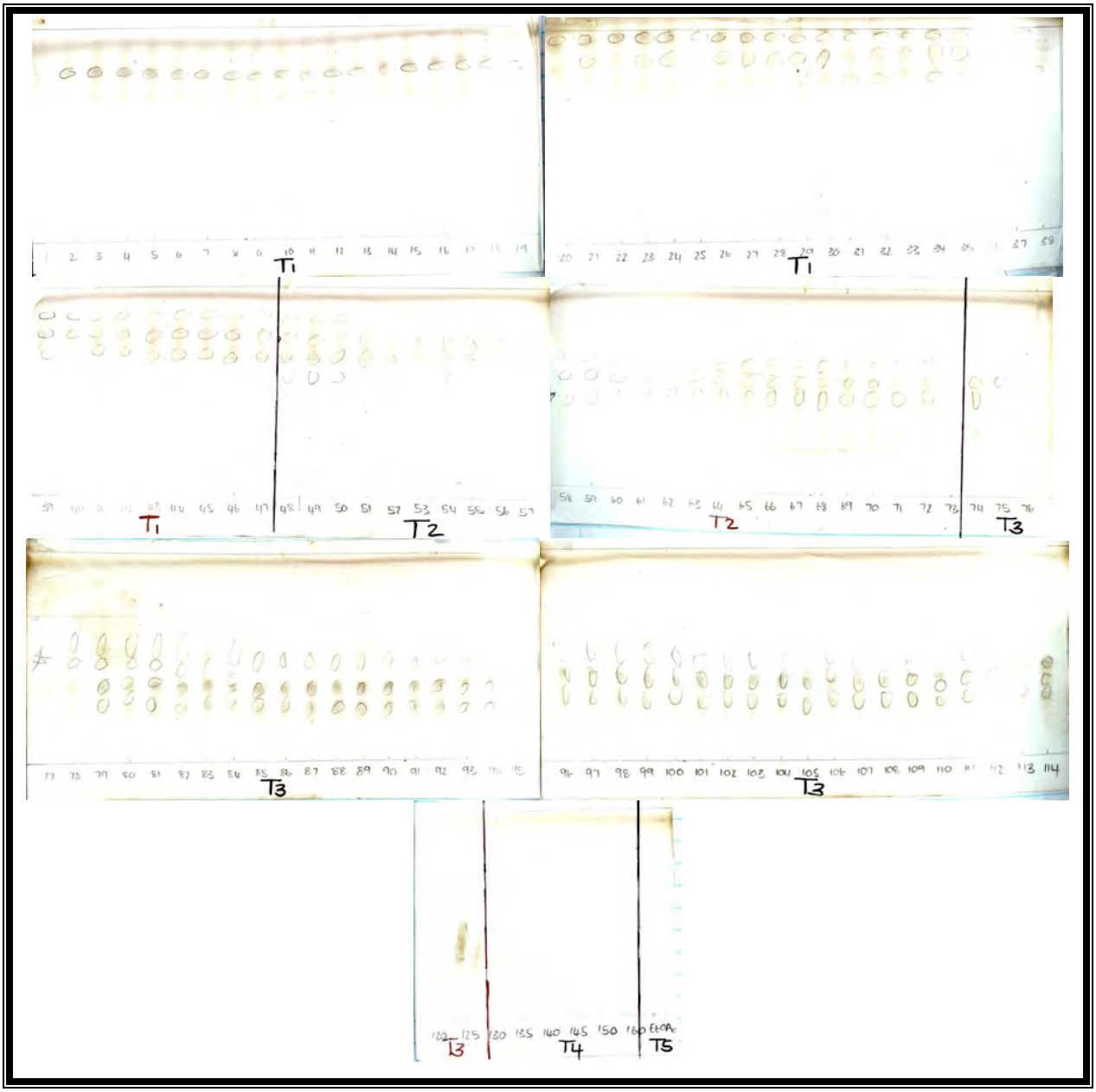

Figure 7.5 TLC chromatograms of fractions collected from column chromatography of fraction S1 with PE:DCM:EtOAc (1:2:8). Fractions were collected from test tubes 1 – 47; 48 – 73; 74 – 125; 126 -160; EtOAc fraction and EtOH fraction. 5% H_2SO_4 was used as the visualizing agent.

Fraction T3 exhibited antioxidant activity (see figures 7.8 & 7.9) and was further purified using column chromatography and PE:DCM:EtOAc (1:2:6) EtOAc:EtOH (1:1) and EtOH

consecutively. 100 fractions of 2 ml each were collected and applied onto the TLC plates. The plates were developed in PE:DCM:EtOAc (1:2:6), dipped in 5% H_2SO_4 and heated at 100 ˚C (see figure 7.6).

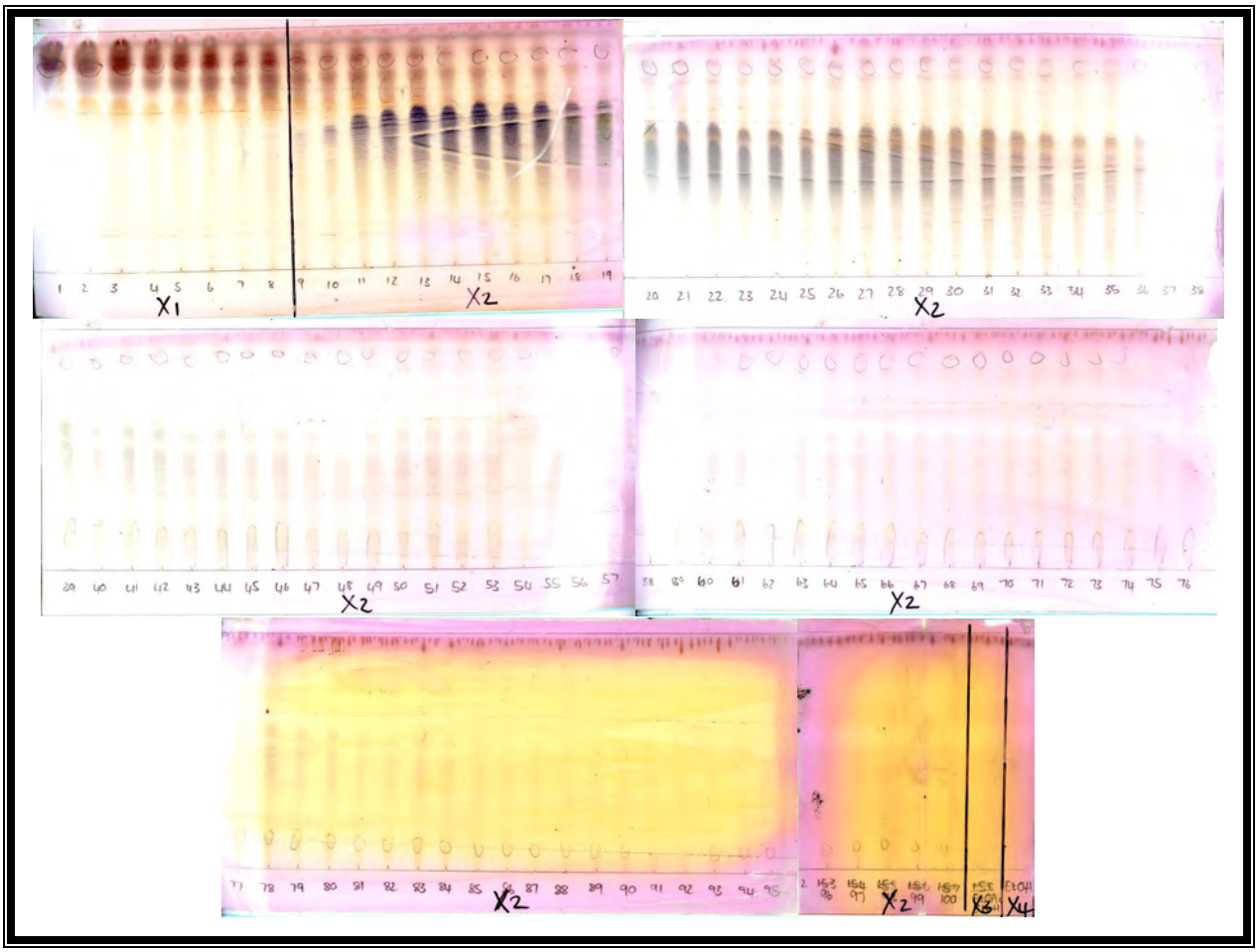

Figure 7.6 TLC of fractions collected from column chromatography of fraction T3 with PE:DCM:EtOAc (1:2:6). Fractions were collected from test tubes 1 – 8; 9 – 100; EtOAc:EtOH (1:1) fraction and EtOH fraction. 5% H_2SO_4 was used as visualizing agent.

Fraction X2 exhibited antioxidant activity (see figures 7.8 & 7.9) and was further purified using column chromatography and PE:EtOAc (4:1); PE:DCM:EtOAc (1:2:6) and EtOH consecutively. 60 fractions of 2 ml each were collected and applied onto the TLC plates. The plates were developed in PE:DCM:EtOAc (1:2:6), dipped in anisaldehyde and heated at 100 ˚C (figure 7.7).

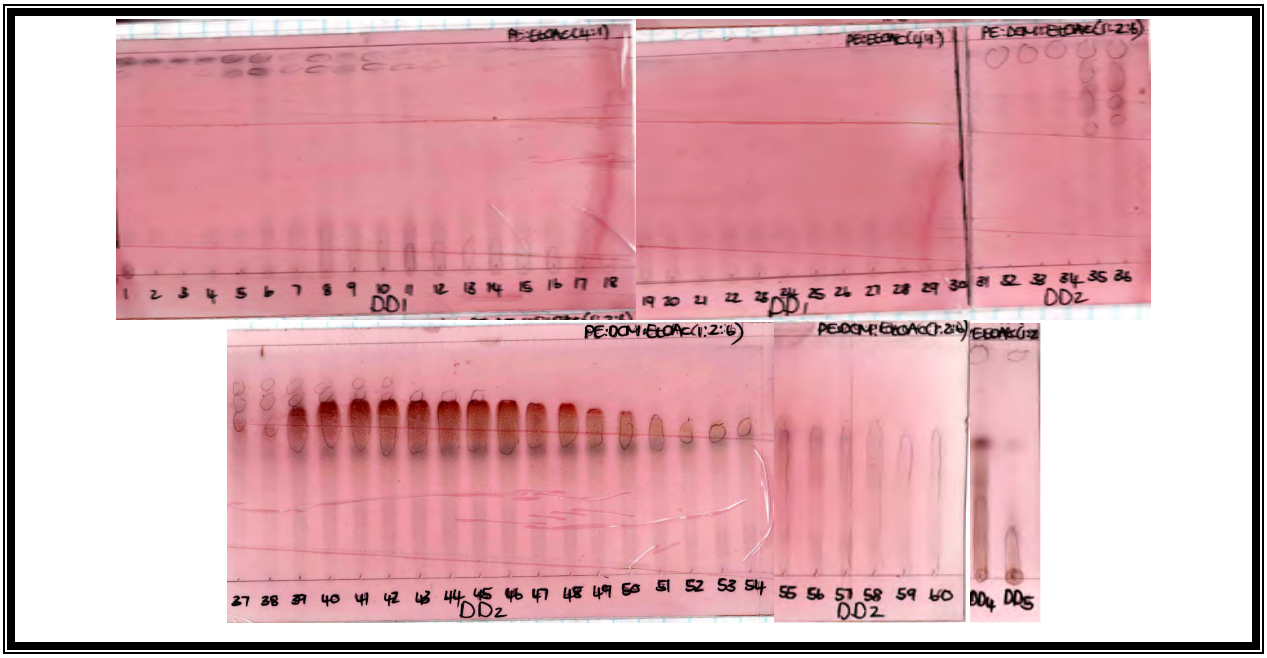

Figure 7.7 TLC chromatograms of fractions collected from column chromatography of fraction X2 with PE:EtOAc (4:1); PE:DCM:EtOAc (1:2:6) and MeOH consecutively. Fractions were collected from test tubes 1 – 30; 31 – 60; EtOH fraction and MeOH fraction. Anisaldehyde was used as visualizing agent.

Fraction DD1 was not absolutely pure. After removing the solvent from fraction DD1, the structure of the substance from fraction DD1 was investigated by means of IR, NMR and MS after which the sample was recovered and used for biological assays.

7.2.2 Antioxidant activity of fractions

7.2.2.1 NBT-assay

The NBT assay was carried out as described in section 6.1. All of the fractions as well as the PE extract of *L. javanica* were tested at a concentration of 1.25 mg/ml to compare the activity.

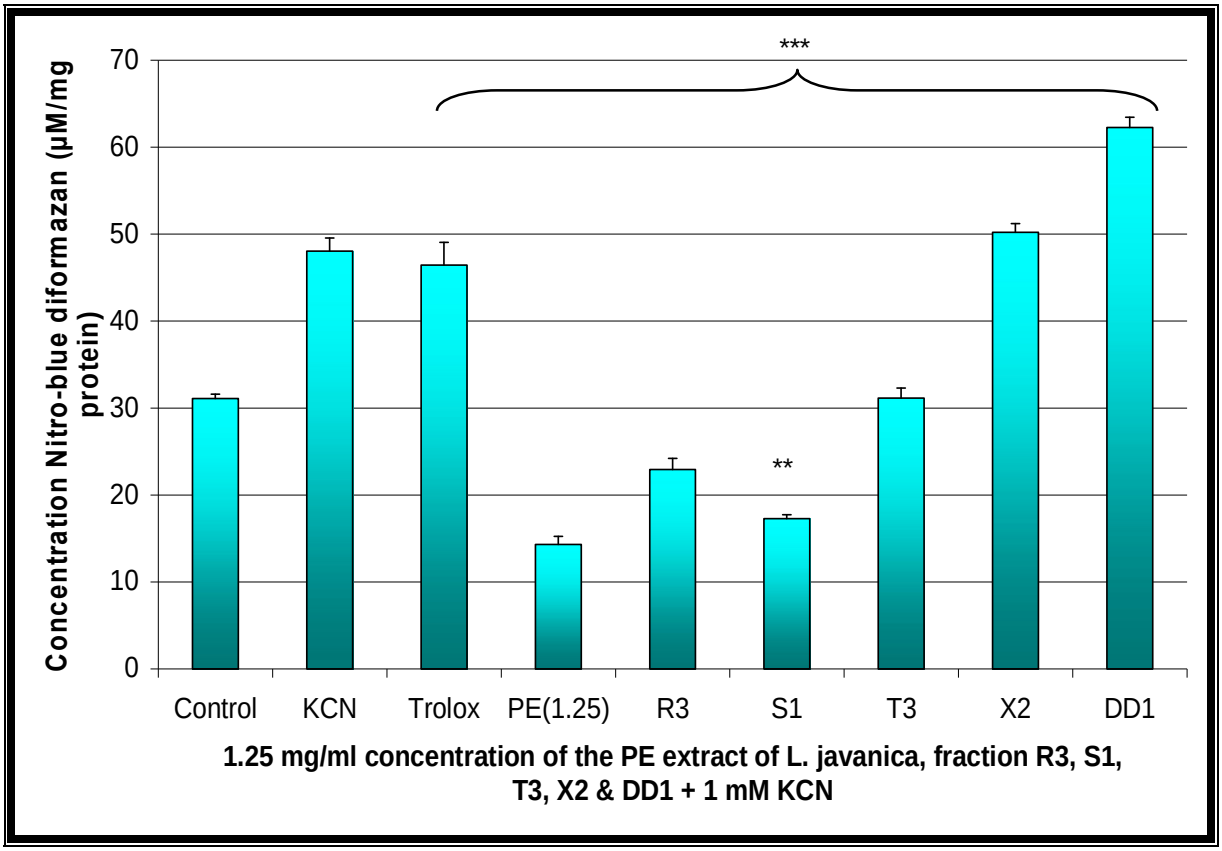

Figure 7.8 The superoxide anion radical scavenging effect of 1.25 mg/ml of the PE extract of the leaves of *L. javanica*, fractions R3, S1, T3, X2 and DD1 on 1 mM KCN-induced superoxide anion generation in rat brain homogenate. Each bar represents the mean ± SEM. n = 5. **p<0.01 compared to fraction R3 and ***p<0.001 in comparison to the control and 1 mM KCN. The 1.25 mg/ml concentration of the PE extract exhibited a non-significant difference compared to fraction S1, the control exhibited a non-significant difference compared to fraction T3, fraction X2 and 1 mM KCN exhibited a non-significant difference compared to 1 mM Trolox and fraction X2 exhibited a non-significant difference when compared to 1 mM KCN (Student-Newman-Keuls Multiple Range Test).

As shown in figure 7.8, treatment of rat brain homogenate with the various fractions of the petroleum ether extract of the leaves of *L. javanica,* resulted in a reduction of nitro-blue diformazan, indicating that these fractions are able to reduce KCN-induced superoxide anion generation. However, none of the fractions exhibited more promising activity than that of the crude extract. The rational for this being that the various compounds present in a sample may exhibit synergistic actions whereas the pure compounds therefore usually exhibit less promising activity than the combination of compounds in a specific fraction. Fraction DD1 exhibited the least promsing activity with the NBT assay.

7.2.2.2 TBARS-assay

The TBARS assay was carried out as described in section 6.2. All of the fractions as well as the PE extract of *L. javanica* were tested at a concentration of 1.25 mg/ml to compare the activity.

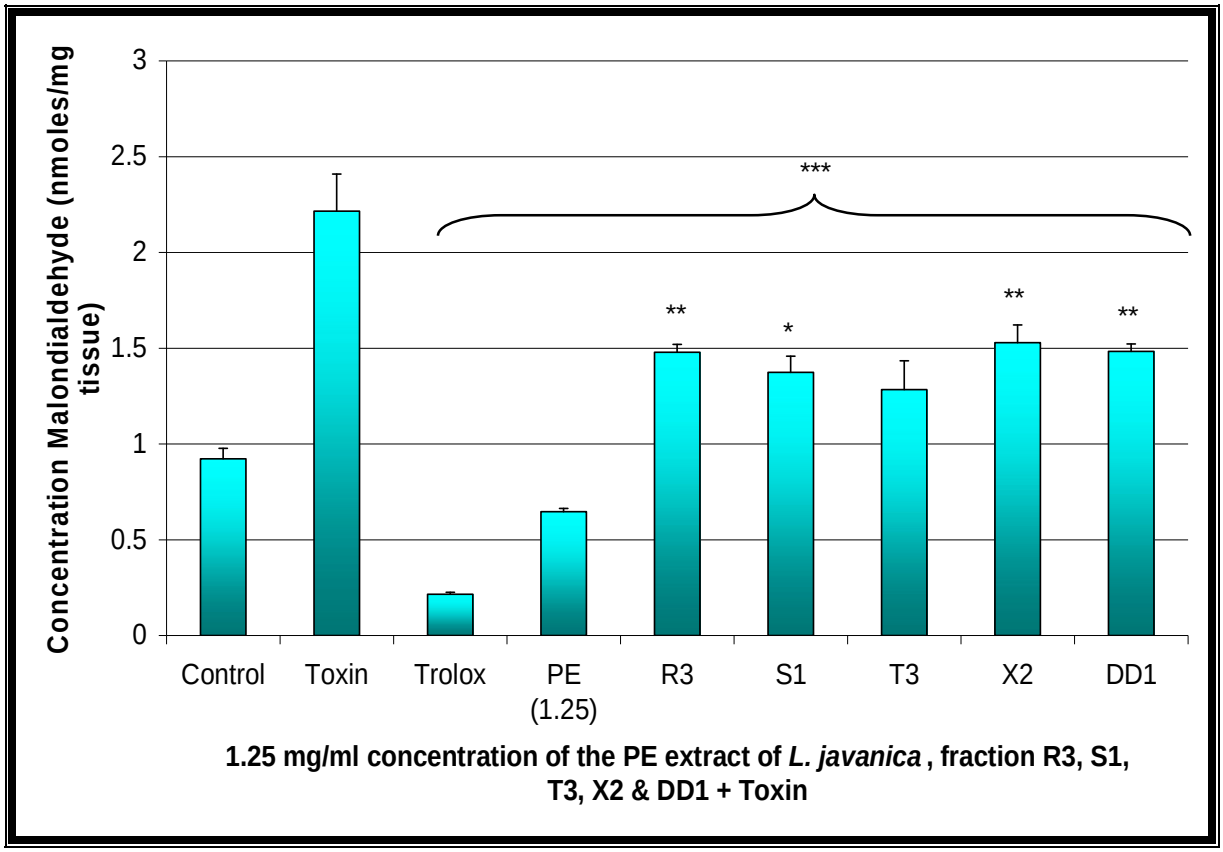

Figure 7.9 The attenuation of lipid peroxidation of 1.25 mg/ml of the PE extract of the leaves of *L. javanica*, fractions R3, S1, T3, X2 and DD1 on toxin (H_2O_2 + $FeCl_3$ + ascorbic acid)-induced lipid peroxidation in rat brain homogenate. Each bar represents the mean ± SEM. n = 5. ***p<0.001 compared to the control and Toxin, **p<0.01 compared to the control and *p<0.05 compared to the control. The 1.25 mg/ml concentration of the PE extract exhibited a non-significant difference when compared to the control. Fraction R3 exhibited a non-significant difference compared to fractions S1, T3, X2 and DD1, fraction S1 exhibited a non-significant difference when compared to fractions T3, X2 and DD1. Fraction X2 exhibited a non-significant difference when compared to fraction T3 and fraction DD1 exhibited a non-significant difference when compared to fractions T3 and X2 (Student-Newman-Keuls Multiple Range Test).

As shown in figure 7.9, treatment of rat brain homogenate with the various fractions of the petroleum ether extract of the leaves of *L. javanica,* resulted in attenuation of toxin-induced lipid peroxidation, indicating that these fractions exhibit activity in the specific assay. None of the fractions however, exhibited more promising activity than that of the crude extract. As

mentioned in section 7.2.2.1, this could be due to the fact that the various compounds present in a fraction possess synergistic properties, causing the fraction to exhibit more promising activity than the pure compound. Fraction DD1 appeared to exhibit similar activity as fraction X2 in the TBARS assay.

7.3 Instrumentation

7.3.1 Nuclear Magnetic Resonance (NMR) Spectroscopy

NMR is a valuable structure elucidation tool for organic molecules. The ^{13}C and ^{1}H nuclear magnetic resonance (NMR) spectra of the isolated compound were recorded on a Bruker avance 600 spectrometer, in a 14.09 Tesla magnetic field using an ultra shield plus magnet. ^{13}C spectra were recorded at a frequency of 150.9126 MHz while the ^{1}H spectra were recorded at a frequency of 600.1724 MHz. All spectra were recorded at 25 ˚C and all chemical shifts are reported in parts per million (ppm) relative to tetramethylsilane ($\delta = 0$) of the solvent shift. NMR samples were dissolved in deutirated chloroform ($CDCl_3$).

7.3.2 Infrared absorption (IR) spectra

The infrared (IR) spectra were recorded on a Nicolet Nexus 470-FT-IR spectrometer over the range 400 – 4000 cm^{-1}. The samples were recorded with the use of KBr pellets and using the diffuse reflectance method.

7.3.3 Mass spectroscopy (MS)

Mass spectroscopy is the most sensitive and selective method for molecular analysis and can yield information on the molecular weight as well as the structure of the molecule. The basis of mass spectrometry is the production of ions that are subsequently separated according to their (m/z) ratio, where m is the mass and z is the number of charges carried by the ion, and then detected. Mass spectra (MS) were recorded on an analytical Micromass Autospec mass spectrometer using electron impact (EI) at 70 eV as ionisation technique.

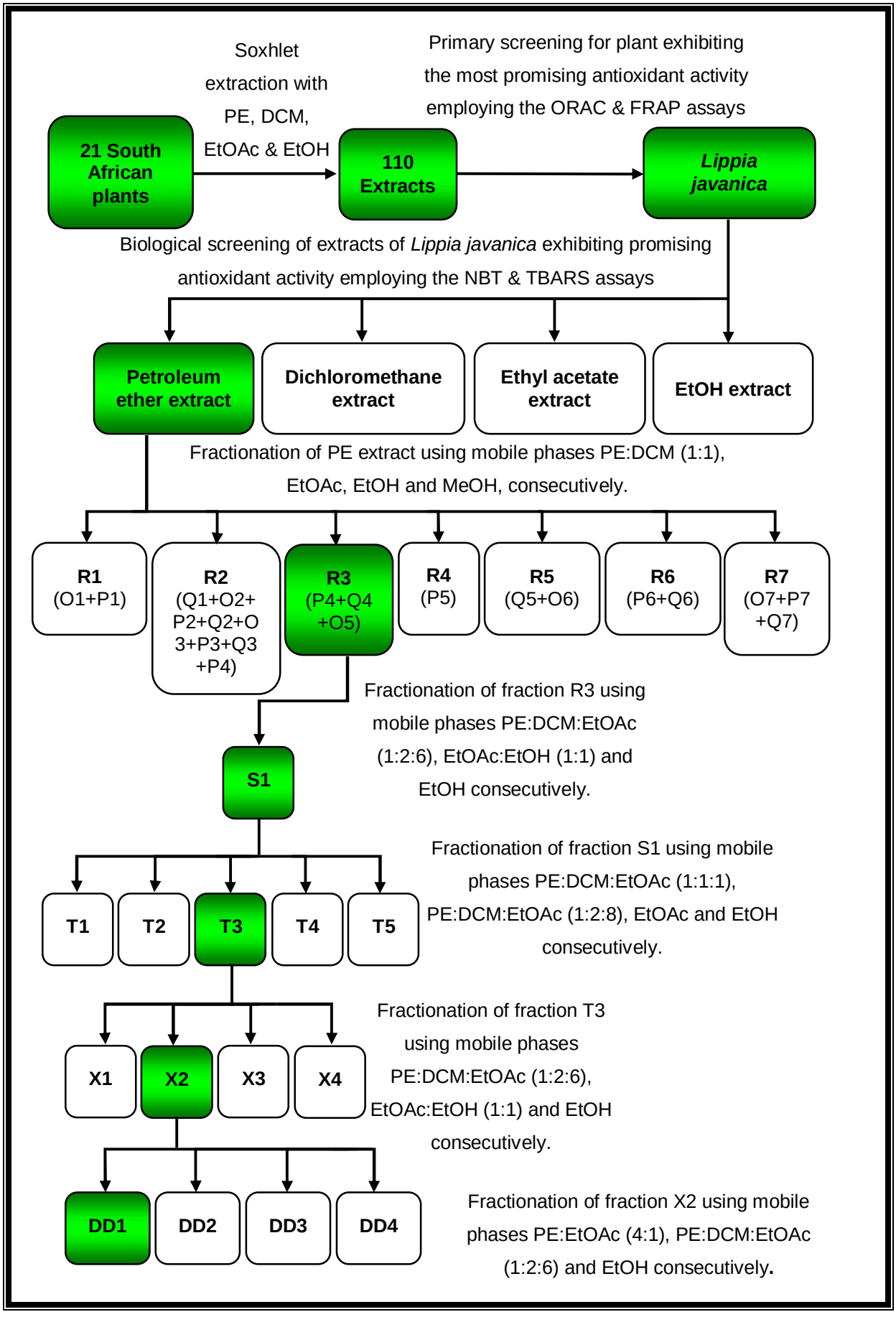

Figure 7.10 Isolation flowchart of fraction DD1 from the PE extract of *L. javanica*.

7.4 Structure elucidation and identification of fraction DD1

7.4.1 Physical data

The substance from fraction DD1 is an amorphous white solid. V_{max} (spectrum **1**, KBr, cm^{-1}): 707.20, 800.40, 953.30, 1 094.50, 1 260.90, 1 316.70, 1 377.10, 1 407.30, 1 459.40, 1 655.00, 2 134.90, 2 853.40, 2 923.60, 2 959.00, 3 367.60. δ_H (spectrum **2**, 600.1724 MHz, $CDCl_3$): 7.240 ($CDCl_3$), 0.857, 1.231 and 1.117. δ_C (spectrum **3**, 150.9126 MHz, $CDCl_3$): 77.004 ($CDCl_3$), 14.115, 22.687, 31.921, 29.688 and 29.688. m/z (%) (spectrum **4**) 62.80(100), 149.20(69), 96.80(31), 279.40(27), 181.20(12), 355.30(10), 231.20(5), 381.30(5).

7.4.2 Characterization of fraction DD1

The IR spectrum (KBr) showed an absorption band at 3 367.60 cm^{-1} (spectrum **1**) which is indicative of the presence of OH-groups and showed no signals in the aromatic region. The ^{1}H NMR and ^{13}C spectrums also showed no signals in the aromatic region and the substance from fraction DD1 is therefore possibly an aliphatic compound (spectra **2** & **3**). EI-MS gave a molecular mass (m/z 381) (spectrum **4**). ESI-MS gave a signal at 408. However, the signal to noise ratio in this spectrum (spectrum **5**) was not of desirable quality and was not considered to be reliable. All spectral data are presented in Appendix A.

The structure elucidation of fraction DD1 could not be completed due to the small quantity isolated as well as impurities. An attempt was made to compare the spectral data of fraction DD1 to that found in literature and it could only be deduced that the substance is possibly an alilphatic compound that contains one or more OH-groups. The specific identity of the substance from fraction DD1 was not confirmed.

7.5 Results and discussion

In an attempt to clarify the structural elements conferring bioactivity in the analyzed systems, fraction DD1 was identified and the spectral data thereof compared to that found in literature.

The IR absorption bands at 1 377.10, 1 459.40, 2 853.40, 2 923.60 and 2 959.00 cm^{-1} (spectrum **1**) could be indicative of the presence of hydrocarbon chains, while the band at 3 367.60 cm^{-1} could indicate the presence of an OH-group. There were no signals in the aromatic region (Mc Murry, 1992).

The ^{13}C signals at δ_C 22.687, 29.352, 29.688 and 31.921 (spectrum **3**) can be an indication of the different CH_2-groups while the signal at δ_C 14.115 can be an indication of CH_3-groups present in the substance from fraction DD1. There also appeared no signals in the aromatic region (Mc Murry, 1992).

The ^{1}H signal at δ_H 0.857 can be an indication of CH_3-groups while the signal at δ_H 1.231 can be indicative of CH_2-groups present in the substance from fraction DD1. Again, there appeared no signals in the aromatic region (Mc Murry, 1992) (spectrum **2**).

Most of the studies conducted on *L. javanica,* have focussed on the volatile oil fraction (Viljoen *et al.,* 2005). This study focussed on the antioxidant activity of extracts made from the leaves of *L. javanica.* Fraction DD1 was subjected to antioxidant screening in the NBT and TBARS assays. Fraction DD1 exhibited no activity with the NBT assay. In effect, fraction DD1 exhibited a pro-oxidant effect with the NBT assay. Fraction DD1 exhibited promising activity with the TBARS assay. Fraction DD1 however exhibited less promising antioxidant activity than the crude petroleum ether extract of the leaves of *L. javanica* with both the NBT and TBARS assays. This may indicate that the main component responsible for the antioxidant activity conferred from the petroleum ether extract was not isolated during this study. The presence of OH-groups in the structure of the substance from fraction DD1 could be the explanation for the observed antioxidant activity in the TBARS assay. It would be interesting to elucidate the structure of the substance from fraction DD1 since antioxidant activity is usually conferred by compounds with complex aromatic structures that contain OH-groups. The substance from fraction DD1 does not contain an aromatic chain or ring. Therefore, the next step would be to isolate the substance from fraction DD1 in sufficient quantity to elucidate the structure. Also, the main component responsible for the promising antioxidant activity should be isolated and the structure thereof elucidated.

CHAPTER 8

Discussion, conclusion and objectives of future research

8.1 Conclusion

ROS are produced as an essential part of human metabolism and all the cells in the body are continually exposed to these ROS. Unrestricted production of ROS and RNS has the potential to damage vital biological systems and have thus been implicated in aging and neurodegenerative diseases (Ames *et al.,* 1993; Halliwell *et al.,* 1992). The brain is especially vulnerable to oxidative damage due to its high oxygen consumption and the presence of high levels of iron and polyunsaturated fatty acids (Zaidi & Banu, 2004). As a result of the detrimental effects of ROS, all cells are equipped with an intricate antioxidant system. Antioxidants have evolved to counteract the damaging effects of ROS (Yu, 1994) and can therefore be useful in reducing the oxidative damage inflicted by ROS. Antioxidants are thus essential in the prevention of these disease states (Wang *et al.,* 1996). Natural antioxidants from plants thus hold great promise in the search for novel therapies in the treatment or prevention of neurodegeneration (Halliwell, 1996; Blaylock, 1998).

The aim of this study was to investigate the antioxidant activity of South African plants using two different screening methods (ORAC and FRAP assays). Twenty one South African plants were analyzed and the plant with the most promising antioxidant profile was chosen. The leaf extracts of this plant, *L. javanica*, were analyzed for biological activity against cyanide-induced superoxide anion generation and a toxin solution (consisting of H_2O_2, $FeCl_3$ and ascorbic acid)-induced lipid peroxidation in rat brain homogenate. The final objective was to isolate and characterize a compound from an extract exhibiting promising antioxidant activity by employing a bioassay-guided approach.

Plants contain a wide variety of free radical scavenging molecules, such as phenolic compounds (phenolic acids, flavonoids, quinones, coumarins, lignans, stilbenes and tannins), nitrogen compounds (alkaloids, betalains and amines), vitamins and terpenoids, which demonstrate antioxidant activity (Zheng & Wang, 2001). It is possible to find similar substances and thus similar activity in species of the same genus or the same family (Hostettmann *et al.,* 2000). In this study 21 plants (Table 3.1) were selected following an extensive literature survey based on their use in traditional medicine as well as their availability and were screened for antioxidant activity. One hundred and ten different extracts were prepared from the leaves of the plants using PE, DCM, EtOAc and EtOH, consecutively. The yields of the extracts were determined by calculating the percentage of

extract per mass of leaf material. The yields (summarized in Table 4.1) varied between 0.47% and 40.58% based on the weight of the dry leaves.

Although care was taken to conduct all experiments in subdued light, exposure to white fluorescent light could not be fully avoided. In addition, the Soxhlet method of extraction was employed in this study and therefore losses of volatile constituents occured due to exposure to elevated temperatures. Ideally, experimental work is to be done in temperature-controlled and dark laboratories in view of the sensitive nature of phytochemicals.

The 21 South African plants were screened using two chemistry-based assays, each of which determines a very different aspect of antioxidant activity. The chosen assays were the ORAC and FRAP assays. The ORAC assay measures the ability of a sample to protect a fluorescent probe from damage by free radicals and the FRAP assay measures the ability of a sample to reduce Fe^{3+} to Fe^{2+}. In the screening project for the search for free radical scavenging agents from natural sources, one hundred and ten extracts (see table 4.1) belonging to twenty one species of ten different plant families (see table 3.1) were studied. Ultimately, arbitrary FRAP values above 3 000 and ORAC values above 30 000 were considered in selecting a plant for further testing. These values are highlighted in pink (table 8.1). Table 8.1 is a combination of the ORAC (table 5.1 and figure 5.6) and FRAP results (table 5.2 and figure 5.9) to allow for easy comparison.

Table 8.1 ORAC and FRAP values for the 21 plants

Plant	Extract	ORAC value (mean) µM TE (n = 3)	Standard error of the mean (SEM)	FRAP value (mean) µM AE (n = 3)	Standard error of the mean (SEM)
Berula erecta	PE	3 604.00	± 791.57	33.28	± 12.83
	DCM	6 804.33	± 497.74	76.51	± 14.85
	EtOAc	8 405.00	± 968.12	126.78	± 16.62
	EtOH	20 318.33	± 497.74	1 460.94	± 98.07
Heteromorpha arborescens	PE	3 340.67	± 1 445.50	30.93	± 34.47
	DCM	6 434.00	± 527.60	48.02	± 18.79
	EtOAc	13 247.00	± 562.93	719.71	± 51.68
	EtOH	10 413.33	± 1 341.90	618.72	± 84.28
Tarchonanthus camphoratus	PE	4 689.67	± 1 648.70	101.08	± 40.21
	DCM	25 822.67	± 1 473.70	1 292.90	± 45.95
	EtOAc	18 347.67	± 1 636.00	846.13	± 90.67
	EtOH	30 422.67	± 1 849.70	4 445.60	± 108.02

Plant	Extract	ORAC value (mean) µM TE (n = 3)	Standard error of the mean (SEM)	FRAP value (mean) µM AE (n = 3)	Standard error of the mean (SEM)
Vernonia oligocephala	PE	5 302.00	± 1 310.30	53.13	± 20.49
	DCM	19 838.67	± 1 631.50	372.35	± 30.43
	EtOAc	24 993.67	± 711.51	1 416.08	± 58.72
	EtOH	18 302.67	± 1 368.50	1 536.43	± 144.69
Gymnosporia buxifolia	PE	11 380.00	± 848.94	105.80	± 57.71
	DCM	21 091.67	± 559.68	432.50	± 49.38
	EtOAc	26 974.67	± 518.36	433.51	± 33.59
	EtOH	72 262.33	± 7 944.70	3 332.04	± 61.34
Acacia karroo	PE	4 465.33	± 282.97	52.65	± 52.65
	DCM	3 838.00	± 218.28	0.00	± 0.00
	EtOAc	4 754.00	± 395.88	15.19	± 8.28
	EtOH	22 406.00	± 980.34	4 641.33	± 73.73
Elephantorrhiza elephantina	PE	8 020.33	± 3 551.30	18.34	± 4.70
	DCM	9 923.33	± 837.96	15.51	± 7.39
	EtOAc	10 413.33	± 329.43	51.25	± 11.87
	EtOH	9 219.67	± 1 899.00	324.40	± 18.32
Erythrina zeyheri	PE	5 250.67	± 538.17	5.40	± 5.40
	DCM	26 486.67	± 425.44	490.13	± 13.61
	EtOAc	11 144.67	± 1 372.90	65.96	± 6.10
	EtOH	64 516.33	± 4 627.50	1 060.04	± 75.90
Leonotis leonurus	PE	3 153.00	± 1 327.00	320.94	± 15.33
	DCM	9 504.33	± 1 780.90	212.18	± 9.49
	EtOAc	13 743.00	± 1 287.00	712.98	± 42.70
	EtOH	12 559.67	± 1 193.40	893.72	± 60.47
Plectranthus ecklonii	PE	6 529.00	± 1 527.60	86.98	± 6.96
	DCM	7 080.00	± 414.10	162.20	± 10.25
	EtOAc	9 484.67	± 741.01	438.17	± 37.00
	EtOH	10 548.67	± 1 392.20	920.08	± 32.78
Plectranthus rehmanii	PE	14 783.00	± 754.14	2 957.87	± 74.08
	DCM	7 302.00	± 889.43	174.90	± 7.97
	EtOAc	8 293.33	± 481.73	274.33	± 17.65
	EtOH	13 987.67	± 1 448.20	3 239.53	± 182.03
Plectranthus venteri	PE	6 380.33	± 3 294.20	127.25	± 3.74
	DCM	13 539.33	± 2 497.80	221.51	± 2.69
	EtOAc	13 068.33	± 1 121.70	1 044.44	± 50.70
	EtOH	7 520.67	± 921.16	1 067.20	± 8.12
Salvia auretia	PE	-1 258.33	± 817.09	123.18	± 2.05
	DCM	8 834.67	± 707.65	1 535.97	± 40.73
	EtOAc	1 757.33	± 168.06	180.07	± 4.31
	EtOH	4 977.33	± 943.95	81.57	± 14.64

Plant	Extract	ORAC value (mean) µM TE (n = 3)	Standard error of the mean (SEM)	FRAP value (mean) µM AE (n = 3)	Standard error of the mean (SEM)
Salvia runcinata	PE	32 218.67	± 556.85	407.09	± 203.73
	DCM	14 915.00	± 7 274.00	0.00	± 0.00
	EtOAc	12 415.67	± 305.09	651.55	± 116.26
	EtOH	26 969.33	± 2 112.40	2 400.53	± 42.82
Solenostemon latifolius	PE	4 234.33	± 1 131.2	2.89	± 2.89
	DCM	9 853.67	± 669.17	678.28	± 58.73
	EtOAc	7 015.00	± 233.60	276.92	± 10.51
	EtOH	9 744.00	± 397.79	801.02	± 36.40
Solenostemon rotundifolius	PE	-1 217.67	± 289.65	130.88	± 19.90
	DCM	-444.67	± 41.83	1 092.07	± 85.48
	EtOAC	4 305.67	± 190.54	110.16	± 5.84
	EtOH	13 245.00	± 1 331.30	1 542.21	± 61.18
Plumbago auriculata	PE	2 198.67	± 1 207.20	28.04	± 20.39
	DCM	6 802.00	± 1 215.00	85.30	± 8.85
	EtOAc	5 406.67	± 870.81	42.84	± 5.60
	EtOH	31 505.67	± 4 673.60	1 983.95	± 45.71
Clematis brachiata	PE	6 426.00	± 1 447.20	128.35	± 26.21
	DCM	12 276.33	± 1 223.90	129.76	± 11.64
	EtOAc	27 462.33	± 598.63	1 976.46	± 47.94
	EtOH	19 058.33	± 708.60	1 334.27	± 24.50
Vangueria infausta	PE	5 203.00	± 1 545.30	59.71	± 8.36
	DCM	10 469.33	± 1 156.00	71.46	± 5.34
	EtOAc	11 581.33	± 403.90	291.02	± 22.42
	EtOH	12 443.00	± 1 450.60	581.10	± 21.70
Physalis peruviana	PE	2 524.67	± 541.51	116.72	± 10.39
	DCM	2 210.00	± 2 031.80	107.67	± 3.52
	EtOAc	13 271.00	± 92.09	744.54	± 45.32
	EtOH	15 468.67	± 1 046.40	1 062.83	± 4.03
Lippia javanica	PE	12 664.33	± 1 549.10	234.45	± 50.37
	DCM	49 147.67	± 8 355.10	698.13	± 39.25
	EtOAc	75 908.00	± 6 545.20	6 742.13	± 24.33
	EtOH	NA	± NA	9 502.13	± 85.47

NA = not available

From Table 8.1, it can be seen that most of the 110 extracts of the leaves of the selected South African plants exhibited activity with the ORAC and FRAP assays. The most promising activity with the ORAC assay was observed for the EtOAc extract of *Lippia javanica* with an ORAC value of 75 908.00 ± 6 545.20 µM Trolox equivalents/10 mg of extract. The EtOH extract of *L. javanica* had a value too high to detect. The most promising activity with the FRAP assay was observed for the EtOAc and EtOH extracts of *L. javanica*,

with FRAP values of 6 742.13 ± 24.33 µM ascorbic acid equivalents/10 mg of plant extract for the ethyl acetate extract and 9 502.13 ± 85.47 µM ascorbic acid equivalents/10 mg of plant extract for the ethanol extract, respectively. *L. javanica* was selected for further investigation based on the results mentioned above. The availability of plant material was also an aspect brought into consideration for choosing *L. javanica*.

A further, more detailed study with other models, the *in vitro* superoxide anion scavenging ability and the *in vitro* attenuation of lipid peroxidation, was conducted on the extracts of the identified plant and bioassay-guided isolation and identification of a compound followed.

The crude leaf extracts of *L. javanica* was subjected to biological screening using the NBT and TBARS assays. The results of the NBT and TBARS assays could be influenced by the absorbance of chlorophyll. The extracts were therefore dissolved in acetone and photobleached using a photochemical reactor prior to the biological screening. The NBT assay measures the ability of a sample to scavenge superoxide anions. The rational for employing the NBT assay is to determine the ability of a sample to scavenge the precursor ROS (superoxide anion) that is formed during the first step of reduction of O_2. A sample exhibiting superoxide anion scavenging activity therefore inhibits formation of further ROS (H_2O_2 and $^{\cdot}OH$). This study has demonstrated that cyanide-induced oxidative stress could be mitigated by the administration of extracts of the leaves of *L. javanica*. The PE and EtOAc extracts of the leaves of *L. javanica* exhibited significant *in vitro* antioxidant activity with the NBT assay (see table 8.2) with values of 8.359 ± 0.562 µM NBD/mg protein for the 2.5 mg/ml concentration of the PE extract and 7.755 ± 0.274 µM NBD/mg protein for the 5 mg/ml concentration of the EtOAc extract. Since there was no significant difference between the NBT values for the 2.5 mg/ml concentration of the PE extract and the 5 mg/ml concentration of the EtOAc extract, it was concluded that the PE extract exhibited similar activity at a lower concentration than the EtOAc extract (2.5 mg/ml compared to 5 mg/ml).

The thiobarbituric acid reactive substances (TBARS) assay was employed as a model to measure lipid peroxidation in whole rat brain homogenate. Studies in our lab showed cyanide to be ineffective to induce lipid peroxidation and was therefore substituted with a toxin-solution that consisted of hydrogen peroxide (H_2O_2), iron(III)chloride ($FeCl_3$) and ascorbic acid (vitamin C) (Linden *et al.,* 2008; Aruoma *et al.,* 1989). Ascorbic acid can interact with metal ions (from $FeCl_3$) *in vitro* to produce hydroxyl and lipid alkoxyl radicals (Carr & Frei, 1999). The combination of chemicals in the toxin-solution induced the Fenton reaction that leads to the production of the hydroxyl radical ($^{\cdot}OH$). $^{\cdot}OH$ initiates lipid peroxidation (figure 2.16). The rational for using the TBARS assay is to assess the ability of a sample to scavenge the hydroxyl radicals that is formed during the Fenton reaction and thereby acts as chain-breaking antioxidants and attenuates lipid peroxidation. The current

study indicates that the toxin solution significantly increased oxidative stress, thereby initiating lipid peroxidation in rat brain homogenate. In contrast, the extracts of *L. javanica* attenuated lipid peroxidation, suggesting a protective role of the extracts of *L. javanica* in toxin-induced lipid peroxidation in rat brain homogenate. All of the extracts of the leaves of *L. javanica* exhibited significant *in vitro* antioxidant activity with the TBARS assay (see table 8.2) with values that ranged from 0.217 ± 0.028 nM MDA/mg tissue for the 5 mg/ml concentration of the EtOAc extract and 0.681 ± 0.031 nM MDA/mg tissue for the 1.25 mg/ml concentration of the DCM extract of the leaves of *L. javanica*. All of the extracts of *L. javanica* exhibited promising antioxidant activity by lowering the MDA formed from lipid peroxidation to values lower than the control. All the extracts are therefore capable of attenuating lipid peroxidation induced by the toxin solution as well as lipid peroxidation that occurs during normal metabolism in the brain. The PE extract was selected for further investigation due to favourable antioxidant profile and much faster extraction time needed to aquire the extract. Table 8.2 is a combination of the NBT (table 6.2 and figure 6.8) and TBARS results (table 6.3 and figure 6.11) to allow for easy comparison.

Table 8.2 NBT and TBARS values of the four leaf extracts of *L. javanica.* Values below that of 1 mM Trolox are highlighted in pink.

Compound/ Extract	Concentration	NBD (µM/mg protein) (n = 5)	Standard error of the mean (SEM)	MDA (nM/mg tissue) (n = 5)	Standard error of the mean (SEM)
Control	-	20.474	± 1.248	0.912	± 0.054
KCN/Toxin	1 mM	33.246	± 1.602	2.188	± 0.192
Trolox	1 mM	18.074	± 0.420	0.213	± 0.010
L. javanica$_{PE}$	1.25 mg/ml	14.305	± 0.947	0.638	± 0.017
	2.5 mg/ml	8.359	± 0.562	0.520	± 0.042
	5 mg/ml	10.493	± 0.557	0.488	± 0.014
L. javanica$_{DCM}$	1.25 mg/ml	22.336	± 0.484	0.681	± 0.031
	2.5 mg/ml	15.980	± 0.338	0.387	± 0.044
	5 mg/ml	11.690	± 0.223	0.313	± 0.017
L. javanica$_{EtOAc}$	1.25 mg/ml	18.433	± 0.484	0.233	± 0.013
	2.5 mg/ml	13.199	± 0.447	0.235	± 0.009
	5 mg/ml	7.755	± 0.274	0.217	± 0.028
L. javanica$_{EtOH}$	1.25 mg/ml	28.267	± 0.569	0.293	± 0.010
	2.5 mg/ml	26.033	± 0.632	0.342	± 0.008
	5 mg/ml	19.930	± 0.374	0.430	± 0.010

From qualitative thin-layer chromatography, it was observed from the profiles of the extracts that the extraction method and solvents chosen for this study resulted in complex crude extracts that contained various plant constituents with a wide range of polarities (see

figure 7.1). Bioassay-guided isolation was used for the purification and isolation of a substance from fraction DD1. The plant extracts were sequentially fractionated and fractions of ample amount were subjected antioxidant screening through the NBT and TBARS assays. Bioassay-guided fractionation indicated that most the fractions exhibited antioxidant activity; some to a greater extent than others (see figures 7.9 & 7.10). Several analytical methods were used in the fractionation of the PE extract. Thin layer chromatography was employed in the selection of the mobile phases to be used with column chromatography. Subsequent fractionation led to the isolation of a substance from fraction DD1. Fraction DD1 was subjected to spectral analysis using ^{1}H and ^{13}C NMR, MS and IR spectrometry (spectra **1, 2, 3, 4** & **5**). Structural elucidation of fraction DD1 was not completed since the NMR experiments revealed that fraction DD1 contained impurities which made it difficult to determine the exact structure. The isolated amount of fraction DD1 was also very small. Structural elucidation of fraction DD1 was also complicated by the fact that the relaxation times of the NMR were not set at optimum levels. From the spectral data of fraction DD1 (spectra **1, 2, 3, 4** & **5**), it was deducted that the substance from fraction DD1 is probably an aliphatic chain with OH substitution.

The results obtained further support the view that plants represent a rich source of bioactive compounds. Medicinal plants are a potential source of new active agents that has the advantage of being relatively non-toxic and therefore more tolerable than designed drugs. Medicinal plants also represent an affordable source of pharmacologically active substances available through cultivation. As our study was conducted on the leaves only, and other parts of the plants were not considered, the results may underestimate the activity of the plant species involved. The petroleum ether extract of the leaves of *L. javanica* exhibited promising antioxidant activity with both the NBT and TBARS assays (sections 7.2.2.1 and 7.2.2.2) while fraction DD1 exhibited less promising acitivity than the crude extract in both assays. Therefore, the substance from fraction DD1 is probably not the main constituent responsible for the observed antioxidant activity from the crude petroleum ether extract of the leaves of *L. javanica*. The main component responsible for the observed antioxidant activity should be isolated and the structure thereof elucidated in future studies.

In conclusion, all of the aims set at the beginning of this research project, were achieved. A substance was isolated from an extract exhibiting promising antioxidant activity (PE extract) of the plant (*L. javanica*) that was chosen from the initial screening of 21 South African plants. The only aim that could not be achieved was the structure elucidation of the substance from fraction DD1.

8.2 Objectives of future research

The objectives of future research should include further work in order to validate the antioxidant effects in additional model antioxidant systems for different radical scavenging mechanisms against different target substrates as well as the antioxidant effect in *in vivo* conditions to ensure their effectiveness. The absence of adverse effects should be determined by toxicity tests. Other objectives should include further fractionation of the PE extract to detect all the compounds that contribute to the antioxidant activity observed with the *in vitro* antioxidant assays and identifying the most active compound. The structures of the isolated compounds should also be elucidated.

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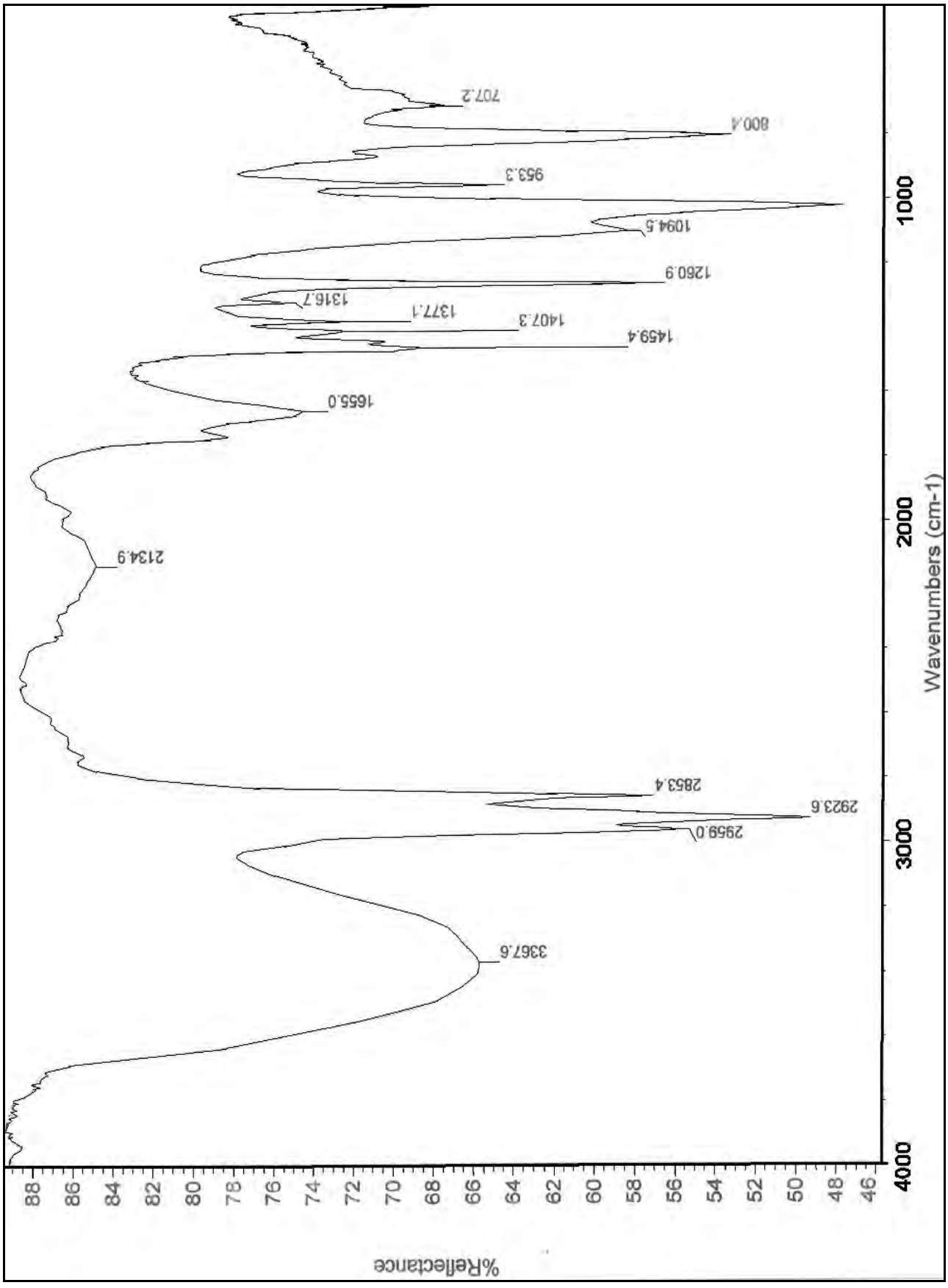
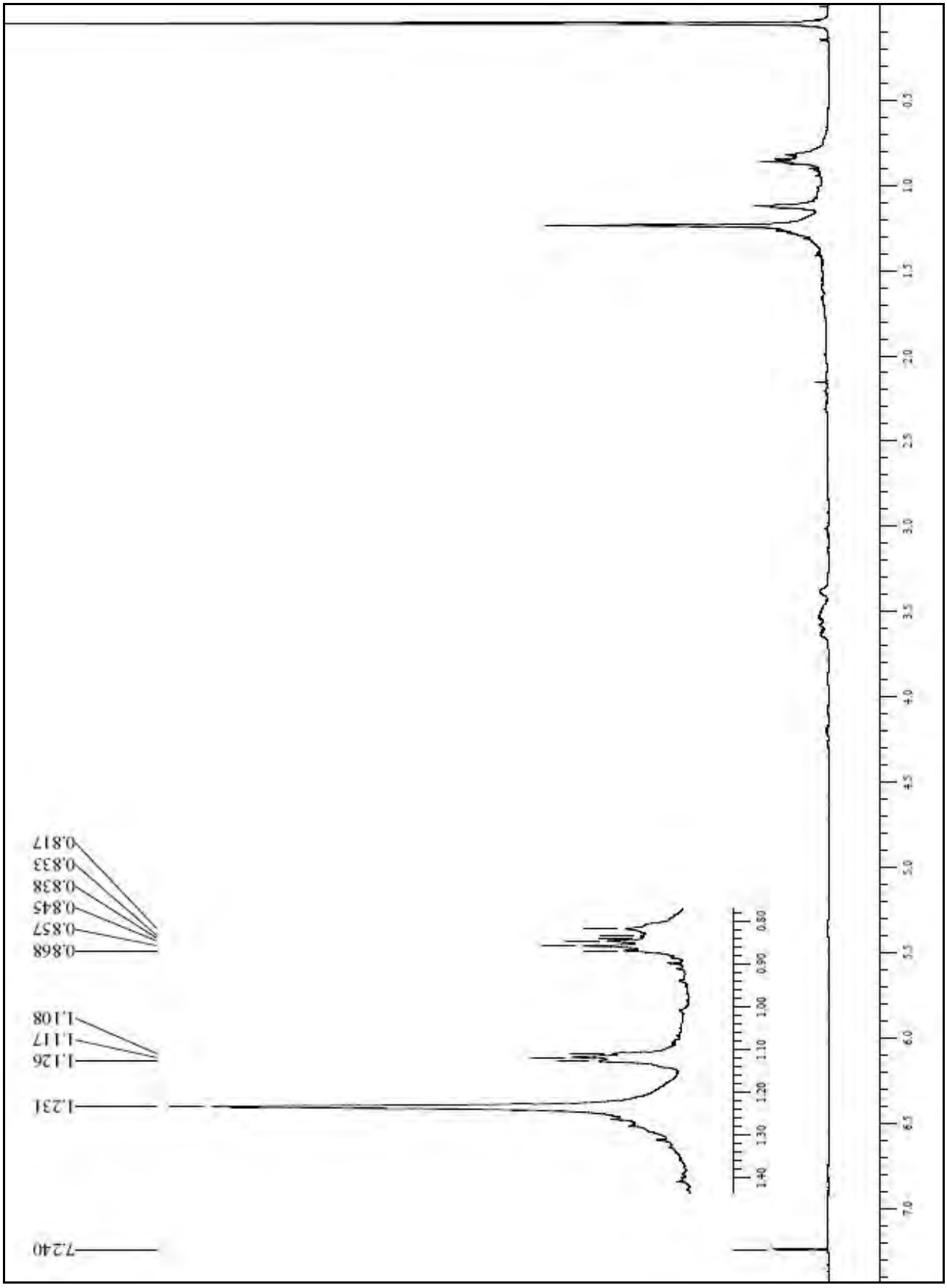
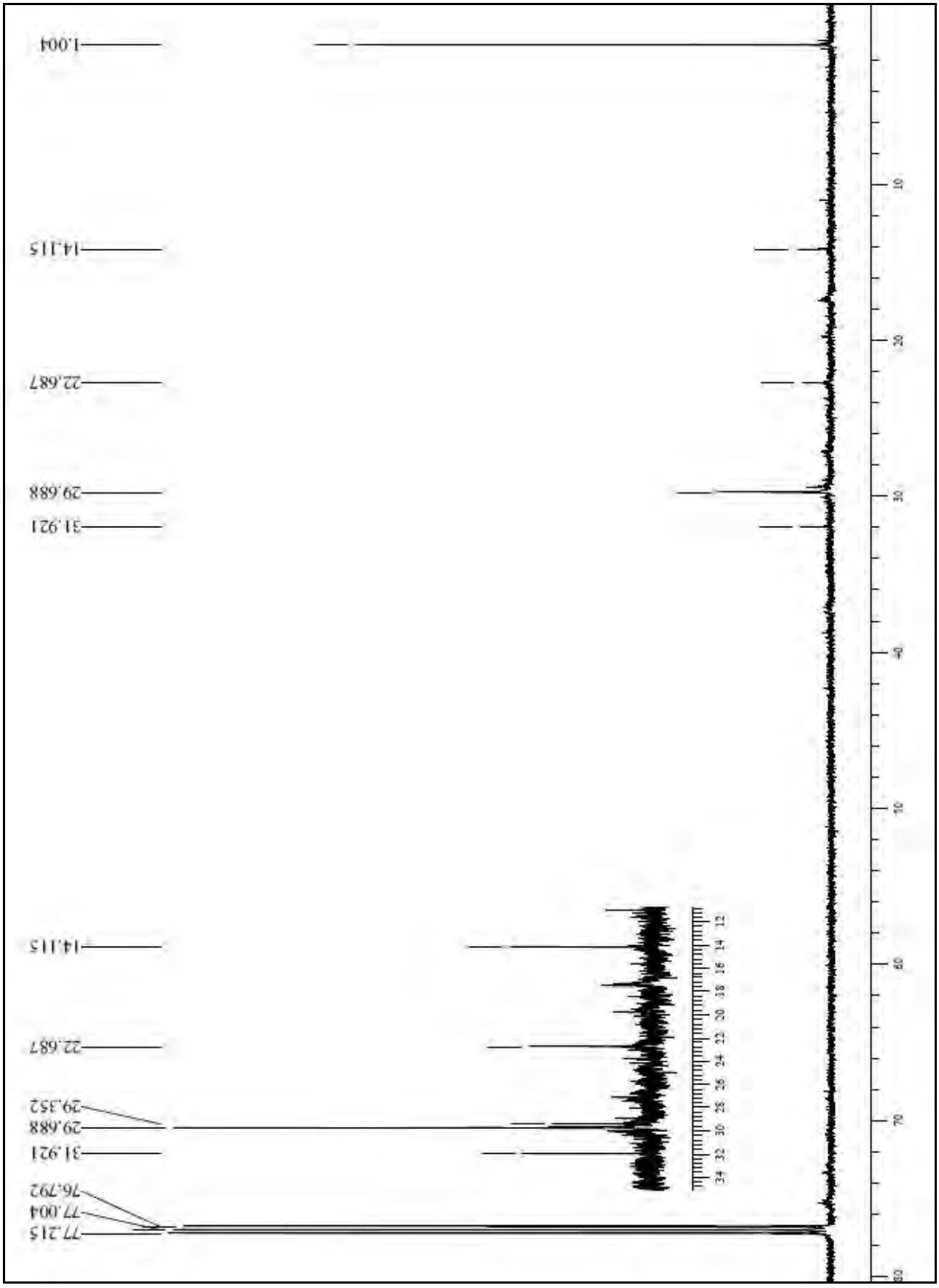
Spectrum 1: IR spectrum of compound DD1



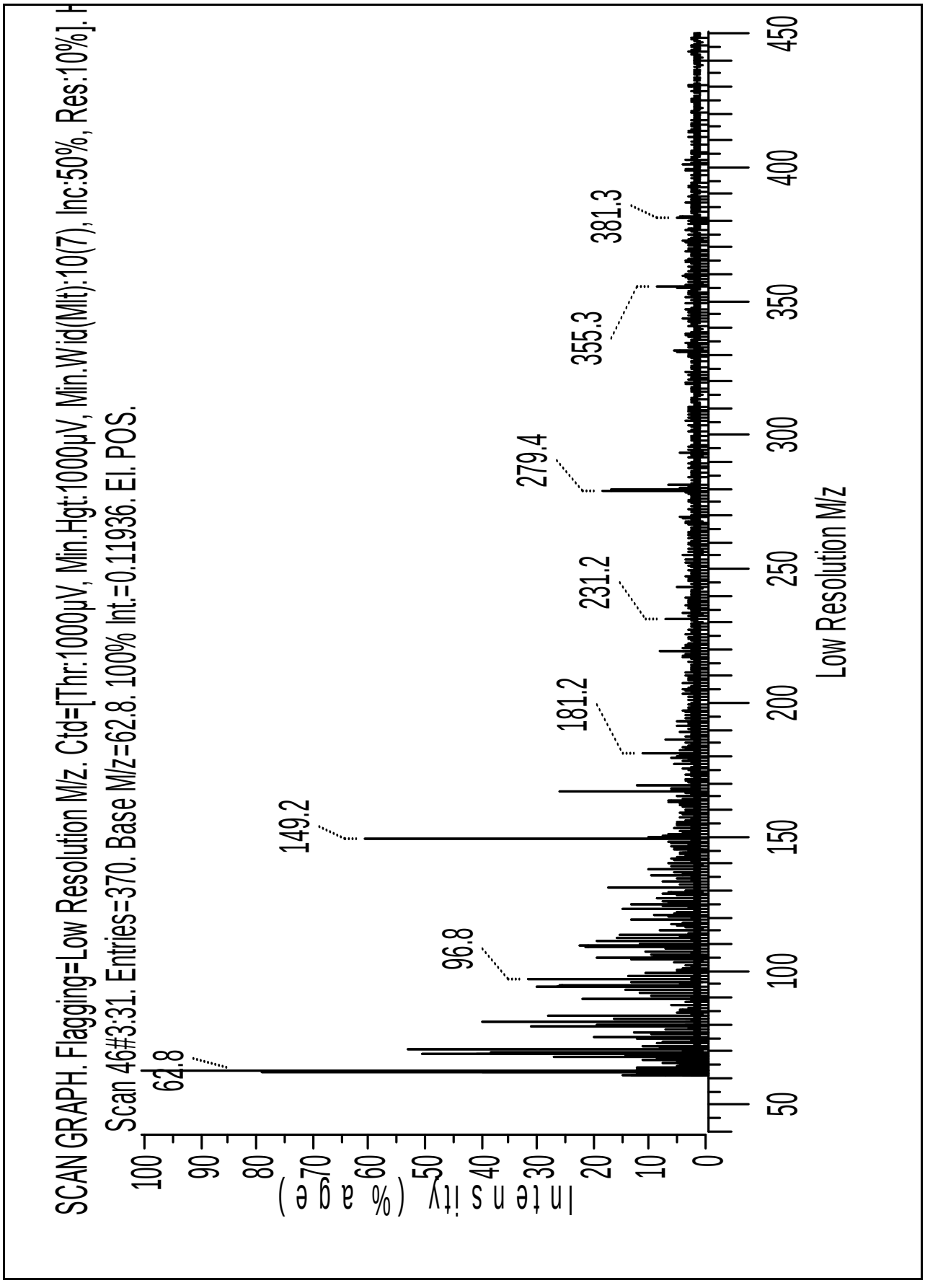
Spectrum 2: ^{1}H-NMR spectrum of compound DD1



Spectrum 3: ^{13}C NMR spectrum of compound DD1



Spectrum 4: EI-MS spectrum of compound DD1



Spectrum 4: ESI-MS spectrum of compound DD1