

**Antioxidant properties of
Gymnosporia buxifolia Szyszyl**

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“We can’t all be Einstein. At the very least, we need a sort of street-smart science: the ability to recognise evidence, gather it, assess it, and act on it.” - Judith Stone

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ABSTRACT

Chemistry of natural products is a research field with endless potential and with the global increase in natural product research, many plants have shown immense potential in therapeutic uses. Questions about the long term safety of synthetic antioxidants have increased the demand for natural antioxidants. Natural antioxidants have better long-term safety and stability and have the capacity to improve food quality and can act as nutraceuticals to terminate free radical chain reactions in biological systems. The primary factor in various degenerative diseases, like Parkinson's disease and Alzheimer's disease, is oxidative stress induced by oxygen radicals. These reactive oxygen species are generated by normal metabolic processes and are capable of damaging a wide range of essential biomolecules. The oxidation of cellular oxidizable substrate can be prevented and delayed by antioxidants. Antioxidants scavenge reactive oxygen species by preventing the generation of reactive oxygen species by activating a battery of detoxifying proteins.

A literature survey was done and 21 plants were selected for screening for antioxidant activity. These plants were selected based on previous studies done on plants in the same families. Plant leaves were collected and dried. The leaves were then extracted by soxhlet extraction using solvents in order of increased polarity (petroleum ether, dichloromethane, ethyl acetate and ethanol). The crude plant extracts were used for screening by assessing the total antioxidant capacity by measurement of the oxygen radical absorbance capacity (ORAC) and the ferric reducing antioxidant power (FRAP). The Frap results in terms of vitamin C equivalents ranged from as low as $0.000 \pm 0.000 \mu\text{M}$ for the *Acacia karroo* petroleum ether and dichloromethane phase to as high as $9009.32 \pm 130.714 \mu\text{M}$ for the *Lippia javanica* ethanol phase. The ORAC results in terms of Trolox equivalent ranges from as low as $-1491.8 \pm 227 \mu\text{M}$ for *Solenostemon rotundifolius* petroleum ether phase to as high as $75908.1 \pm 1336 \mu\text{M}$ for *Lippia javanica* ethyl acetate phase. The higher the results the better it is. *Gymnosporia buxifolia* was selected due to high ORAC and FRAP values and the availability of large quantities of plant material.

The four crude extracts, from the soxhlet extraction of *Gymnosporia buxifolia*, were tested using the nitroblue tetrazolium assay and the thiobarbaturic assay (lipid peroxidation). Nitroblue tetrazolium (NBT) is reduced to nitroblue diformazan (NBD) in the presence of the superoxide anion radical. The capacity of the crude plant extract to scavenge the superoxide radical anion determines the antioxidant capacity of the extract. The thiobarbaturic assay is one of the most widely used methods for lipid peroxidation in biological samples. The principle of this assay is based on the reduction of malondialdehyde equivalents with thiobarbaturic acid to form a pink colour complex. The ethanol crude plant extract of

Gymnosporia buxifolia showed the best transformation of nitroblue tetrazolium to nitroblue diformazan indicating a reduction in superoxide radical anions. It reduced the KCN from 88.791 ± 6.34 diformazan ($\mu\text{M}/\text{mg}$ protein) to 24.273 ± 5.29 diformazan ($\mu\text{M}/\text{mg}$ protein) which is very good. It also illustrated the best reduction in lipid peroxidation. It reduced the Toxin from 0.009931 ± 0.000999 malondialdehyde (nmol/mg tissue) to 0.000596 ± 0.000221 malondialdehyde (nmol/mg tissue) which is very good. The ethanol extract was chosen for isolation of active compound(s).

Two compounds were isolated using column chromatography, thin layer chromatography, solid phase extraction and selective precipitation. D-mannitol or dulcitol (galactitol) or a combination of the two and a compound with a dihyro- β -agarofuran sesquiterpenoid core skeleton is proposed by comparing spectra generated with nuclear magnetic resonance, mass spectrometry and infrared spectrometry.

The antioxidant activity of the two compounds was assessed using lipid peroxidation with both showing activity.

OPSOMMING

Vrae oor die langtermyn veiligheid van sintetiese antioksidante het tot gevolg dat daar 'n verhoging is in die aanvraag na natuurlike antioksidante. Dit het daartoe gelei dat die navorsingsgebied, oor die chemie van natuurlike produkte, oneindig baie potensiaal toon. Natuurlike produkte het beter stabiliteit, veiligheid en 'n verhoogde kapasiteit vir voedselkwaliteit. Dit het ook die vermoë om as 'n antioksidant op te tree en die vryradikaal kettingreaksie te staak. Die primêre faktor in 'n verskeidenheid degeneratiewe siektes, soos Parkinson- en Alzheimer se siekte, is oksidatiewe stres wat veroorsaak word deur suurstofradikale. Hierdie reaktiewe suurstofspesie word gegenereer deur normale metaboliese prosesse en is in staat daartoe om 'n wye reeks essensiële biologiese molekules te beskadig. Antioksidante kan die oksidasie van oksideerbare substrate verhoed en uitstel. Antioksidante ruim reaktiewe suurstofspesies op deur te verhoed dat die reaktiewe suurstofspesie gegenereer word. Dit word gedoen deur die aktivering van detoksifiserende proteïene.

'n Literatuurstudie is gedoen waarna 21 plante geselekteer is vir die siftingsproses vir antioksidante. Die blare van die plante is versamel en gedroog. Die blare is geëkstraheer deur soxhletekstaksie met die hulp van oplosmiddels in volgorde van verhoogde polariteit (petroleumeter, dichloormetaan, etielasetaat en etanol). Die rou ekstrak is gebruik vir die siftingsproses van die plante. Dit is gedoen deur die bepaling van die totale antioksidantkapasiteit deur twee toetse, ORAC en FRAP. Die FRAP resultate in terme van vitamien C strek van so laag as $0.000 \pm 0.000 \mu\text{M}$ vir *Acacia karroo* se petroleumeter en dichlorometaan fase tot so hoog as $9009.32 \pm 130.714 \mu\text{M}$ vir *Lippia javanica* se etanol fase. Die ORAC resultate in terme van Trolox strek van so laag as $1491.8 \pm 227 \mu\text{M}$ vir *Solenostemon rotundifolius* se petroleumeter fase tot so hoog as $75908.1 \pm 1336 \mu\text{M}$ vir *Lippia javanica* se etielasetaat fase. Hoe hoër die resultate hoe beter is dit. Die mees belowende plant is gekies vir verdere studie. *Gymnosporia buxifolia* is gekies as gevolg van sy goeie resultate met beide die toetse en omdat dit beskikbaar was in groot hoeveelhede.

Lipiedperoksidase en NBT is gebruik om die biologiese *in vitro* toetse op die vier rou ekstrakte, vanaf die soxhlet, van *Gymnosporia buxifolia* te doen. NBT word omgeskakel na NBD in nabyheid van die superoksiedanioonradikaal. Die vermoë van die rou plant ekstrak om die superoksiedanioonradikaal op te ruim bepaal sy antioxidantkapasiteit. Die tiobarbatuursuurmetode word die meeste gebruik om vir lipiedperoksidase te toets in biologiese monsters. Die prinsiep van die metode is gebaseer daarop dat dat tiobarbatuursuur die maloondialdehydekswaivalent verlaag en die hele kompleks pienk kleur. Die rou etanolekstrak van *Gymnosporia buxifolia* het die beste resultate in beide metodes

getoon en is daarom gekies vir isolasie van die aktiewe komponente. Met die NBT metode het die etanolekstrak die KCN verlaag vanaf 88.791 ± 6.34 diformazaan ($\mu\text{M}/\text{mg}$ proteïen) na 24.273 ± 5.29 diformazaan ($\mu\text{M}/\text{mg}$ proteïen) wat baie goed is. Met die lipiedperoksidase metode het die etanolekstrak die toksien verlaag vanaf 0.009931 ± 0.000999 maloondialdehyd (nmol/mg tissue) na 0.000596 ± 0.000221 maloondialdehyd (nmol/mg tissue) wat baie goed is.

Verbindings is geïsoleer deur gebruik te maak van kolomchromatografie, dunlaagchromatografie plaatjies, selektiewe persipitasie en vastefase ekstaksie. Die voorgestelde strukture is d-mannitol of dulsitol (galaktitol), of 'n kombinasie van die twee en 'n struktuur met 'n dihidro- β -agarofuraan seskwiterpeenstruktuur. Die strukture is geïdentifiseer met behulp van kernmagnetiese resonansie, massaspektrometrie en infrarooispektroskopie.

Antioksidantaktiwiteit van die komponente is getoets met behulp van lipiedperoksidase. Al twee komponente het aktiwiteit gewys. Die rou etanolekstrak het steeds beter getoets wat tot die afleiding lei dat daar nog steeds 'n baie goeie antioksidant in die ekstrak is wat nie geïsoleer is nie.

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LIST OF ABBREVIATIONS

°C	-	degrees Celsius
%	-	percentage
µg	-	microgram
µl	-	microlitre
µM	-	micromolar
AAPH	-	2,2'-azinobis(2-amidinopropane)dihydrochloride
AD	-	Alzheimer's disease
ADP	-	adenosine diphosphate
ATP	-	adenosine triphosphate
B.C.	-	before Christ
BHT	-	buthylated hydroxytoluene
B-PE	-	B-phycoerythrin
BSA	-	bovine serum albumin
CAT	-	catalase
•CCl ₃	-	trichloromethyl radical
CCl ₄	-	carbon tetrachloride
CHCl ₃	-	chloroform
Cl ⁻	-	chloride anion
cm ⁻¹	-	per centimetre
Cu ⁺	-	copper (I)
Cu ₂ ⁺	-	copper (II)
CuSO ₄	-	copper (II) sulphate
CuZnSOD	-	copper zinc superoxide dismutase
DCM	-	dichloromethane
DMSO	-	dimethyl sulfoxide
EC-SOD	-	extracellular superoxide dismutase
EtOAc	-	ethyl acetate
EtOH	-	ethanol
f ₀	-	initial fluorescence
Fe ²⁺	-	ferrous (iron II)
Fe ³⁺	-	ferric (iron III)
FeCl ₃	-	ferric chloride

FeCl ₃ .6H ₂ O	-	Iron (III) chloride hexahydrate
fi	-	fluorescence at time i
FRAP	-	ferric reducing antioxidant power
g	-	gram(s)
g	-	relative centrifuge force
GPx	-	glutathione peroxidase
GR	-	glutathione reductase
GSH	-	glutathione
GSSG	-	oxidised glutathione
H ⁺	-	hydrogen
HCl	-	hydrochloric acid
H ₂ O ₂	-	hydrogen peroxide
HO ₂ [•] /HOO [•]	-	protonated hydroxyl radical
HOCl	-	hypochlorous acid
H ₂ SO ₄	-	sulphuric acid
IR	-	infrared
KBr	-	potassium bromide
KCl	-	potassium chloride
KCN	-	potassium cyanide
kDa	-	kilo Daltons
KH ₂ PO ₄	-	potassium dihydrogen orthophosphate
K ₂ HPO ₄	-	dipotassium phosphate
l	-	litre
L	-	lipid radical
L-Arg	-	L-Arginine
L-Cit	-	L-Citrulline
LDL	-	low density lipoprotein
LH	-	lipid substrate
LO	-	lipid alkoxyl
LOO	-	lipid peroxy radical
LOOH	-	polyunsaturated fatty acid
LOOH	-	lipid hydroperoxide
M	-	molar concentration (mole.l ⁻¹)
m	-	meter

MDA	-	malondialdehyde
MeOH	-	methanol
mg	-	milligram
ml	-	millilitre
MHz	-	megahertz
mm	-	millimetre
mM	-	millimolar
MnSOD	-	manganese superoxide dismutase
MS	-	mass spectroscopy
Na	-	sodium
NaAc.3H ₂ O	-	sodium acetate trihydrate
NaCl	-	sodium hydroxide
NAD• or NAD ⁺	-	nicotinamide adenine dinucleotide
NADH	-	nicotinamide adenine dinucleotide (reduced)
NADPH	-	nicotinamide adenine dinucleotide phosphate (reduced)
Na ₂ HPO ₄	-	disodium hydrogen orthophosphate anhydrous
NaH ₂ PO ₄	-	sodium dihydrogenphosphate
NaOH	-	sodium hydroxide
NBD	-	nitroblue diformazan
NBT	-	nitroblue tetrazolium
nm	-	nanometre
NMR	-	nuclear magnetic resonance spectroscopy
NO•	-	nitric oxide
•NO ₂	-	nitrogen dioxide anion
NOS _s	-	specific nitric oxide synthases
NRP	-	nonradical product
O ₂	-	oxygen molecule
O ₂ ^{-•}	-	superoxide anions
O ₃	-	ozone
•OH	-	hydroxyl radical
OH ⁻	-	hydroxide
ONOO•	-	peroxynitrate
ONOO ⁻	-	peroxynitrate anion
ORAC	-	oxygen radical absorbance capacity

PA	-	Parkinson's disease
PBS	-	phosphate buffered saline
PE	-	petroleum ether
pH	-	power of hydrogen
pKa	-	acid dissociation constant
ppm	-	parts per millions
RNS	-	reactive nitrogen species
ROO•	-	peroxyl radical
ROS	-	reactive oxygen species
RS•	-	thiyl radicals
RSH	-	thiol compounds
s	-	seconds
SNpc	-	substantia nigra pars compacta
SOD	-	superoxide dismutase
SPE	-	solid phase extraction
Stdev	-	standard deviation
TBA	-	thiobarbituric acid
TCA	-	trichloroacetic acid
TEP	-	1,1,3,3-tetramethoxypropane
TLC	-	thin layer chromatography
TMS	-	tetra methylsilane
TPTZ	-	2,4,6-tripyridyl-s-triazine
v/v/v	-	volume/volume/volume
W	-	Watt
w/v	-	weight / volume
WWF	-	world wildlife foundation
UV	-	ultra violet
ZnSe	-	zink selenium

CHAPTER 1: INTRODUCTION

1.1. Introduction

The industrialised world's population is growing increasingly older, with increases in both life expectancy and in age-related neurodegenerative disorders such as Alzheimer's disease (AD) and Parkinson's disease (PD). Approximately 15% over the age of 65 years are afflicted with AD and 1% by PD. Aging and age-associated neurodegenerative diseases are associated with various degrees of behavioural impairments that significantly decrease the quality of life and severely tax the health care system (Cantuti-Castelvetri *et al.*, 2000).

The prime candidates responsible for producing the neuronal changes mediating these behavioural deficits appear to be free radicals and the oxidative stress they generate (Cantuti-Castelvetri *et al.*, 2000). When an organism's natural defences are overwhelmed by an excessive generation of reactive oxygen species (ROS), a situation of "oxidative stress" occurs, in which cellular and extra cellular macromolecules (proteins, lipids and nucleic acids) can suffer oxidative damage, causing tissue damage (Bektasoglu *et al.*, 2006). In humans, oxidative DNA damage is considered an important promoter of neurological diseases and aging (Trushina and McMurray, 2007). Age related changes occur as a result of an inability to cope with oxidative stress that occurs throughout the life span. The brain is very vulnerable to oxidative stress; it exhibits reduced free radical scavenging ability and utilises high amounts of oxygen. Normal and pathological aging, AD and PD have been associated with increased sensitivity to reactive oxygen species, probably the result of pro-oxidant mediators (iron) and a decrease in antioxidants. Precisely how oxidative stress causes its deleterious effects is not known, but some of this damage may include lipid and protein peroxidation and increases in DNA oxidation products. This all may eventually lead to cell death (Cantuti-Castelvetri *et al.*, 2000).

There have been a great number of studies which have examined the putative and positive benefits of antioxidants in altering, reversing or forestalling these neuronal/behavioural decrements with varying degrees of success. Additional experiments have examined the effects of diets rich in fruits and vegetables in reducing certain types of cancer and cardiovascular diseases. These kinds of diets are particularly rich in antioxidants such as vitamins A, C, E and bioflavonoids (such as flavones, tannins, anthocyanins and quercetin), and thus there may be synergistic effects among them. Therefore it might be important to examine the impact of antioxidants contained in different food and plants on various neuronal and behavioural parameters known to change with age (Cantuti-Castelvetri *et al.*, 2000).

It is estimated that 70 – 80% of people worldwide rely mainly on traditional, largely herbal medicine to meet their primary healthcare needs. The global demand for herbal medicine is not only large, but growing. Factors contributing to the growth in demand for traditional medicine include the increasing human population and the frequently inadequate provision of Western (allopathic) medicine in developing countries (Hamilton, 2003).

Plants have contributed hugely to Western medicine, through providing ingredients for drugs or having played central roles in drug discovery. Some drugs, having botanical origins, are still extracted directly from plants and others are made through transformation of chemicals found within them, while yet others are today synthesised from inorganic material, but have their historical origins in research in the active compounds found in plants. There are undoubtedly many more secrets still hidden in the world of plants. The estimated number of flora species used medicinally includes about 35,000-70,000 or 53,000 worldwide out of the estimated 297,000-510,000 total native species of flora (Hamilton, 2003).

1.2. Aim and objectives of this study

The aim of this study was to screen and identify specific plants with possible free radical scavenging effects and then to isolate and characterise the active compounds responsible for this activity.

As screening methods, the ferric reducing antioxidant power (FRAP) and the oxygen radical absorbance capacity (ORAC) assays to determine the oxidising/reducing ability of the selected extracts were used. Extracts that are able to reduce free radical generation, will reduce oxidative stress and also oxidative damage. After initial antioxidant screening of 21 plants, *Gymnosporia buxifolia* was selected for further investigation.

The study then focused on the biological evaluation. The neuroprotective properties of the extracts were examined by measuring the ability of the extract to reduce superoxide anion levels and malondialdehyde levels. Superoxide anion levels and malondialdehyde concentration were assessed using the nitroblue tetrazolium and lipid peroxidation assays.

The most promising extract was then selected for isolation and characterisation of the compound(s) with possible antioxidant activity.

To reach the aim of this study the following objectives were proposed:

- Thorough discriminative literature screening to select South African plants species with described antioxidant activity available in the Potchefstroom area.

- Screening methods to determine *in vitro* antioxidant activity using the FRAP and ORAC assays.
- Selection of the most promising plant and determination of the ability of extracts to reduce superoxide anions *in vitro* using the nitroblue tetrazolium assay.
- Determination of the ability of extracts to reduce malondialdehyde concentration *in vitro* using the lipid peroxidation assay.
- Selection of the most promising extract and isolation of compound(s) by chromatographic techniques.
- Characterisation of the compound(s) responsible for antioxidant activity from the active extract of *Gymnosporia buxifolia* by spectrometric methods.

CHAPTER 2: LITERATURE REVIEW

2.1. Plants and medicine

It is estimated by The World Health Organisation that up to 80% of the world's population relies mainly on herbal medicines either in part or entirely for primary health care (Blyth, 1999; WWF, 2006). Many people cannot afford the high cost of pharmaceutical drugs and others are just seeking natural alternatives with fewer side effects (Blyth, 1999). 40% of urban and 90% of rural patients in China are largely treated with traditional medicine from around 5,000 plants. In India traditional health care is widely practised, there are 400,000 registered traditional medical practitioners compared to the 332,000 registered doctors (WWF, 2006).

Uses of plants as medicine can be traced back as far as 3000 B.C. where Babylonians imported myrrh for medicinal uses and trade between Babylon and Egypt was documented on a tablet by 2250 B.C. Medicinal plants were also mentioned in the earliest Chinese monographs (2700 B.C.) and in India (1500 B.C. in *Rig Verda*). Hippocrates and Theophrastus (Greeks), Galen and Dioscorides (Roman) and Avicenna (Arabic) are just five of the famous ancient physicians who used plant medicines. All five of them also have plant genera named in their honour. It was only late in the 19th century that botany became an academic discipline in its own right at universities and botanical gardens. Botanists like Linnaeus were also physicians (Gibson, 1999).

Uses of plants by traditional healers date back at least 10,000 years for hallucinogens and are even more ancient among hunter-gatherer societies (Gibson, 1999). South Africa has an estimated 200,000 indigenous traditional healers and up to 60% of South Africans consult these healers, usually in addition to using modern biomedical services (Van Wyk *et al.*, 2000). This information on traditional medicine systems have not yet been systematised and are passed on by word of mouth from one generation to the next (Van Wyk *et al.*, 2000; Collins, 2001). This knowledge of plant uses accumulated over thousands of years through trial and error and is the key to indigenous plant uses, but it is disappearing at an increasing rate as skilled herbalist and practitioners die (Collins, 2001).

Southern Africa has well over 30,000 species of higher plants. The Cape Floral Kingdom alone has nearly 9,000 species and is the most diverse temperate flora on earth rivalling the tropical rainforests in terms of species richness. In South Africa approximately 3,000 species of plants are used as medicines and 350 species of plants are most commonly used and traded as medicinal plants (Van Wyk *et al.*, 2000). A few South African plants that contribute

to the world medicine include Cape aloe (*Aloe ferox*), buchu (*Agathosma betulina*) and devil's claw (*Harpagophytum procumbens*). There is a growing interest in natural and traditional medicines as a source of new commercial products (Van Wyk *et al.*, 2000). Scientific testing of herbal medicines is increasing. Understanding and reporting their efficacy and possible side effects from trials is important (WWF, 2006). A single plant chemical can at one concentration be curative, at another be potentially addictive and at a higher concentration be harmful or be a lethal poison (Gibson, 1999). The continued testing of herbal medicine is an essential and growing part of the international pharmacopeias and it will make them an increasingly safe alternative or a preferred option to western medicine (WWF, 2006).

More than 7,000 compounds produced by pharmaceutical industries are contributed by plants in industrialised countries. This includes ingredients in heart drugs, laxatives, anticancer agents, hormones, contraceptives, diuretics, antibiotics, decongestants, analgesics, anaesthetics, ulcer treatments and anti-parasitic compounds. Of all prescription drugs dispensed by western pharmacists one in four contains ingredients derived from plants. These include: Reserpine from *Rauvolfia serpentina*; Levodopa from *Mucuna deeringiana*; Ephedrine from *Ephedra sinica*; Picrotoxin from *Anamirta cocculus* just to mention a few (WWF, 2006).

There are also examples of widely used drugs that was first extracted from plants and then later inspired research into the active principals in plants and that are now being synthesised. Aspirin is the best known example of this; it is chemically related to the compound that was first extracted from the bark of the willow tree, *Salix alba*, and a herb meadowsweet (WWF, 2006). These are just a few examples of many and new contributions are made daily. The use of plant medicine is not in the past it is the future (Van Wyk *et al.*, 2000).

Free radical production and lipid peroxidation are actively involved in the pathogenesis of a wide number of diseases including atherosclerosis, carcinogenesis, neurodegenerative disorders and in the aging process. Plant derived antioxidants such as vitamin E, vitamin C, polyphenols including phenolic acids, phenolic diterpenes, flavonoids, catechins, procyanidins and anthocyanins are increasingly suggested as important dietary factors (Luximon-Ramma *et al.*, 2002). They can act as free radical scavengers, neutralising dangerous reactive oxygen species and metal ion chelators (Hashim *et al.*, 2005). The growing interest in the substitution of synthetic food antioxidants by natural ones has fostered research on plant sources and the screening of raw materials for identifying new antioxidants (Luximon-Ramma *et al.*, 2002).

2.2. Free radicals and reactive oxygen species

The Gersham's free radical theory of oxygen toxicity (1954) was one of the first publications which stated that the toxicity of oxygen is due to partially reduced forms of oxygen. In the same year Commoner, Townsend and Pake (1954) observed a weak electron paramagnetic resonance signal and attributed it to the presence of free radicals in a variety of lyophilised biological materials. Soon thereafter in 1956 the world of free radicals in biological systems was explored by Denham Harman who proposed the concept of free radicals playing a role in the aging process. In 1969 McCord and Fridovich discovered the enzyme superoxide dismutase (SOD) and provided evidence about the importance of free radicals in systems. In 1977 Mittal and Murad provided evidence that the hydroxyl radical, $\cdot\text{OH}$, stimulates activation of guanylate cyclase and the formation of the "second messenger" cyclic guanosine monophosphate. Evidence has shown since then that there is not only a coexistence with free radicals in the living systems, but in various physiological functions there are mechanisms for advantageous use of free radicals (Valko *et al.*, 2007).

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are produced by normal cellular metabolisms. They are also well recognised for playing a dual role as both deleterious and beneficial species, since they can be harmful or beneficial to living systems (Valko *et al.*, 2007).

Beneficial effects: ROS/RNS occurs in low/moderate concentrations and is involved in physiological roles in cellular responses to noxia, as for example in defence against infectious agents and in the function of a number of cellular signalling systems. It is also beneficial in the induction of a mitogenic response (Valko *et al.*, 2007).

Harmful effect: Free radicals causing biological damage is termed oxidative stress and nitrosative stress. This occurs when there is an overproduction of ROS/RNS in the biological system compared to a deficiency of enzymatic and non-enzymatic antioxidants on the other side. The excess ROS can damage cellular lipids, proteins or DNA, inhibiting their normal function (Valko *et al.*, 2007).

The delicate balance between beneficial and harmful effects is a very important aspect of living organisms because oxidative stress has been implicated in a number of human diseases and as well as the aging process (Valko *et al.*, 2007).

Free radicals can be defined as molecules of molecular fragments containing one or more unpaired electrons in atomic or molecular orbital (Young and Woodside, 2001; Valko *et al.*, 2007). This unpaired electron results in certain common properties that are shared by most

2.2.1. Sources of free radicals

Reactive oxygen species are found intracellular and extracellular and may be produced endogenously or be exogenous i.e. taken from the environment (Goodall, 2007; Young and Woodside, 2001).

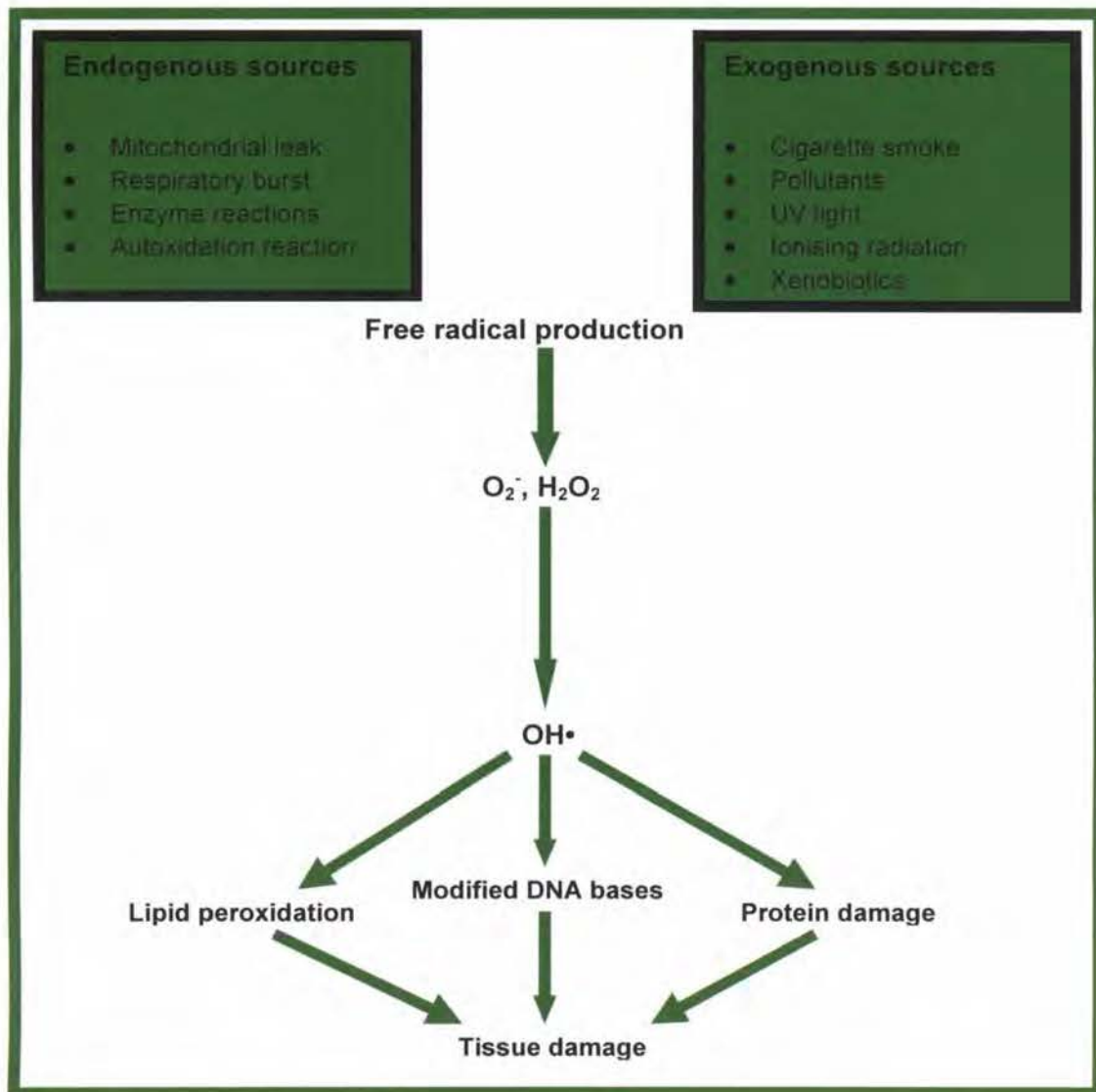


Figure 2.1: Major sources of free radicals in the body and the consequences of free radicals (Young and Woodside, 2001).

radicals. For example they are paramagnetic because they are weakly attracted to a magnetic field (Young and Woodside, 2001). This unpaired electron usually gives a considerable degree of reactivity to the free radical and it can either donate an electron to or extract an electron from other molecules, therefore behaving like oxidants or reductants (Young and Woodside, 2001; Valko *et al.*, 2007). A result of this high reactivity is that the radical has a very short half life (10^{-6} seconds or less) in biological systems, although some species may survive for much longer (Young and Woodside, 2001). Free radicals are more reactive than non-radicals and will react with them to produce new free radicals in a chain reaction. It is these chain reactions that can lead to damage to molecules in the body. A reaction between two free radicals will result in the pairing of their unpaired electrons and therefore non-radicals are formed (Goodall, 2007). Radicals derived from oxygen represent the most important class of radical species generated in living systems (Valko *et al.*, 2007; Young and Woodside, 2001)

2.2.1.1. Endogenous sources

2.2.1.1.1. Autoxidation

Autoxidation is a by product of the aerobic internal milieu. Catecholamins, haemoglobin, myoglobin, reduced sytochrome C and thiol are just some molecules that undergo autoxidation. Any of these molecules in a reaction result in reduction of oxygen diradicals and the formation of reactive oxygen species. The process of autoxidation primarily forms a superoxide radical but ferrous ion (Fe^{2+}) can also have an electron withdrawn from it by oxygen to produce superoxide and Fe^{3+} (Fouad, 2003).

2.2.1.1.2. Enzymatic oxidation

Xanthine oxidase (activated in ischemia reperfusion), prostaglandin synthase, lipoxygenase, aldehyde oxidase, acid oxidase and a variety of enzyme systems are capable of generating significant amounts of free radicals. The enzyme myeloperoxidase utilises hydrogen peroxide to oxidise chloride ions into the powerful oxidant hypochlorous acid (HOCl) produced in activated neutrophils (Fouad, 2003).

2.2.1.1.3. Respiratory burst

Respiratory burst describes the process by which cells consume large amounts of oxygen during phagocytosis. Superoxide production can account for between 70% and 90% of this oxygen consumption. These phagocytic cells possess a membrane bound flavoprotein cytochrome-b-245 NADPH oxidase system. Exposures to immunoglobulin-coated bacteria, immune complexes, complement 5a or leukotriene activate the enzyme NADPH-oxidase, which exist in an inactive form in the cell membrane. This activation initiates the production of superoxide from the respiratory burst of the cell membrane. H_2O_2 is then formed from superoxide by dismutation with subsequent generation of OH and HOCl by bacteria (Fouad, 2003).

2.2.1.1.4. Subcellular organelles

Organelles such as mitochondria, chloroplast, microsomes, peroxisomes and nuclei have been shown to generate O_2^- . This is easily demonstrated after endogenous superoxide dismutase has been washed away (Fouad, 2003).

Mitochondria are the main source of reduced oxygen species in the cell and are the main cellular organelle for cellular oxidation reactions. Leaks in the mitochondrial electron transport system allow O_2 to accept a single electron forming O_2^- . The production of

superoxide in the mitochondria increases if the oxygen concentration is greatly increased or when the respiratory chain becomes fully reduced (Fouad, 2003).

Microsomes are responsible for 80% of the H_2O_2 production *in vivo* at 100% known sites (Fouad, 2003). Under physiological conditions peroxisomes are known to produce H_2O_2 , but not O_2^- . Organs that contain peroxisomes are exposed to these H_2O_2 -generating mechanisms, although the liver is the primary organ where peroxisomal contributions to the overall H_2O_2 production are significant. Peroxisomal oxidation of fatty acids has recently been recognised as a potentially important source of H_2O_2 production with prolonged starvation (Fouad, 2003).

2.2.1.1.5. Transition metal ions

Transition metal ions like iron and copper play a major role in generation of free radical injury and the facilitation of lipid peroxidation. It participates in the Harber-Weiss reaction that generates OH from O_2^- and H_2O_2 . This reaction accelerates the nonenzymatic oxidation of molecules such as epinephrine and glutathione that generates O_2^- and H_2O_2 and subsequently OH (Fouad, 2003).

2.2.1.1.6. Ischemia reperfusion injury

A number of effects contributing to the production of free radicals are conserved in ischemia. Xanthine oxidase is known to catalyse the reaction of hypoxanthine to xanthine and subsequently xanthine to uric acid. An electron acceptor as a cofactor is required in this reaction. Two processes occur during ischemia (i) the production of xanthine and xanthine oxidases are greatly enhanced, (ii) there is a loss of both superoxide dismutase and glutathione peroxidase. The molecular oxygen supplied on reperfusion serves as an electron acceptor and cofactor for xanthine oxidase causing the generation of the O_2^- and H_2O_2 (Fouad, 2003).

2.2.1.2 Exogenous sources

2.2.1.2.1. Drugs

The production of free radicals can be increased by a number of drugs in the presence of increased oxygen tension. The rate of damage appears to be accelerated by the agents that act additively to hyperoxia. These drugs include antibiotics that depend on quinoid groups or bound metal for activity (nitrofuratoin), antineoplastic agents such as bleomycin, anthracyclines (adriamycin) and methotrexate, which possess pro-oxidant activity. Radicals derived from penicillamine, phenylbutazone, some fenamic acids and the aminosalicylate

component of sulphasalazine might inactivate protease and deplete ascorbic acid, accelerating lipid peroxidation (Fouad, 2003).

2.2.1.2.2. Radiation

Radiotherapy may cause tissue injury that is caused by free radicals. Primary radicals is generated by electromagnetic radiation (X rays, gamma rays) and particulate radiation (electrons, photons, neutrons, alpha and beta particles) by transferring their energy to cellular components such as water. These primary radicals can undergo secondary reactions with dissolved oxygen or with cellular solutes (Fouad, 2003).

2.2.1.2.3. Tobacco smoking

Sufficient amounts of oxidants exist in tobacco to suggest that they play a major role in injury of the respiratory tract. Intracellular antioxidants in the lung cells, *in vivo*, are severely depleted by tobacco smoke oxidants by a mechanism that is related to oxidant stress. It has been estimated that there are an enormous amount of oxidant materials in each puff of smoke. These include aldehydes, epoxides, peroxides and other free radicals that may be sufficiently long lived as to survive till they cause damage to the alveoli. In addition nitric oxide, peroxy radicals and carbon centred radicals are present in the gas phase. Semiquinone moieties derived from various quinones and hydroquinones are some of the relatively stable radicals that are present in the tar phase. Micro-haemorrhages are most probably the cause for iron deposition found in smoker's lung tissue. This form of iron leads to the formation of the lethal hydroxyl radicals from hydrogen peroxide. Further elevation of the concentration of free radicals could be contributed to the elevated amounts of neutrophils in the lower respiratory track of smokers (Fouad, 2003).

2.2.1.2.4. Inorganic particles

Inhalation of inorganic particles also known as mineral dust (e.g. asbestos, quartz, silica) can lead to lung injury that seems at least in part to be mediated by free radical production. Increased risk of developing pulmonary fibrosis (asbestosis), mesothelioma and bronchogenic carcinoma has been linked to asbestos inhalation. Silica particles and asbestos are phagocytosed by pulmonary macrophages. Increased production of free radicals and other reactive oxygen species are caused by the rupturing of these cells and the release of proteolytic enzymes and chemotactic mediators causing infiltration by other cells such as neutrophils, thus initiating the inflammatory process (Fouad, 2003).

2.2.1.2.5. Ozone

Ozone (O₃) is not a free radical but a very powerful oxidizing agent that contains two unpaired electrons. It degrades under physiological conditions to OH which suggests that free radicals are formed when ozone reacts with biological substrates. Ozone can generate lipid peroxidation *in vitro*, although similar findings *in vivo* have not been demonstrated (Fouad, 2003).

2.2.1.2.6. Others

Fever, excess glucocorticoid therapy and hyperthyroidism decrease oxygen tolerance in experimental animals. The decrease is attributed to the increased generation of oxygen-derived radicals that accompanies increased metabolism. A wide variety of environmental agents including photochemical air pollutants such as pesticides, solvents, anaesthetics, exhaust fumes and the general class of aromatic hydrocarbons, also cause free radical damage to cells (Fouad, 2003).

2.2.2. Types of free radicals

The term reactive species is also used to describe free radicals and other molecules that are themselves easily converted to free radicals or are powerful oxidising agents. More specifically the terms reactive oxygen species (e.g. superoxide, hydrogen peroxide) and reactive nitrogen species (e.g. nitric oxide, dinitrogen tetroxide) are often used (Goodall, 2007).

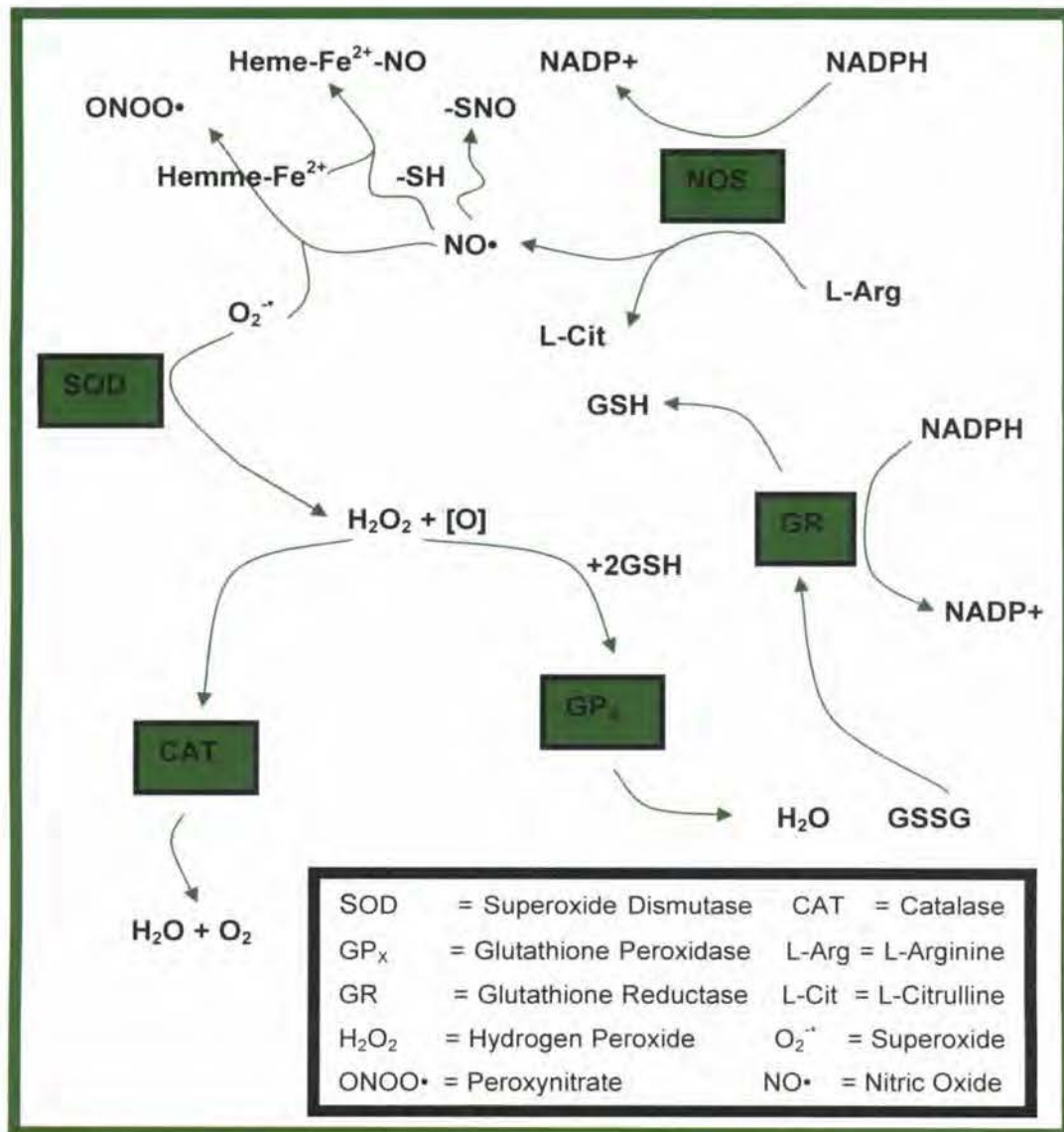


Figure 2.2: Schematic representation of the free radical and antioxidant network (Merck biosciences, 2007).

2.2.2.1. Reactive oxygen species (ROS)

2.2.2.1.1. Superoxide ($O_2^{\cdot-}$)

Superoxide is produced by the addition of a single electron to oxygen and several mechanisms exist by which it can be produced *in vivo* (Young and Woodside, 2001; Cyberlipid, 2007). It arises either through metabolic processes or following oxygen “activation” by physical irradiation. The production occurs mostly in the mitochondria of a cell. The mitochondrial electron transport train is the main source of ATP in the mammalian cell and thus essential for life (Valko *et al.*, 2007). During energy transduction, a small number of electrons “leak” to oxygen prematurely, forming the oxygen free radical superoxide, which is implicated in the pathophysiology of a variety of diseases. One to three percent of electrons in the transport chain generate superoxide instead of contributing to the reduction of oxygen to water (Young and Woodside, 2001; Valko *et al.*, 2007; Cyberlipid, 2007).

Superoxide is considered the “primary” ROS and can further interact with other molecules to generate “secondary” ROS, either directly or prevalently through enzyme- or metal-catalysed processes (Valko *et al.*, 2007). Superoxide can release Fe^{2+} from iron-sulphur proteins and ferritin. It also undergoes dismutation to form H_2O_2 spontaneously or by enzymatic catalysis. It is also a precursor for metal-catalysed $\cdot OH$ formation (Rice-Evans and Gopinathan, 1995).

Superoxide can be directly toxic but it has limited reactivity with lipids, raising questions about its toxicity. Superoxide action is frequently considered to result from secondary production of far more reactive $\cdot OH$ species by the iron-catalysed Harber-Weiss reaction. It is also proposed that nitric oxide reaction with $O_2^{\cdot-}$ generates secondary cytotoxic species (peroxynitrate anion) (Cyberlipid, 2007).

2.2.2.1.2. Hydrogen peroxide (H_2O_2)

Hydrogen peroxide is mainly produced by enzymatic reactions. These enzymes are located in microsomes, peroxisomes and mitochondria (Cyberlipid, 2007; Valko *et al.*, 2007). The hydrogen peroxide production is relatively important, even in normoxia, and leads to a constant cellular production of between 10^{-9} and 10^{-7} M (Cyberlipid, 2007). Superoxide dismutase is able to produce H_2O_2 by dismutation of $O_2^{\cdot-}$, thus contributing to the lowering of oxidative reactions, which is then used to oxidise a variety of molecules (Cyberlipid, 2007; Valko *et al.*, 2007). Several enzymatic reactions, including those catalysed by glycolate oxidase and D-amino acid oxidase, might produce hydrogen peroxide directly. Hydrogen peroxide is usually included under the general heading of ROS but is not a free radical itself.

It is a weak oxidising agent that might directly damage proteins and enzymes containing reactive thiol groups (Young and Woodside, 2001).

The most vital property of hydrogen peroxide is its ability to cross cell membranes freely, because it is lipid soluble, something that superoxide generally cannot do (Cyberlipid, 2007; Young and Woodside, 2001). Therefore hydrogen peroxide formed in one location might diffuse a considerable distance before decomposing to yield the highly reactive oxygen species the hydroxyl radical, which is likely to mediate most of the toxic effects ascribed to hydrogen peroxide. Hydrogen peroxide acts as a conduit to transmit free radical induced damage across cell compartments and between cells. Myeloperoxidase will generate hypochlorous acid and singlet oxygen in the presence of hydrogen peroxide, a reaction that plays an important role in the killing of bacteria by phagocytes (Young and Woodside, 2001).

Hydrogen peroxide has a true cellular antioxidant activity because of the natural combination of dismutase and catalase that contributes to remove H_2O_2 (Cyberlipid, 2007). When peroxisomes are damaged H_2O_2 consuming enzymes down regulate and H_2O_2 is released into the cytosol which significantly contributes to oxidative stress (Valko *et al.*, 2007).

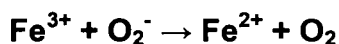
2.2.2.1.3. Hydroxyl radical ($OH^\bullet$)

The hydroxyl radical, $OH^\bullet$ is the neutral form of the hydroxyl ion. It has a very high reactivity, making it a very dangerous radical. It has a very short *in vivo* half life of 10^{-9} s and because of that it reacts very close to the site of formation (Valko *et al.*, 2007). It is probably the final mediator of most free radical induced tissue damage. The ROS described above exert most of their pathological effect by giving rise to hydroxyl radical formation. The reason for this is that it reacts, with an extremely high rate constant, with almost every type of molecule found in living cells including sugars, amino acids, lipids and nucleotides. The most important of all the mechanisms *in vivo* is likely to be the transition metal (mostly iron and copper) catalysed decomposition of superoxide and hydrogen peroxide (Young and Woodside, 2001).

The redox state of the cell is largely linked to an iron redox couple and is maintained within strict physiological limits. Under stress conditions and in the presence of an excess of superoxide “free iron” is released from iron-containing molecules (Valko *et al.*, 2007). Hydrogen peroxide can react with iron II (or copper I) to generate hydroxyl radicals. This reaction was first described by Fenton in 1894 (Young and Woodside, 2001; Valko *et al.*, 2007; Cyberlipid, 2007):



Superoxide and hydrogen peroxide can react to produce hydroxyl radicals. The rate constant for this reaction in aqueous solution is virtually zero. However, a reaction sequence is established that can proceed at a rapid rate if transition metal ions are present (Young and Woodside, 2001; Valko *et al.*, 2007):



Net result:



This net result reaction sequence illustrated above is known as the Harber-Weiss reaction (Young and Woodside, 2001; Valko *et al.*, 2007). The iron-catalysed decomposition of oxygen peroxide is considered one of the most prevalent reactions in the biological system. It is also the source of various deleterious lipid peroxidation products. Hydroxyl radicals are also produced (with $\bullet\text{NO}_2$) by the decay of peroxynitrite or peroxylnitrous acid. Another important $\bullet\text{OH}$ production process in the neutrophils during phagocytosis is the reaction involving myeloperoxidase and Cl^- (Cyberlipid, 2007).

2.2.2.1.4. Peroxyl radical ($\text{ROO}^\bullet$)

Oxygen can also help to form additional reactive radicals in the living system like the peroxyl radical ($\text{ROO}^\bullet$). The simplest radical is $\text{HOO}^\bullet$, which is the protonated form (conjugate acid: $\text{pK}_a = 4.8$) of superoxide ($\text{O}_2^{\bullet-}$) and is usually termed either hydroperoxyl radical or perhydroxyl radical. The hydroperoxyl radical initiates fatty acid peroxidation by two parallel pathways: fatty acid hydroperoxide (LOOH)-independent and LOOH -dependent. The LOOH -dependent pathway of $\text{HO}_2^\bullet$ initiates fatty acid peroxidation and may be relevant to mechanisms of lipid peroxidation initiation *in vivo*. Xanthine oxidase and xanthine dehydrogenase are inconvertible forms of the same enzyme, known as xanthine oxidoreductase (Valko *et al.*, 2007).

2.2.2.1.5. Singlet oxygen (O_2)

Singlet oxygen has a unique electronic configuration and is itself not a true radical but is reported to be an important ROS in reactions to ultraviolet exposition. An addition of one electron to dioxygen forms the superoxide anion radical ($\text{O}_2^{\bullet-}$) (Valko *et al.*, 2007; Cyberlipids, 2007). When appropriate photoexcitable compounds (sensitizers) are present with molecular oxygen, its toxicity is reinforced. Tetrapyrroles (bilirubin), flavins, chlorophyll,

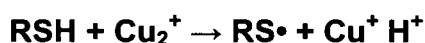
haemoproteins and reduced pyridine nucleotides are some of the natural sensitizers that are known to catalyse oxidative reactions. The presence of metals contributes to increase the production of singlet oxygen, as well as anion superoxide. This accelerates the oxidation of unsaturated lipids generating hydroperoxides (Cyberlipids, 2007).

2.2.2.1.6. Ozone (O₃)

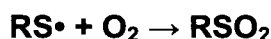
The photochemical reaction between hydrocarbons and nitrogen oxides forms ozone. It is present in the lower atmosphere of our polluted cities. It is also a natural compound in the higher atmosphere. Ozone is not a free radical but as singlet oxygen, may produce them, stimulates lipid peroxidation and thus induces damages at the lipid and protein levels (Cyberlipids, 2007).

2.2.2.1.7. Thiyl radicals (RS•)

The thiol compounds (RSH) are frequently oxidised in the presence of iron or copper ions:



These thiyl radicals have strong reactivity in combining with O₂:



They are also able to oxidise ascorbic acid and NADH to NAD• and to generate various free radicals (OH• and O₂•⁻). Homolytic fission of disulfide bonds in proteins may also form these thiyl radicals (Cyberlipids, 2007).

2.2.2.1.8. Carbon-centred radicals

When cells are treated with CCl₄ the formation of these free radicals are observed. The action of the cytochrome P450 system generates the trichloromethyl radical (•CCl₃) which is able to react with oxygen to give several peroxy radicals (i.e. •O₂CCl₃) (Cyberlipids, 2007).

2.2.2.2. Reactive nitrogen species

2.2.2.2.1. Nitric oxide (•NO)

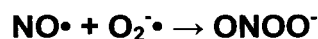
NO• is a small molecule that contains one unpaired electron on the antibonding orbital and is therefore a radical. It is generated in biological tissue by specific nitric oxide synthases (NOS), which metabolise arginine to citrulline with the formation of NO• via a five electron oxidative reaction (Ghafourifar and Cadenas, 2005).

Nitric oxide is an abundant reactive radical that acts as an important oxidative biological signalling molecule in a large variety of diverse physiological processes, including neurotransmission and blood pressure regulation, defence mechanisms, smooth muscle relaxation and immune regulations (Bergendi *et al.*, 1999). Nitric oxide has a half life of a few seconds in an aqueous environment and has greater stability in an environment with lower oxygen concentration. Nitric oxide is both soluble in aqueous and in lipid media and it readily diffuses through cytoplasm and plasma membranes (Valko *et al.*, 2007). It has effects on neuronal transmission as well as on synaptic plasticity in the central nervous system. In the extracellular milieu nitric oxide reacts with oxygen to form nitrate and nitrite anions (Klatt and Lamas, 2000).

Overproduction of nitrogen species is called nitrosative stress. It can lead to nitrosylation reactions that can alter the structure of proteins and so inhibit the normal function (Klatt and Lamas, 2000). Nitric oxide and the superoxide anion may react to produce significant amounts of a much more oxidatively active molecule, the peroxylnitrate anion. The reaction of nitric oxide and superoxide has one of the highest rate constants known and nitric oxide's toxicity is predominantly linked to its ability to combine with superoxide anions (Valko *et al.*, 2007; Cyberlipids, 2007).

2.2.2.2. Peroxynitrate anion (ONOO⁻)

The rapid reaction of O₂^{-•}, produced in different biological states, with NO[•] gives the extremely reactive peroxynitrate (ONOO⁻).



It mediates oxidation, nitrosation and nitration reactions. In alkaline solutions it is stable but decays rapidly once protonated into peroxylnitrous acid. It is a potent oxidising agent that can cause DNA fragmentation and lipid oxidation (Valko *et al.*, 2007; Cyberlipids, 2007).

2.3. Antioxidants

An antioxidant is defined as any substance that when present in low concentrations, compared to that of an oxidisable substrate, significantly delays or inhibits the oxidation of that substrate. This suggests that the physiological role is to prevent damage to cellular components arising as a consequence of chemical reactions involving free radicals (Young and Woodside, 2001; Fouad, 2007).

Antioxidants are substances that react with free radicals and other oxygen species within the body to protect it from damaging oxidation reactions, hence hindering the process of oxidation. Antioxidant supply is not unlimited because it can only react with a single free radical and during the reaction the antioxidant sacrifices itself by becoming oxidised. Therefore, there is a constant need to replenish antioxidant sources (Fouad, 2007).

An extensive range of antioxidant defences, both endogenous and exogenous, are present to protect cellular components due to the fact that radicals have the capacity to react in an indiscriminate manner leading to damage of almost any cellular components. Antioxidants can be divided into three main groups: antioxidant enzymes, chain breaking antioxidants and transition metal binding proteins (Young and Woodside, 2001).

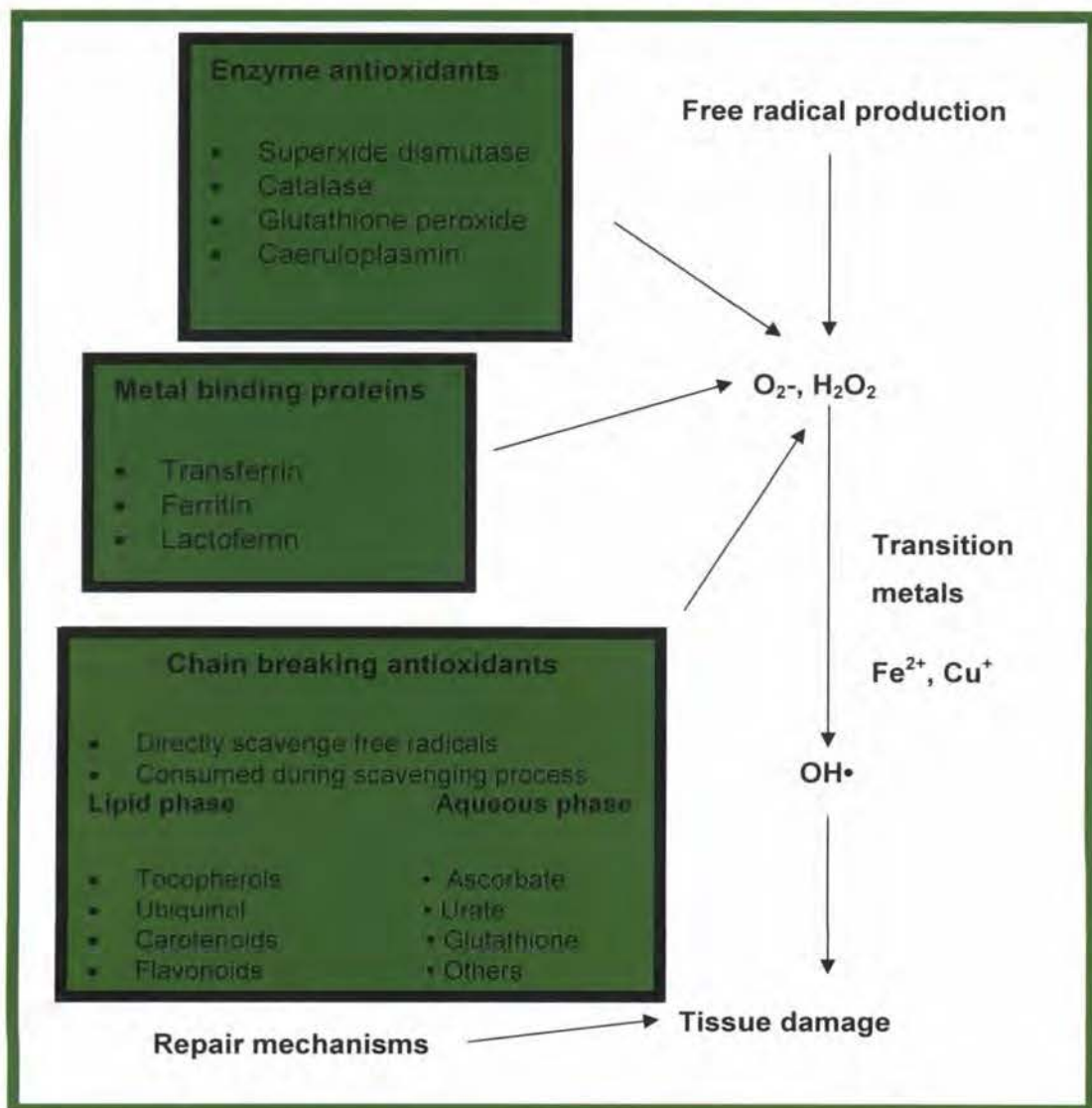


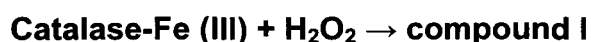
Figure 2.3: Antioxidant defences against free radical attack (Young and Woodside, 2001).

Antioxidant enzymes catalyse the breakdown of free radical species, usually in the intracellular environment. Transition metal binding proteins prevent the interaction of transition metals such as iron and copper with hydrogen peroxide and superoxide producing highly reactive hydroxyl radicals. Chain breaking antioxidants are powerful electron donors and react preferentially with free radicals before important target molecules are damaged. In doing so, the antioxidant is oxidised and must be generated or replaced. By definition, the antioxidant radical is relatively unreactive and unable to attack further molecules (Young and Woodside, 2001).

2.3.1. Antioxidant enzymes

2.3.1.1. Catalase

Catalase is the enzyme that characterised and catalyses the two stage conversion of hydrogen peroxide to water and diatomic oxygen (Fouad, 2007; Young and Woodside, 2001):



It consists of four protein subunits, each containing a haem group and a molecule of NADPH. The reaction described above has an extremely high rate constant that implies that it is virtually impossible to saturate the enzyme *in vivo* (Young and Woodside, 2001). An increase in the production of superoxide dismutase without the subsequent elevation of catalase or glutathione peroxidase will lead to the accumulation of hydrogen peroxide, which gets converted to hydroxyl radical (Fouad, 2007).

Most of the enzymes capable of generating hydrogen peroxide and catalase are largely located within cells in peroxisomes. Peroxisomes are easily ruptured during manipulation of cells, which makes the amount of catalase in the cytoplasm and other subcellular compartments unclear (Young and Woodside, 2001). Catalase is present in all body organs but is especially concentrated in the liver and erythrocytes. There are only low amounts present in the brain, heart and skeletal muscle (Fouad, 2007; Young and Woodside, 2001).

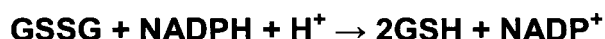
2.3.1.2. Glutathione peroxidases and glutathione reductase

Glutathione peroxidases catalyse the oxidation of glutathione (GSH) at the expense of a hydroperoxide. It might also be a hydrogen peroxide or another species such as a lipid hydroperoxide (Young and Woodside, 2001; Fouad, 2007).



Other peroxides can also act as substrates for these enzymes, including lipid hydroperoxides, which might play a role in the repairing of damage resulting from lipid peroxidation (Nakane *et al.*, 1998). Glutathione peroxidase is a tetrameric protein; it has four atoms of selenium bound as seleno-cysteine moieties that confers the catalytic activity (Fouad, 2007; Nakane *et al.*, 1998). Deficiency might occur in the presence of severe selenium deficiency (Nakane *et al.*, 1998). Several glutathione peroxidase enzymes are encoded by discrete genes (Brigelius-Floch , 1999). The kidney is believed to be the main synthesiser of the plasma form of the glutathione peroxidase (Young and Woodside, 2001). The highest concentrations, within cells, are found in the liver although glutathione peroxidase is widely distributed in almost all tissues. The cytosol and mitochondria are the predominant subcellular sites of glutathione peroxidase, suggesting that glutathione peroxidase is the main scavenger of hydrogen peroxide in these subcellular compartments (Holben and Smith, 1999).

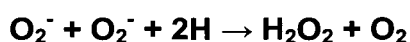
Rereduction of the oxidised form of glutathione (GSSG) is catalysed by glutathione reductase. The availability of reduced glutathione determines the activity of the enzyme. The activity of the enzyme glutathione reductase usually keeps the ratio between the reduced to oxidised glutathione very high (Fouad, 2007; Young and Woodside, 2001):



The pentose phosphate pathway provides the supply of NADPH that is required by the enzyme to replenish the supply of reduced glutathione. The action of glutathione peroxidase can be impaired by any competing pathway that utilises NADPH (such as the aldose reductase pathway) that might lead to a deficiency of reduced glutathione. Glutathione reductase is a flavine nucleotide dependent enzyme and has a similar tissue distribution as glutathione peroxide (Young and Woodside, 2001).

2.3.1.3. Superoxide dismutase

Superoxide dismutase catalyses the dismutations of superoxide to hydrogen peroxide:



Catalase and glutathione peroxidase must then be used to remove the hydrogen peroxide, as described above. Superoxide dismutase in mammalian tissues has three forms, each with a specific subcellular location and a different tissue distribution (Fouad, 2007; Young and Woodside, 2001).

Copper zinc superoxide dismutase (CuZnSOD): It is found in the cytoplasm and organelles of virtually all mammalian cells. It has two protein subunits that each contain a catalytically active copper and zinc atom. It has a molecular mass of approximately 32,000 kDa (Fouad, 2007; Young and Woodside, 2001).

Manganese superoxide dismutase (MnSOD): It is found in the mitochondria of almost all cells (Fouad, 2007; Young and Woodside, 2001). It has four protein subunits containing a single manganese atom. It has a molecular mass of 40,000 kDa. In a mixture of two enzymes MnSOD activity can be distinguished from CuZnSOD because their amino acid sequence is entirely dissimilar and the MnSOD is not inhibited by cyanide (Young and Woodside, 2001).

Extracellular superoxide dismutase (EC-SOD): It is a secretor of copper and zinc containing SOD distinct from the CuZnSOD described above (Fouad, 2007; Young and Woodside, 2001). It is synthesised by only a few cell types which include fibroblasts and endothelial cells. It is expressed on the cell surface where it is bound to the heparin sulphates. EC-SOD is the major SOD detectable in extracellular fluids and following an injection of heparin it is released into the circulation from the surface of vascular endothelium. It might also play a role in regulation of vascular tone, because endothelial derived relaxing factor (nitric oxide or closely related compound) is neutralised in the plasma by superoxide (Young and Woodside, 2001).

There is a feedback system, which is tightly regulated, of the respective enzymes that interacts with superoxides and H_2O_2 . Excessive superoxide inhibits glutathione peroxidase and catalase to modulate the equation from H_2O_2 to H_2O . Likewise, increased H_2O_2 slowly inactivates CuZnSOD. Meanwhile, catalase and glutathione peroxidase conserve SOD by reducing H_2O_2 . The SOD also conserves catalases and glutathione peroxidase by reducing superoxide. Through this feedback system, steady low levels of SOD, glutathione peroxidase and catalase, as well as superoxide and H_2O_2 are maintained. This keeps the entire system in a fully functioning state. By reducing O_2^- that would otherwise lead to the reduction of Fe^{3+} to Fe^{2+} and thereby promoting OH formation, the SOD exhibits antioxidant activity. The produced SOD will increase the tissue oxidant activity if the catalase activity is insufficient to metabolise the H_2O_2 produced. Antioxidant enzymes function as a tightly balanced system. Any disruption of this system will lead to promotion of oxidation (Fouad, 2007).

2.3.2. Chain breaking antioxidants

If a free radical interacts with another molecule they may generate a secondary radical, this radical can react with other targets to produce yet more radical species. An example of such

a chain reaction is lipid peroxidation and this chain reaction will continue to propagate until two radicals combine to form a stable product or the radicals are neutralised by a chain breaking antioxidant. These antioxidants are small molecules that can receive or donate an electron to a radical with the formation of stable by-products. The resulting products will not readily accept or donate an electron to another molecule because the charge associated with the presence of an unpaired electron becomes dissociated over the scavenger. This prevents further propagation of the chain reaction. These antioxidants can be divided into aqueous phase and lipid phase antioxidants (Young and Woodside, 2001).

2.3.2.1. Lipid chain breaking antioxidants

These antioxidants scavenge radicals in membranes and lipoprotein particles that are crucial in preventing lipid peroxidation (Young and Woodside, 2001).

2.3.2.1.1. Vitamin E

Vitamin E is the most important antioxidant in the lipid phase. In nature there are eight different forms of vitamin E and all of them differ greatly in their degree of biological activity. The tocopherols (alpha-, beta-, gamma-, delta-) have a chromanol ring and a phytyl tail and differ in the number and position of the methyl groups on the ring. The tocotrienols (alpha-, beta-, gamma-, delta-) are structurally similar but have unsaturated tails (Fouad, 2007; Young and Woodside, 2001). The tocopherols and the tocotrienols are lipid soluble and have pronounced antioxidant properties. Their reaction with peroxy radicals is more rapid than the reaction of polyunsaturated fatty acids with peroxy radicals. This reaction acts to break the chain reaction of lipid peroxidation. Vitamin E also might have a role in the structural stabilising of the membranes (Young and Woodside, 2001).

The essential antioxidant function of vitamin E in the cell membranes and lipoproteins is to trap peroxy radicals and to break the chain reaction of lipid peroxidation. Vitamin E minimizes the formation of secondary radicals but does not prevent the initial formation of carbon centred radicals in a lipid rich environment (Young and Woodside, 2001).

2.3.2.1.2. Carotenoids

This group is based on an isoprenoid carbon skeleton. At least 20 different forms of carotenoids may be present in membranes and lipoproteins of which the β -carotene is the most important. They are particularly efficient scavengers of singlet oxygen, but can also at low oxygen pressure trap peroxy radicals with efficiency at least as great as that of alpha-tocopherol (Fouad, 2007; Young and Woodside, 2001).

The ability of the carotenoids is limited because the carotenoid itself can be oxidised during the process. At high concentrations carotenoids may function as pro-oxidants and can activate proteases (Fouad, 2007).

The carotenoids might play a role in preventing *in vivo* lipid peroxidation because these conditions prevail in many biological tissues (Young and Woodside, 2001). Certain carotenoids are also precursors of vitamin A (retinol) that also has antioxidant properties, which do not, however, show dependency on oxygen concentration (Young and Woodside, 2001; Fouad, 2007).

2.3.2.1.3. Flavonoids

It is a large group of polyphenolic antioxidants found in many fruits, vegetables and beverages such as tea and wine. Over 4000 flavonoids have been identified. They are divided into several groups according to their chemical structure. Some examples are flavonols (quercetin and kaempferol), flavanols (the catechins), flavones (apigenin), and isoflavones (genistein). An inverse relationship between flavonoid intake and the incidence of chronic diseases such as coronary heart diseases is suggested by epidemiological studies. Little is known about the absorption and metabolism of flavonoids and epidemiological associations might be a consequence of confounding by other factors. Bioavailability of many flavonoids is poor and plasma values very low, although there is some evidence that augmenting the intake of flavonoids might improve biochemical indices of oxidative damage. Other dietary phenolic compounds, apart from flavonoids, might also make a small contribution to total antioxidant capacity (Young and Woodside, 2001).

2.3.2.1.4. Ubiquinol-10

It is the reduced form of coenzyme Q10. It protects the body from destructive free radicals and enhances immune defences (Fouad, 2007). Even though it presents in lower concentrations than alpha-tocopherol, it can scavenge lipid peroxyl radicals with higher efficiency than either alpha-tocopherol or the carotenoids. It also regenerates membrane bound alpha-tocopherol from the tocopheryl radical. Ubiquinol-10 is the first antioxidant to be consumed whenever plasma or isolated low density lipoprotein cholesterol is exposed to radicals that are generated in the lipid phase. This suggests that it might be of particular importance in preventing the propagation of lipid peroxidation (Young and Woodside, 2001).

2.3.2.2. Aqueous phase chain breaking antioxidants

These antioxidants will directly scavenge radicals present in the aqueous compartment (Young and Woodside, 2001).

2.3.2.2.1. Vitamin C (ascorbate)

It is qualitatively the most important antioxidant of this type. It is an essential cofactor for several enzymes catalysing hydroxylation reactions in humans. It provides electrons for enzymes that require prosthetic metal ions in a reduced form to achieve full enzymatic activity. In the synthesis of collagen it is best known for the role of cofactor for prolyl and lysyl oxidases (Young and Woodside, 2001). The other major function of ascorbate is a key chain breaking antioxidant in the aqueous phase (Fouad, 2007; Young and Woodside, 2001). Ascorbate has been shown to scavenge superoxide, hydrogen peroxide, the hydroxyl radical, hypochlorous acid, aqueous peroxy radicals and singlet oxygen (Young and Woodside, 2001).

Ascorbate undergoes two-electron reductions during its antioxidant action; initially one electron is donated by ascorbate and gives the semi-dehydroascorbate radical. Then it reacts rapidly with OH to give dehydroascorbate (Fouad, 2007; Young and Woodside, 2001).

2.3.2.2.2. Uric acid

It is an antioxidant present in the plasma in high concentrations. Radicals like the singlet oxygen, peroxy radical and nitric oxide radical are efficiently scavenged by uric acid and in the process it is converted to allantoin (Fouad, 2007; Young and Woodside, 2001). Urate might be particularly important in providing protection against certain oxidising agents, such as ozone. The formation of stable non-reactive complexes with iron might partly be attributed to the antioxidant effect of urate, but it is also a direct free radical scavenger (Young and Woodside, 2001).

2.3.2.2.3. Thiol groups

The protein bound thiol group is the other major chain breaking antioxidant in plasma. The sulphhydryl groups present on plasma proteins can function as chain breaking antioxidants, with the resultant formation of a protein thiyl radical. *In vitro* work has shown that the protein thiyl radicals can themselves act as a potential source of reactive oxidants. The process lipid peroxidation can be initiated by the thiyl radical that abstracts an electron from a polyunsaturated fatty acid. This reaction can be inhibited by ascorbate and retinol (Young and Woodside, 2001).

2.3.2.2.4. Albumin bound bilirubin

It is an efficient radical scavenger. It has a particularly crucial role in protecting the neonate from oxidative damage, because deficiency of other chain breaking antioxidants is common in a newborn (Young and Woodside, 2001). Albumin contains 17 disulphide bridges and has

a single remaining cysteine residue. It is this residue that is responsible for the capacity of albumin to react with and neutralise peroxy radicals. Albumin plays an important role in the transporting of free fatty acids in the blood (Young and Woodside, 2001). Albumin also has the capacity to bind copper ions and will inhibit copper dependent lipid peroxidation and hydroxyl radical formation. It also provides the main plasma defence against and scavenges the oxidant of the phagocytic product hypochlorous acid (Young and Woodside, 2001; Fouad, 2007).

Albumin has been viewed as a sacrificial molecule that prevents damage occurring to more vital species. The high plasma concentration of albumin and the relatively short half life mean that any damage suffered is unlikely to be of biological importance (Young and Woodside, 2001; Fouad, 2007).

2.3.2.2.5. Glutathione (GSH)

Reduced glutathione is a major source of thiol groups in the intracellular compartments but is of little importance in the extracellular space (Young and Woodside, 2001). It might function directly as an antioxidant, scavenging hydroxyl radicals and singlet oxygen, as well as acting as an essential factor for glutathione peroxidase (Young and Woodside, 2001; Fouad, 2007). Glutathione is synthesised in the liver and approximately 40% is secreted in the bile for defence against dietary xenobiotics, lipid peroxidation in the lumen of the gut and protection of the intestinal epithelium from oxygen radical attack (Fouad, 2007)

2.3.2.3. Interactions between chain breaking antioxidants

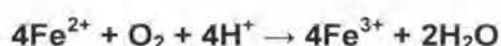
It is vital to remember that *in vivo* complex interactions between antioxidants are likely to occur. It is likely that ascorbate will recycle the tocopheryl radical in the aqueous-lipid interface, so regenerating tocopherol. This might be crucial in ensuring that the tocopherol concentration is maintained in lipoproteins and membranes. Glutathione can regenerate ascorbate from dehydroascorbate in a similar manner. It is difficult to predict how antioxidants will function *in vivo*, because a complex interplay is likely to exist between antioxidants (Young and Woodside, 2001).

Chain breaking antioxidants can also act as pro-oxidants. This means that in certain circumstances, the presence of an antioxidant might paradoxically lead to increase oxidative damage. It has been reported that an increase in oxidative damage can sometimes follow from administration of vitamin C. Similarly it was shown that in the absence of an aqueous phase antioxidant such as ascorbate *in vitro* the tocopherol might promote LDL oxidation. It is still unclear if any of these reactions are important *in vivo*. The possibility that antioxidants

may have pro-oxidant effects *in vivo* must be considered when designing and interpreting the results of clinical trials of antioxidant supplementation (Young and Woodside, 2001; Fouad, 2007).

2.3.3. The transition metal binding proteins

The transition metal binding proteins (ferritin, transferrin, lactoferrin and caeruloplasmin) act as crucial components of the antioxidant defence system, by sequestering iron and copper so that they are not available to drive the formation of the hydroxyl radical. Caeruloplasmin, the main copper binding protein, might also function as an antioxidant enzyme that can catalyse the oxidation of divalent iron.



The Fenton reaction is driven by the Fe^{2+} form of iron. It is rapidly oxidised from Fe^{2+} to Fe^{3+} and therefore has an antioxidant effect (Young and Woodside, 2001).

2.4. Oxidative stress

The term oxidative stress was defined by Sies in 1991 as "a disturbance in the pro-oxidant – antioxidant balance in favour of the former, leading to potential damage". One of the following scenarios must be present for a disturbance of the balance (Goodall, 2007):

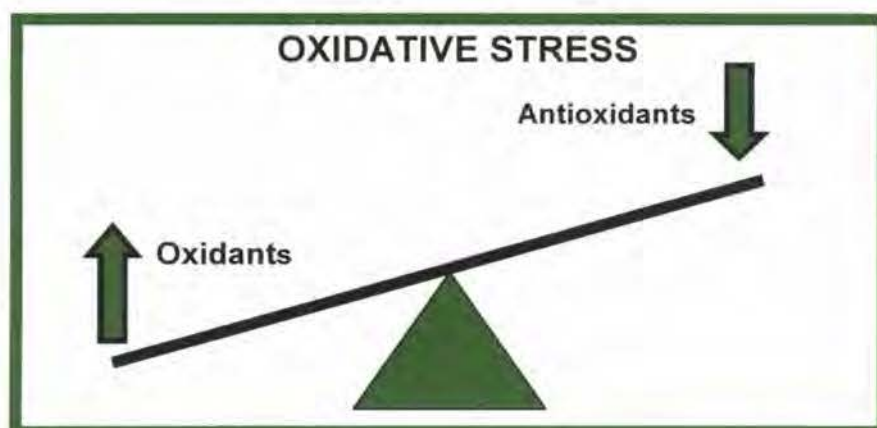


Figure 2.4: The balance of oxidants and antioxidants (Goodall, 2007).

A reduction in antioxidants: It can be brought on by three main mechanisms. The first is malnutrition where there is inadequate intake of essential antioxidant nutrients. For example, in cases of neurodegeneration in patients with faulty fat absorption, it can lead to vitamin E deficiency. The second mechanism is that many drugs are conjugated with glutathione in

preparation for their excretion from the body, leading to reduced glutathione levels. The final mechanism for a reduction in antioxidants is genetic mutation. It may adversely affect antioxidant systems leading to reduced antioxidant action (Goodall, 2007).

An increase in reactive species: This is thought to be the most common causes of oxidative stress in the human body. Excessive formation of reactive oxygen species such as H_2O_2 and $\text{OH}^\bullet$ can be caused by increased O_2 concentration. Also the cytochrome p450 plays a role in the detoxification of toxins in the body. But in some cases the products of the enzyme are free radicals which may be more damaging than the primary toxin and cause oxidative stress. Excessive activation of phagocytic cells is an important cause of oxidative stress. Many different reactive oxygen species are formed by the activation of phagocytic cells and they impose oxidative stress on tissues. Direct exposure to toxins from our environment may also play an important role in the generation of oxidative stress (Goodall, 2007).

2.4.1. Damage caused by oxidative stress

An imbalance between free radical production and antioxidant defences arise from oxidative stress. It is associated with damage to a wide range of molecular species including lipids, proteins and nucleic acids (Young and Woodside, 2001; Valko *et al.*, 2007; Fouad, 2003).

2.4.1.1. Nucleic acid (DNA)

Oxidative damage to DNA is a result of the interaction of DNA with reactive oxygen species, in particular the hydroxyl radical. Wide spectrums of oxidative base modifications occur with reactive oxygen species (Valko *et al.*, 2007; Kow, 2007).

Oxygen derived species including free radicals causes the most frequent type of DNA damage encountered by aerobic cells. This oxidative DNA damage can produce a multiplicity of modifications in DNA including base and sugar lesions, strand breaks, DNA-protein cross-links and base-free sites (Dizdar and Jaruga, 2005; Kow, 2007). DNA damage plays an important role in a number of disease processes. It is implicated in carcinogenesis, mutagenesis and neurodegenerative diseases such as Alzheimer's disease. There is also strong evidence for the role of this type of DNA damage in the aging process (Dizdar and Jaruga, 2005; Valko *et al.*, 2007; Young and Woodside, 2001; Salmon *et al.*, 2004).

Free radical attack causes fragmentation of DNA and this causes the activation of the poly (ADP-ribose) synthetase enzyme. This enzyme splits the NAD^+ to aid in the repair of DNA. If the damage is extensive the NAD^+ levels may become depleted to the extent that the cell may no longer be able to function and will die (Goodall, 2007).

2.4.1.2. Lipids

It is known that metal-induced generation of ROS results in an attack not only on DNA, but also on other cellular components involving polyunsaturated fatty acid residues of phospholipids, which are extremely sensitive to oxidation (Valko *et al.*, 2007; Fouad, 2007). The polyunsaturated fatty acids are most often affected because they contain double bonds, in between which lie methylene groups that possess especially reactive hydrogens. These lipoprotein particles or membranes characteristically undergo the process of lipid peroxidation (Young and Woodside, 2001; Fouad, 2007). Lipid peroxidation in the cell membrane can also adversely damage cell membranes by disrupting fluidity and permeability. It can also adversely affect the function of membrane bound protein and receptors (Goodall, 2007). The reaction of lipid peroxidation consists of three major steps: initiation, propagation and termination (Cyberlipids, 2007).

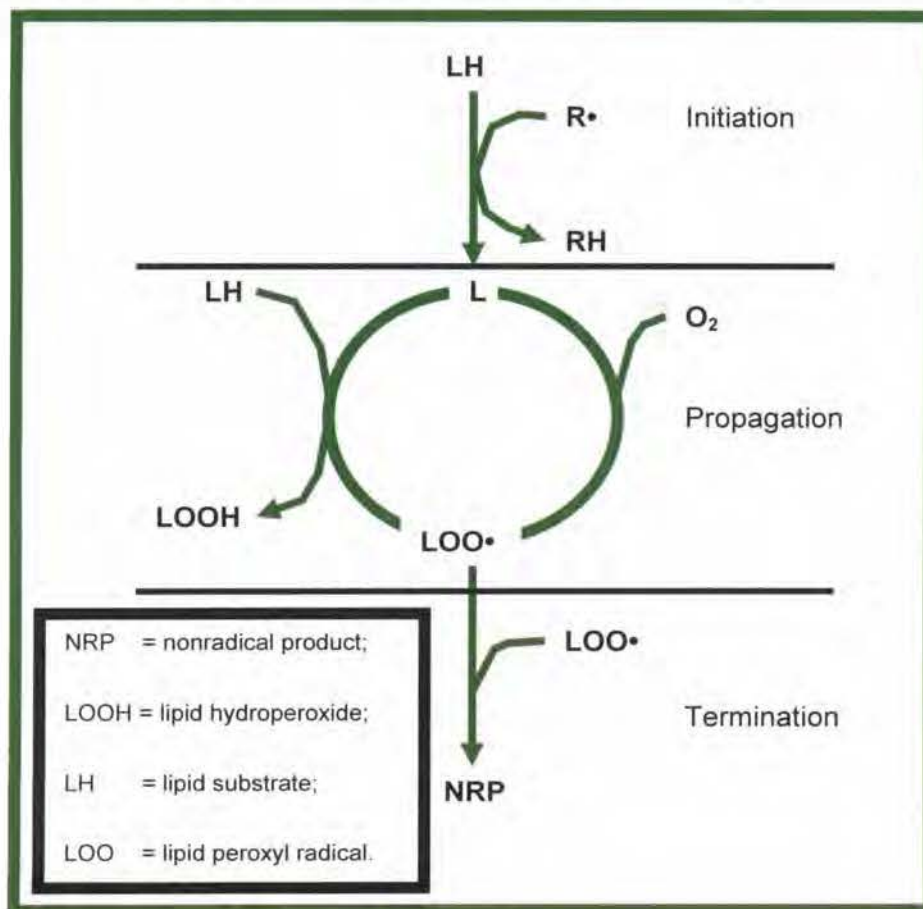


Figure 2.5: An overview of lipid peroxidation (Kelly *et al.*, 1998).

Initiation: This phase may proceed by the reaction of an activated oxygen species such as singlet oxygen ($^1O_2$), O_2^- or OH with a lipid substrate or by the breakdown of pre-existing lipid

hydro peroxides by transition metals. Peroxidation occurs by abstraction of a hydrogen atom from a methylene carbon in the lipid substrate (LH) to generate a highly reactive carbon-centred lipid radical (L) (Kelly *et al.*, 1998).

Propagation: Molecular oxygen adds rapidly to L at a diffusion rate to produce the lipid peroxy radical (LOO). This peroxy radical can abstract a hydrogen atom from a number of *in vivo* sources, such as DNA and proteins to form the primary oxidation product, a lipid hydroperoxide (LOOH). Alternatively antioxidants can act as excellent hydrogen atom donors. In the absence of antioxidants or other inhibitors, LOO can abstract hydrogen from another lipid molecule (LH), producing another highly reactive carbon centred radical (L) which then propagates the radical chain (Kelly *et al.*, 1998).

Transition metals are of particular interest to lipid peroxidation. OH is thought to be the primary initiating radical in transition metal-catalysed lipid peroxidation. However, both ferrous (Fe^{2+}) and ferric (Fe^{3+}) iron, in addition to increasing the production of initiating hydroxyl radicals, can catalyse the propagation of the lipid peroxidation chain by decomposing LOOH oxidation products. The resulting alkoxy (LO) and peroxy (LOO) radicals are able to initiate new radical chains by interacting with additional lipid molecules (Kelly *et al.*, 1998).

Termination: This step only happens with the coupling of two radicals to form non-radical products. These products are stable and unable to propagate lipid peroxidation (Kelly *et al.*, 1998).

The major aldehyde products of lipid peroxidation is malondialdehyde (MDA) and 4-hydroxy-2-nonenal (Young and Woodside, 2001; Valko *et al.*, 2007). MDA is mutagenic in bacteria and mammalian cells and carcinogenic in rats. Hydroxy-nonenal is weakly mutagenic but appears to be the major toxic product of lipid peroxidation (Valko *et al.*, 2007). Other products that are formed are alkanes, alkenes conjugated dienes and a variety of hydroxides and hydroperoxides. Many of these products can be measured as markers of lipid peroxidation (Young and Woodside, 2001).

2.4.1.3. Proteins

Free radicals cause direct damage to proteins. This interferes with enzyme activity and the function of structural proteins (Goodall, 2007). Mechanisms involved in the oxidation of proteins by ROS were elucidated by studies in which amino acids, simple peptides and proteins were exposed to ionising radiation under conditions where hydroxyl radicals or mixture of hydroxyl/superoxide radicals are formed. The side chains of all amino acid

residues of proteins, in particular cysteine and methionine residues of protein are susceptible to oxidation by the action of ROS/RNS. Oxidation of cysteine residues may lead to the reversible formation of mixed disulphides between protein thiol groups and low molecular weight thiols (Valko *et al.*, 2007).

2.4.2. Consequences of damage caused by oxidative stress

The precise site of tissue damage by free radicals is dependant on the tissue and the reactive species involved. The overall damage caused by oxidative stress is often an accumulation of damage to many sites. Extensive damage can lead to death of the cell (Goodall, 2007). Neuronal cells with a few exceptions do not renew themselves, thus gradual reduction throughout life is unavoidable and that makes the central nervous system highly susceptible to damage (Koutsilieri *et al.*, 2002). Cell death associated with oxidative stress can be either apoptotic or necrotic (Koutsilieri *et al.*, 2002; Goodall, 2007).

Apoptotic cell death: It is characterised by caspase cleavage, chromatin condensation, DNA fragmentation, cellular shrinkage and formation of apoptotic bodies. Cells dying of apoptosis do not affect the neighbouring cells. Various stimuli trigger apoptotic processes and it is often associated with changes in the cellular redox state. Apoptosis can be triggered by oxidative stress but it is not a general prerequisite for apoptotic cell death. NO-dependent apoptosis has been observed in several experimental models and certain pathologies. It is associated with decreased activity in the mitochondrial electron transport chain and release of mitochondrial cytochrome c into the cytoplasm. It can be inhibited by an elevated intracellular glutathione concentration (Koutsilieri *et al.*, 2002).

Necrotic cell death: It is characterised by a rapid loss of membrane integrity as a consequence of a breakdown in membrane potential. This leads to swelling of the organelles and to rupture of the cell membranes. This results in the release of cellular protein into neighbouring tissue. As a consequence, necrotic cell death is accompanied by signs of inflammation. Only high levels of oxidative stress cause necrotic cell death where as, mild alterations in redox state lead to modulation of intracellular signalling cascades (Koutsilieri *et al.*, 2002).

2.4.3. Oxidative stress and diseases

Iron rusts, butter turns rancid and apples brown. This is all everyday signs of oxidative stress and destruction caused by free radicals. None of these however compare to what these unstable molecules can do inside the body especially to the cells of the brain. The oxygen molecule's unpaired electron is the source of this devastating action because it is unstable

and electrically charged. It reacts with the nearest available molecule and have no prejudices, and it targets proteins, lipids or even DNA. Free radical actions can damage molecules they react with and can even sometimes cause cell death. Scientists are trying to stop the free radicals by studying the various pathways the molecules take when it corrodes the cells of the brain (Society of Neuroscience, 1996).

For oxidative stress to be in the primary role in a particular setting there should be a plausible mechanism by which free radical production or a decrease in antioxidant defences might occur. The evidence of oxidative stress should also be detectable before the onset of tissue damage and augmentation of antioxidant status at an early stage should either prevent or greatly reduce tissue damage (Young and Woodside, 2001).

Humans are equipped with a series of defences, or antioxidants, that control free radical molecules and mend damage. For example radical scavengers such as vitamin E mop up free radicals and help prevent damage to critical cell structures. The imbalance in free radical production and internal defences may result in ailments characterised by loss of neurons such as Parkinson's disease, Lou Gehrig's disease and Huntington's disease. The accumulation of glutamate and related amino acids in the brain is believed to trigger oxidative stress and neurotoxicity in Huntington's disease and amyotrophic lateral sclerosis (Society of Neuroscience, 1996; Young and Woodside, 2001).

2.5. Neurodegenerative diseases

The brain accounts for only ~2% of body mass but it processes 20% of basal oxygen consumption in order to support the neuronal electric activity (Sorg, 2004). Throughout life the brain is exposed to oxidative stress and certain diseases of the brain and nervous system are thought to involve free radical processes and oxidative damage. This involvement is either primary or a consequence of disease progression (Gilgun-Sherki *et al.*, 2001). Nitric oxide is produced by three kinds of nitric oxide synthases (neuronal, endothelial and inducible) and plays a crucial role as a biological messenger. The brain produces high levels of superoxide and nitric oxide. They can react to form one of the most reactive oxygen species, peroxylnitrite. These are some of the reasons why oxidative stress plays a pivotal role in neurodegenerative diseases. Two of the major neurodegenerative diseases, in which the link with oxidative stress has been extensively studied, are Alzheimer's disease and Parkinson's disease (Sorg, 2004).

Alzheimer's disease is a progressive neuropsychiatric disorder with unknown etiology (Gilgun-Sherki *et al.*, 2001). Alzheimer's disease is one of several disorders that causes the

gradual loss of brain cells and is the leading cause of dementia (Sorg, 2004). It is characterised by neuronal degeneration and cognitive deterioration, especially in the elderly. The finding of several characteristics, such as enhanced lipid peroxidation, in specific areas of the brain in post-mortem studies has implicated oxidative stress in the pathogenesis of Alzheimer's disease. Investigation detected an increase in the activity of catalase, superoxide dismutase, glutathione peroxidase and glutathione reductase in the hippocampus and amygdale. It was suggested that oxygen radical formation with resultant neurodegeneration and possibly plaque formation in the central nervous system was caused by oxidative stress (Gilgun-Sherki *et al.*, 2001).

The brain of Alzheimer's disease patients is associated with many markers of oxidative stress and oxidative stress increases the severity of symptoms. But oxidative stress as a cause of the disease is still being debated (Sorg, 2004).

Parkinson's disease is a degenerative disorder of the central nervous system that often impairs the sufferer's motor skills and speech. Parkinson's disease is a movement disorder and is characterised by muscle rigidity, tremors, a slowing of physical movements (bradykinesia) and in extreme cases a loss of physical movement (akinesia). The primary symptoms are the result of decreased stimulation of the motor cortex by the basal ganglia, normally caused by the insufficient formation and action of dopamine, which is produced in the dopaminergic neurons in the brain (Jankovic, 2008).

Oxidative stress plays an important role in neural degeneration of the pigmented dopaminergic neurons in the substantia nigra pars compacta (SNpc) (Gilgun-Sherki *et al.*, 2001; Sorg, 2004). The SNpc of Parkinson's disease patients show a significant reduction in glutathione and a moderate increase in oxidised glutathione levels in post-mortem studies. This could be a critical primary event, leading to a weakening or deficiency of the natural antioxidative cellular defence mechanisms and thereby triggering degeneration of the nigral neurons, causing Parkinson's disease (Gilgun-Sherki *et al.*, 2001).

2.6. Aging

Aging appears to be the result of normal development and metabolic processes responsible for greying of the hair, decreases in the rate of wound healing and increases in susceptibility to disease and death (Gilgun-Sherki *et al.*, 2001). Two general theories explain intrinsic aging: genetic theories and the theories about the accumulation of cellular damages (Sorg, 2004).

Evidence is shown of oxidative damage to macromolecules (DNA, lipids and proteins) especially in the brain of elderly subjects. This supports the hypothesis that oxidative injury might directly cause the aging process. Direct biochemical measurements of mitochondrial function demonstrate age-dependent increases in mitochondrial deletions, point mutations and oxidative damage to the DNA. The mitochondrial DNA in the elderly is particularly susceptible due to its close proximity to the respiratory chain, limited repair mechanisms, few non-coding sequences and absence of histones (Gilgun-Sherki *et al.*, 2001).

CHAPTER 3: PRIMARY SCREENING

3.1. Selection of plants

Following an extensive literature study of antioxidant activity in plants, a list of the families where antioxidant activities were found was made. An alphabetical list of South African plants, belonging to the same families, was compiled. From the list, 21 plants were identified as being available in the Botanical garden of the North–West University by the curator Mr. Peter Mortimer.

3.1.1. The plant families selected

Table 3.1: Plant species identified from the literature search and South African plants selected.

Family	Plant species	South African plants available
Apiaceae	<i>Ferula orientalis</i> (Kartal <i>et al.</i> , 2007), <i>Chaerophyllum libanoticum</i> (Demirci <i>et al.</i> , 2007) <i>Coriandrum sativum</i> (Hashim <i>et al.</i> , 2005) <i>Angelica gigas</i> (Kang <i>et al.</i> , 2005)	<i>Heteromorpha arborescens</i> <i>Berula erecta</i>
Asteraceae	<i>Inula britannica</i> (Kim <i>et al.</i> , 2002) <i>Senecio ambavilla</i> (Aruoma <i>et al.</i> , 2003) <i>Achyrocline satureioides</i> (Arredondo <i>et al.</i> , 2003) <i>Taraxacum officinale</i> (Williams <i>et al.</i> , 1996)	<i>Tarchonanthus camphoratus</i> <i>Vernonia oligocephala</i>
Celastraceae	<i>Celastrus paniculatus</i> (Godkar <i>et al.</i> , 2004; Godkar <i>et al.</i> , 2006; Russo <i>et al.</i> , 2001)	<i>Gymnosporia buxifolia</i>

Family	Plant species	South African plants available
Fabaceae	<i>Sutherlandia frutescens</i> (Fernandes <i>et al.</i> , 2004) <i>Cassia fistula</i> (Luximon-Ramma <i>et al.</i> , 2002) <i>Bauhinia variegata</i> , <i>Bauhinia candida</i> (Silva <i>et al.</i> , 2005)	<i>Acacia karroo</i> <i>Erythrina zeyheri</i> <i>Elephantorrhiza elephantina</i>
Lamiaceae	<i>Hyptis fassiculata</i> (Silva <i>et al.</i> , 2005) <i>Marrubium globosum</i> (Sarikurkcü <i>et al.</i> , 2008) <i>Rosemary</i> (Aruoma <i>et al.</i> , 1996) <i>Ocimum sanctum</i> (Yanpallewar <i>et al.</i> , 2004)	<i>Leonotis leonurus</i> <i>Plectranthus ecklonii</i> <i>Plectranthus rehmanii</i> <i>Plectranthus verticillatus</i> <i>Salvia auritia</i> <i>Salvia runcinata</i> <i>Solenostemon latifolia</i> <i>Solenostemon rotundifolius</i>
Plumbaginaceae	<i>Plumbago zeylanica</i> (Tilak <i>et al.</i> , 2004)	<i>Plumbago auriculata</i>
Ranunculaceae	<i>Delphinium linearilobum</i> (Kolak <i>et al.</i> , 2006) <i>Aconitum anthora</i> (Mariani <i>et al.</i> , 2008)	<i>Clematis brachiata</i>
Rubiaceae	<i>Uncaria tomentosa</i> (Goncalves <i>et al.</i> , 2005) <i>Chassalia coriacea</i> (Aruoma <i>et al.</i> , 2003) <i>Rubia cordifolia</i> (Rawal <i>et al.</i> , 2004)	<i>Vangueria infausta</i>
Solanaceae	<i>Withania somnifera</i> (Russo <i>et al.</i> , 2001)	<i>Physalis peruviana</i>
Verbenaceae	<i>Premna corymbosa</i> (Aruoma <i>et al.</i> , 2003)	<i>Lippia javanica</i>

3.1.2. The 21 plants selected

The voucher specimens are kept at the A.P. Goosen Herbarium (PUC), North-West University, Potchefstroom.

3.1.2.1. *Acacia karroo*

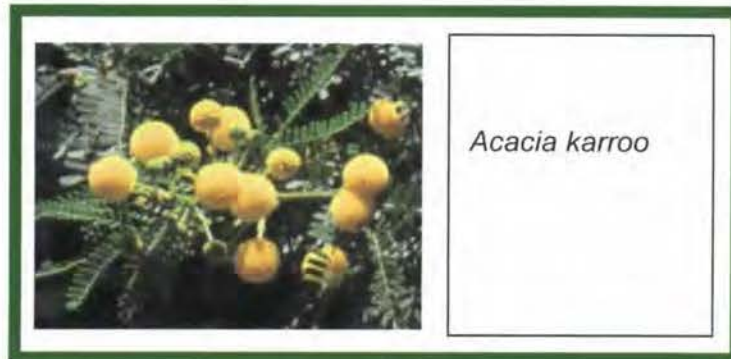


Figure 3.1: *Acacia karroo* (Aubrey and Reynolds, 2002)

Common names: Sweet thorn

Family: Fabaceae

Herbarium: PUC 8763 (Van Heerden, M.)

Traditional medicinal uses: The sweet thorn has many medicinal uses ranging from wound poultices to eye treatments and cold remedies. The bark, leaves and gum are usually used. It is also used to treat cattle which have tulp poisoning (Aubrey and Reynolds, 2002).

3.1.2.2. *Berula erecta*



Figure 3.2: *Berula erecta* (Miles, 2007)

Family: Apiaceae

Herbarium: PUC 6804 (Schoeman, L.)

Traditional medicinal uses: The plant is used externally in the treatment of rheumatism. An infusion of the whole plant can be used as a wash for swellings, rashes and athletes foot infection (Morris, 2004).

3.1.2.3. *Clematis brachiata*



Figure 3.3: *Clematis brachiata* (Viljoen, 2002)

Common name: Traveller's joy, Old man's beard, Wild clematis.

Family: Ranunculaceae

Herbarium: PUC 8765 (Manyakara, B.)

Traditional medicinal uses: The leaves are packed into the shoes to ease blisters and aches and pains and packed under saddles to prevent saddle sores on horses. Fresh leaves packed into the crown of a hat in the heat keeps the wearer cool and protected against the effects of the sun. A tea made of the leaves is not only refreshing but is used to ease headaches, coughs, colds, chest ailments and abdominal upsets. It is also a soothing wash for aching feet, soothes cracked skin and blisters and when cooled down it is used as eyewash for tired red eyes (Viljoen, 2002).

3.1.2.4. *Elephantorrhiza elephantina*

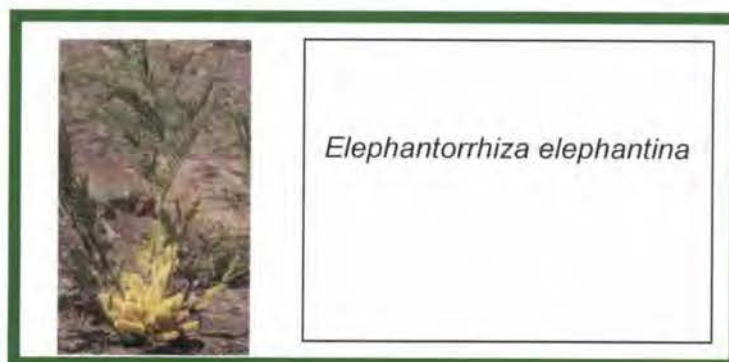


Figure 3.4: *Elephantorrhiza elephantina* (Ithaka Harbors, 2006a)

Common name: Elephant-root

Family: Fabaceae

Herbarium: PUC 7720 (Davoren, E.)

Traditional medicinal uses: The rhizome is used as a general remedy for intestinal and abdominal complaints like diarrhoea, dysentery and stomach-ache in humans and in animals and painful menstruation in humans. It is also used as a relief for heart troubles and haemorrhoids and to cure skin diseases and acne (Jansen, 2005).

3.1.2.5. *Erythrina zeyheri*



Figure 3.5: *Erythrina zeyheri* (Lithudzha et al., 2004)

Common name: Harrow-breaker, Plough-breaker

Family: Fabaceae

Herbarium: PUC 8767 (Killian, C.)

Traditional medicinal uses: In the colonial days the underground portion of this plant was smoked by asthma sufferers (Lithudzha *et al.*, 2004).

3.1.2.6. *Gymnosporia buxifolia*



Figure 3.6: *Gymnosporia buxifolia* (Hyde and Wursten, 2007)

Common name: Common spike thorn

Family: Celastraceae (Hyde and Wursten, 2007)

Herbarium: PUC 8766 (Killian, C.)

Traditional medicinal uses: the spines are used as needles and for extracting the thorns of other plants from the skin, they are said to have an irritant and toxic effect (Schmidt, 2007).

3.1.2.7. *Heteromorpha arborescens*



Figure 3.7: *Heteromorpha arborescens* (Harris, 2003)

Common name: Parsley tree

Family: Apiaceae

Herbarium: Van Wyk, A.E. 1433

Traditional medicinal uses: With an infusion of the leaves used as an enema the plant is used to treat abdominal pain and intestinal worms in children. It is also used to treat nervous and mental disorders. A decoction of the root is used to treat shortness of breath, coughs and dysentery. Headaches are also treated by inhaling the smoke from the burning plant (Harris, 2003).

3.1.2.8. *Leonotis leonurus*

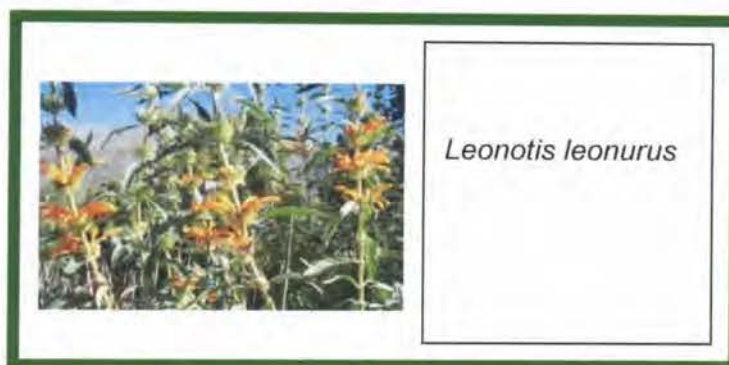


Figure 3.8: *Leonotis leonurus* (Turner, 2001)

Common name: Wild dagga, Lion's ear

Family: Lamiaceae

Herbarium: Thompson, M. 68

Traditional medicinal uses: It is widely used in traditional medicine to treat fevers, headaches, coughs, dysentery and many other conditions. It is also used as a remedy for snake bite and as charm to keep snakes away (Turner, 2001).

3.1.2.9. *Lippia javanica*



Figure 3.9: *Lippia javanica* (Le Roux, 2004)

Common name: Fever tea, Lemon bush

Family: Verbenaceae

Herbarium: De Feijter, C. 33

Traditional medical uses: It is drunk in a weak infusion as a tea and in stronger infusion for the treatment of coughs, colds, and bronchial problems in general. It is also used as a disinfectant for meat that has been infected with anthrax. This herb is also effective against fever, especially in cases of malaria, influenza and measles and as a prophylactic against lung infections. The inhalation of smoke from the herb is effective against asthma, chronic coughs and pleurisy. Skin disorders, such as heat rash and other rashes, as well as scratches, stings and bites can also be treated by this herb (Le Roux, 2004).

3.1.2.10. *Physalis peruviana*

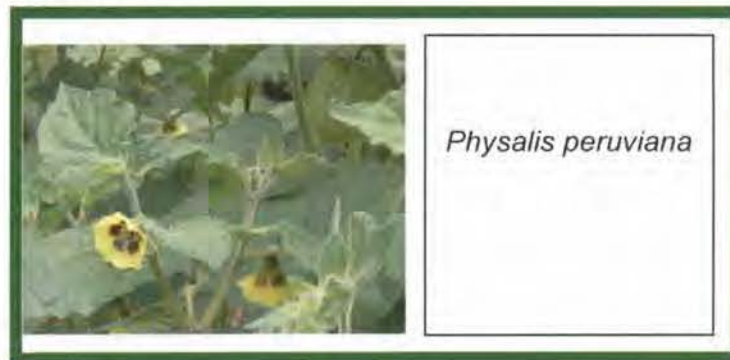


Figure 3.10: *Physalis peruviana* (Herbison-Evans and Ashe, 2007)

Common name: Cape gooseberry, Ground-cherry, Golden berry

Family: Solanaceae

Traditional medicinal uses: The leaves are used in treatment of worms and bowel complaints and as a diuretic (Plants of the future, 2006). It also prevents swelling from sprains and is an antidandruff (Long, 2005).

3.1.2.11. *Plectranthus*



Figure 3.11: *Plectranthus ecklonii* (Van Jaarsveld, 2001) (left); *Plectranthus verticillatus* (Starr and Starr, 2001) (right)

Plants: *Plectranthus ecklonii* (Van Jaarsveld, 2001), *Plectranthus rehmanii*, *Plectranthus verticillatus* (Starr and Starr, 2001).

Common name: Groot spoorsalie (*Plectranthus ecklonii*), Swedish ivy (*Plectranthus verticillatus*)

Family: Lamiaceae

Traditional medicinal uses: The leaves of *Plectranthus ecklonii* is a remedy for headaches and hay fever (Long, 2005). Other medicinal uses for *Plectranthus* include the digestive system, skin conditions, respiratory conditions infections and fever and many more (Lukhoba *et al.*, 2006).

3.1.2.12. *Plumbago auriculata*



Figure 3.12: *Plumbago auriculata* (Aubrey, 2001)

Common name: Cape leadwort

Family: Plumbaginaeae

Herbarium: PUC 9764 (Manyakara, B.)

Traditional medicinal uses: It is used to treat warts, broken bones and wounds. It is also taken as a snuff for headaches and as an emetic to dispel bad dreams (Aubrey, 2001).

3.1.2.13. *Salvia*



Figure 3.13: *Salvia runcinata* (Van Wyk *et al.*, 1988)

Plants: *Salvia auritia*, *Salvia runcinata*

Family: Lamiaceae

Traditional medicinal uses: Some tribes use it for treating bed sores, herpes lesions, stinging nettle rash and swelling due to insect or mosquito bites and wasp stings. They are all used as decoctions, teas or simple lotions (Dweck, 2008).

3.1.2.14. *Solenostemon*



Figure 3.14: *Solenostemon rotundifolius* (Bihrmann, 2007)

Plants: *Solenostemon latifolia*, *Solenostemon rotundifolius* (Bihrmann, 2007)

Common name: Hausa potato, Chinese potato, country potato (*Solenostemon rotundifolius*)

Family: Lamiaceae

Traditional medicinal uses: *Solenostemon rotundifolius* can be used as an eye treatment and for diarrhoea and dysentery (Intaka Harbors, 2006b).

3.1.2.15. *Tarchonanthus camphoratus*



Figure 3.15: *Tarchonanthus camphoratus* (Letsela *et al.*, 2002)

Common name: Camphor bush

Family: Asteraceae

Herbarium: PUC 8761 (Van Heerden, M.)

Traditional medicinal uses: It is used for blocked sinuses and headaches by inhaling the smoke from the burning green leaves. Drinking a boiled mixture of leaves and water can help treat coughing, toothache, abdominal pain and bronchitis. It is also used for massaging body stiffness (Letsela *et al.*, 2002).

3.1.2.16. *Vangueria infausta*



Figure 3.16: *Vangueria infausta* (Behr, 2004)

Common name: Wild medlar

Family: Rubiaceae

Herbarium: PUC 8762 (Van Heerden, M.)

Traditional medicinal uses: An infusion of the roots and the leaves has been used to treat malaria, chest ailments like pneumonia, as a purgative and to treat ringworms. Toothache is also relieved by an infusion of the leaves. There is also the treatment of swelling of the limb; the affected part is bathed in a decoction of the pounded leaves and small twigs, especially in children (Behr, 2004).

3.1.2.17. *Vernonia oligocephala*



Figure 3.17: *Vernonia oligocephala* (Intaka Harbors, 2006c)

Family: Asteraceae (Intaka Harbors, 2006c)

Herbarium: Bredenkamp, C. 175

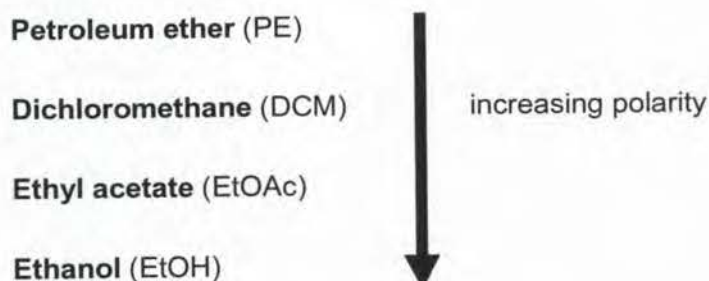
Traditional medicinal uses: The leaves and twigs can be used to treat diabetes, rheumatism and malaise (Long, 2005).

3.1.3. Collection and storage of plant material

Plants were collected from the Botanical garden at the North-West University and in the area around Potchefstroom in March 2005. The plant specimens were positively identified by Mr. Peter Mortimer, curator of the Botanical garden, North-West University Potchefstroom campus. The leaves were separated from the rest of the plant and dried for 5 days. The dried leaves were grounded to obtain smaller particle sizes, thus ensuring more efficient extraction.

3.1.4. Preparation of extracts and solvent extractions

Soxhlet extraction is one of the methods used to extract compounds from plants. If the type of compound being extracted is known, a selective solvent extraction will make the process more effective. But the usual way is to start with a non-polar solvent and exhaustively extract the macerate, followed by a series of more polar solvents, until several extracts are obtained with increasing solute polarity. These are then be tested for activity. The solvents used in this study are:



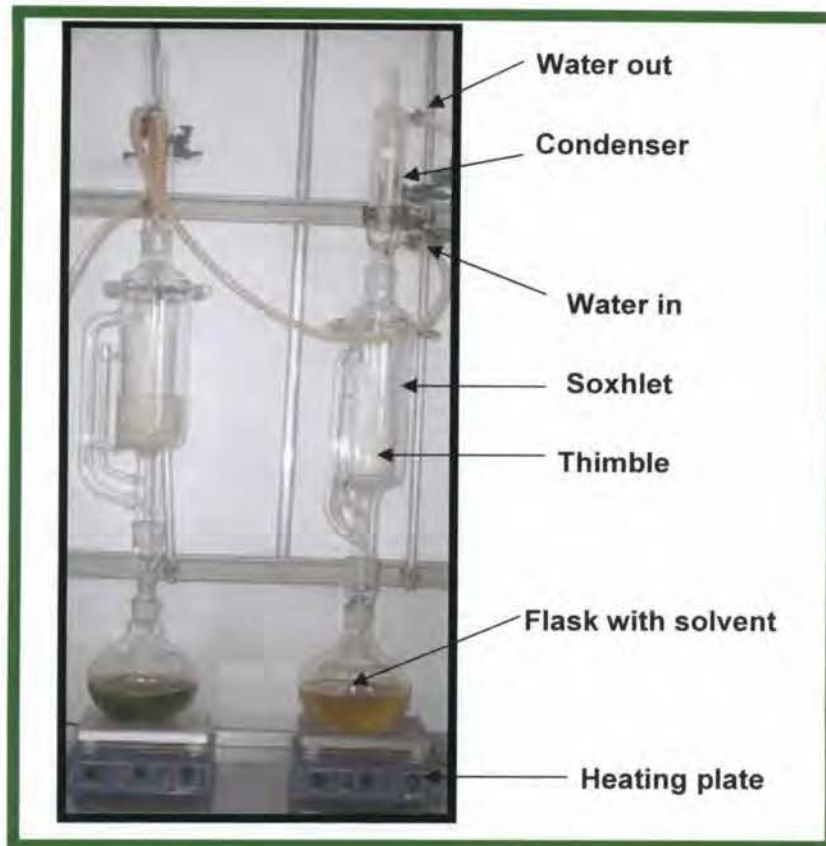


Figure 3.18: Soxhlet apparatus

After the plant material is extracted with each solvent, the extract is concentrated by using a rotary evaporator and allowed to dry completely in a fume hood (Schantz *et al.*, 1998; Marshall, 2004).

There are a couple of advantages to soxhlet extractions. One is that the sample is repeatedly brought into contact with fresh portions of the solvent. This prevents the possibility of the solvent becoming saturated with extractable material and enhances the removal of the analyte from the matrix. Another advantage is that the temperature of the system is close to boiling point of the solvent providing energy in the form of heat that helps to increase the extraction kinetics of the system. There is also no filtration required of the solvent because all the pieces of the plant stay in the thimble (Garcia-Ayuso *et al.*, 1998).

The disadvantages of soxhlet extraction include that it requires several hours or days of extraction, samples are diluted in large volumes and losses of compounds due to thermal degradation and volatilisation of the heat supplied occur (Garcia-Ayuso *et al.*, 1998).

3.1.5. Extracts obtained

Table 3.2: The percentage of extract yielded by the 21 plants.

Plant	Mass of plant material (grams)	Solvent	Mass of extract (grams)	Yield (%)
<i>Acacia karroo</i>	36.3	PE	0.4529	1.25
		DCM	0.386	1.06
		EtOAc	0.5192	1.43
		EtOH	2.0605	5.68
<i>Berula erecta</i>	3.45	PE	0.017	0.49
		DCM	0.065	1.88
		EtOAc	0.0547	1.59
		EtOH	0.0944	2.74
<i>Clematis brachiata</i>	8.4	PE	0.0786	0.94
		DCM	0.1054	1.25
		EtOAc	0.1371	1.63
		EtOH	0.8364	9.96
<i>Elephantorrhiza elephantina</i>	24.2	PE	1.0643	4.4
		DCM	0.5904	2.44
		EtOAc	0.6182	2.55
		EtOH	0.7317	3.02
<i>Erythrina zeyheri</i>	25.1	PE	0.3495	1.39
		DCM	0.348	1.39
		EtOAc	0.3734	1.49
		EtOH	0.4069	1.62
<i>Gymnosporia buxifolia</i>	28.7	PE	0.8858	3.09
		DCM	0.3747	1.31
		EtOAc	0.2034	0.71
		EtOH	0.8214	2.86
<i>Heteromorpha arborescens</i>	8.75	PE	0.1509	1.72
		DCM	0.0489	0.56
		EtOAc	0.1326	1.52
		EtOH	3.5506	40.58
<i>Leonotis leonurus</i>	11.91	PE	0.3311	2.78
		DCM	0.5884	4.94
		EtOAc	0.1007	0.85
		EtOH	0.6753	5.67

Plant	Mass of plant material (grams)	Solvent	Mass of extract (grams)	Yield (%)
<i>Lippia javanica</i>	4.17	PE	0.0425	1.02
		DCM	0.0829	1.99
		EtOAc	0.1068	2.56
		EtOH	0.3877	9.3
<i>Physalis peruviana</i>	6.69	PE	0.1303	1.95
		DCM	0.0959	1.43
		EtOAc	0.0942	1.41
		EtOH	0.2651	3.96
<i>Plectranthus ecklonii</i>	8.66	PE	0.1692	1.95
		DCM	0.2742	3.17
		EtOAc	0.1069	1.23
		EtOH	0.1731	2
<i>Plectranthus rehmanii</i>	4.83	PE	0.1817	3.76
		DCM	0.1182	2.45
		EtOAc	0.0832	1.72
		EtOH	0.387	8.01
<i>Plectranthus verticillatus</i>	2.76	PE	0.0782	2.83
		DCM	0.0436	1.58
		EtOAc	0.0284	1.03
		EtOH	0.1234	4.47
<i>Plumbago auriculata</i>	6.16	PE	0.0447	0.73
		DCM	0.0292	0.47
		EtOAc	0.0805	1.31
		EtOH	0.5472	8.88
<i>Salvia auritia</i>	2.08	PE	0.0872	4.19
		DCM	0.0429	2.06
		EtOAc	0.0226	1.09
		EtOH	0.1799	8.65
<i>Salvia runcinata</i>	7.43	PE	0.1358	1.83
		DCM	0.3462	4.66
		EtOAc	0.0975	1.31
		EtOH	0.0638	0.86
<i>Solenostemon latifolia</i>	1.16	PE	0.017	1.47
		DCM	0.0314	2.71
		EtOAc	0.02	1.72
		EtOH	0.026	2.24

Plant	Mass of plant material (grams)	Solvent	Mass of extract (grams)	Yield (%)
<i>Solenostemon rotundifolius</i>	2.64	PE	0.0721	2.73
		DCM	0.0557	2.11
		EtOAc	0.0595	2.25
		EtOH	0.2975	11.27
<i>Tarchonanthus camphoratus</i>	5.55	PE	0.206	3.71
		DCM	0.176	3.17
		EtOAc	0.0421	0.76
		EtOH	0.32	5.77
<i>Vagueria infausta</i>	53.8	PE	0.3071	0.57
		DCM	0.6821	1.27
		EtOAc	0.4194	0.78
		EtOH	0.8837	1.64
<i>Vernonia oligocephala</i>	10.12	PE	0.1911	1.89
		DCM	0.7631	7.54
		EtOAc	0.2674	2.64
		EtOH	0.468	4.62

3.2. Screening

Several methods to assess the total antioxidant capacity of various biological samples, particularly complex matrices such as plasma, serum, wine, fruits and animal tissues have been developed. It was needed due to the difficulty in measuring each antioxidant component separately and the potential interactions among different antioxidant components in complex biological samples (Cao and Prior, 1999). Two different total antioxidant tests were used in this study. The oxygen radical absorbance capacity (ORAC) assay is based on the scavenging of free radicals. In this study the peroxy radical is generated from the organic molecule AAPH (2,2'-Azobis(2-amidino-propane)dihydrochloride) and attacks a fluorescent molecule, generating an emission of fluorescence, which is monitored (Perez-Jimenez and Saura-Calixto, 2006; Ou *et al.*, 2001). The ferric reducing antioxidant power (FRAP) assay is based not on the free radical scavenging capacity but on the reducing ability of the compound (Perez-Jimenez and Saura-Calixto, 2006).

3.2.1. Ferric reducing antioxidant power (FRAP)

The FRAP assay is a direct test of “total antioxidant power”. Other tests used to date are indirect methods to test total antioxidant power that measures the ability of antioxidants in the sample to inhibit the oxidative effects of reactive species purposefully generated in the reaction mixture. The FRAP assay is simple, speedy, inexpensive and robust. It uses antioxidants as reductants in a redox-linked colorimetric method, employing an easily reduced oxidant present in stoichiometric excess. The FRAP assay does not use a lag phase type measurement like many of the indirect radical scavenger tests designed to measure antioxidant power. Sample pre-treatment in the FRAP assay is not required, stoichiometric factors are constant, linearity is maintained over a wide range, reproducibility is excellent and sensitivity is high. There is no need for highly specialised equipment or skills, or critical control of timing and reaction conditions in the FRAP assay (Benzie and Strain, 1999).

Principle: Any substance, when present at low concentrations compared to those of an oxidisable substrate, which significantly delays or prevents oxidation of a substrate, is defined as a biological antioxidant. However, unless an antioxidant prevents the generation of an oxidising species, for example, by metal chelating or enzyme-catalysed removal of a potential oxidant, a redox reaction still generally occurs, even in the presence of an antioxidant. An antioxidant reduces the oxidant because the oxidizing species reacts with the antioxidant instead of the “substrate”. Electron donating antioxidants can be described as reductants, and inactivation of antioxidants can be described as redox reactions in which one reactive species is reduced while another is oxidised. In this context, therefore “total antioxidant power” may be referred to analogously as total reducing power (Benzie and Strain, 1999).

The reduction of a ferric tripyridyltriazine complex to the ferrous form takes place at a low pH and produces a blue colour. This can be monitored by measuring the change in absorption at 593 nm. This is a non-specific reaction in that any half-reaction that has a less-positive redox potential, under reaction conditions, than that of ferric/ferrous half reaction will drive the ferric to ferrous reaction. The change in absorbance, therefore, is directly related to the combined or “total” reducing power of the electron donating antioxidants present in the reaction mixture (Benzie and Strain, 1999).

3.2.1.1. Experimental

3.2.1.1.1. Chemicals

All chemicals and reagents, including glacial acetic acid, 2,4,6-tripyridyl-s-triazine (TPTZ), vitamin C and $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ were obtained from Sigma Chemical Co. All the reagents were analytical grade.

3.2.1.1.2. Preparation of reagents

- Acetate buffer (300 mM)
(1.55 g $\text{NaAc} \cdot 3\text{H}_2\text{O}$ + 8 ml acetic acid add water to 500 ml)
- FRAP reagents
 - A 40 mM HCl (200 μ l HCl (c) in 50 ml H_2O)
 - B TPTZ (10 mM) (31.24 mg TPTZ in 10 ml HCl (40 mM))
 - C 4.56 mg $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ in 10 ml FRAP buffer
 - D FRAP reagent: 10:1:1 of A,B and C
- Vitamin C standard (1 mM)
Freshly prepared aqueous solution of a pure antioxidant, vitamin C, in the range of 0-1000 μM were used for the calibration of the FRAP assay.

(Benzie and Strain, 1996)

3.2.1.1.3. Preparation of samples

During soxhlet extraction each plant was extracted with four solvents and dried. For this experiment 10 mg of each of the four crude plant extracts from all 21 plants were used. It was dissolved in its respective solvents.

3.2.1.1.4. Reaction

This test is a colorimetric assay and therefore a transparent 96-well microtitre plate was used. The standards (vitamin C) were placed in the first row of the plate. It was used to determine the antioxidant potential of the compounds using a standard curve of increasing concentrations (0-1000 μM). Ten microlitre of each test solution was then placed in the other wells. 90 μ l H_2O and 200 μ l FRAP reagent were also placed in each well. The Bio Tek FI 600 reader was used to determine accurate readings at 593 nm and the reaction was carried out at 37 °C. All solutions were used on the day of preparation (Benzie and Strain, 1996).

3.2.1.1.5. Results

The data were analysed with Microsoft Excel according to the regression equation obtained from the calibration curve of the vitamin C standard solution. The absorbance reached at a fixed point is interpolated in a vitamin C calibration curve, and thus the results are expressed as vitamin C equivalents (Perez-Jimenez and Saura-Calixto, 2006).

Frap values of the 21 plants tested are presented in Table 3.3 and Figure 3.19.

The Frap values measured in terms of vitamin C equivalents ranged from as low as $0.000 \pm 0.000 \mu\text{M}$ for the *Acacia karroo* petroleum ether and dichloromethane phase to as high as $9009.32 \pm 130.714 \mu\text{M}$ for the *Lippia javanica* ethanol phase.

In this study the plant of interest is the one with the highest total antioxidant power. The higher the vitamin C equivalent the better the results because the higher the total antioxidant power.

Table 3.3: Frap value of the different phases of the 21 tested plants. Frap values is in terms of μM vitamin C equivalent $\pm$ standard deviation (Stdev) and samples were done in triplicate (n=3)

Compounds	μM Vitamin C equivalents $\pm$ standard deviation (Stdev) n=3			
	Petroleum ether	Dichloromethane	Ethyl acetate	Ethanol
<i>Acacia karroo</i>	0.000 $\pm$ 0.000	0.000 $\pm$ 0.000	21.0878 $\pm$ 14.215	4421.69 $\pm$ 120.524
<i>Berula erecta</i>	39.035 $\pm$ 12.720	81.9089 $\pm$ 14.730	131.762 $\pm$ 16.472	1454.99 $\pm$ 97.263
<i>Clematis brachiata</i>	138.297 $\pm$ 44.655	139.686 $\pm$ 19.832	1955.92 $\pm$ 81.664	1324.32 $\pm$ 41.236
<i>Elephantorrhiza elephantina</i>	30.100 $\pm$ 8.009	27.3258 $\pm$ 12.587	62.4674 $\pm$ 20.214	331.114 $\pm$ 31.214
<i>Erythrina zeyheri</i>	7.362 $\pm$ 12.751	490.817 $\pm$ 17.326	71.4402 $\pm$ 8.548	1057.37 $\pm$ 106.460
<i>Gymnosporia buxifolia</i>	163.419 $\pm$ 56.056	434.979 $\pm$ 69.266	435.977 $\pm$ 47.108	3310.74 $\pm$ 86.043
<i>Heteromorpha arborescens</i>	31.874 $\pm$ 34.425	48.9404 $\pm$ 18.768	719.694 $\pm$ 51.604	618.849 $\pm$ 84.159
<i>Leonotis leonurus</i>	321.483 $\pm$ 26.515	212.878 $\pm$ 16.418	712.974 $\pm$ 73.861	893.464 $\pm$ 104.585
<i>Lippia javanica</i>	238.552 $\pm$ 70.665	698.434 $\pm$ 55.046	6692.87 $\pm$ 34.128	9009.32 $\pm$ 130.714
<i>Physalis peruviana</i>	121.791 $\pm$ 17.843	112.815 $\pm$ 6.041	744.463 $\pm$ 77.848	1060.14 $\pm$ 6.917
<i>Plectranthus ecklonii</i>	97.609 $\pm$ 11.852	171.591 $\pm$ 17.459	44.01 $\pm$ 63.022	916.962 $\pm$ 55.836
<i>Plectranthus rehmanii</i>	2954.720 $\pm$ 128.136	175.644 $\pm$ 13.789	274.941 $\pm$ 30.520	3235.99 $\pm$ 314.852
<i>Plectranthus verticillatus</i>	128.064 $\pm$ 6.459	222.192 $\pm$ 4.653	1043.97 $\pm$ 87.699	1066.7 $\pm$ 14.048
<i>Plumbago auriculata</i>	28.662 $\pm$ 35.658	86.1759 $\pm$ 15.307	43.7684 $\pm$ 9.689	1982.16 $\pm$ 79.068
<i>Salvia auritia</i>	133.215 $\pm$ 3.490	1522.69 $\pm$ 69.393	189.163 $\pm$ 7.343	92.0596 $\pm$ 24.659
<i>Salvia runcinata</i>	618.640 $\pm$ 19.234	0.000 $\pm$ 0.000	652.236 $\pm$ 199.722	2387.2 $\pm$ 73.795
<i>Solenostemon latifolia</i>	3.212 $\pm$ 5.563	678.322 $\pm$ 101.583	277.528 $\pm$ 18.182	800.891 $\pm$ 62.961
<i>Solenostemon rotundifolius</i>	131.687 $\pm$ 34.425	1091.53 $\pm$ 147.856	110.998 $\pm$ 10.096	1496.74 $\pm$ 100.011
<i>Tarchonanthus camphoratus</i>	111.479 $\pm$ 39.551	1283.63 $\pm$ 45.195	861.936 $\pm$ 89.177	4384.31 $\pm$ 106.233
<i>Vagueria infausta</i>	70.790 $\pm$ 14.236	82.3502 $\pm$ 9.096	298.288 $\pm$ 38.199	583.579 $\pm$ 36.969
<i>Vernonia oligocephala</i>	64.317 $\pm$ 20.150	378.277 $\pm$ 29.925	1404.78 $\pm$ 57.753	1523.15 $\pm$ 142.306

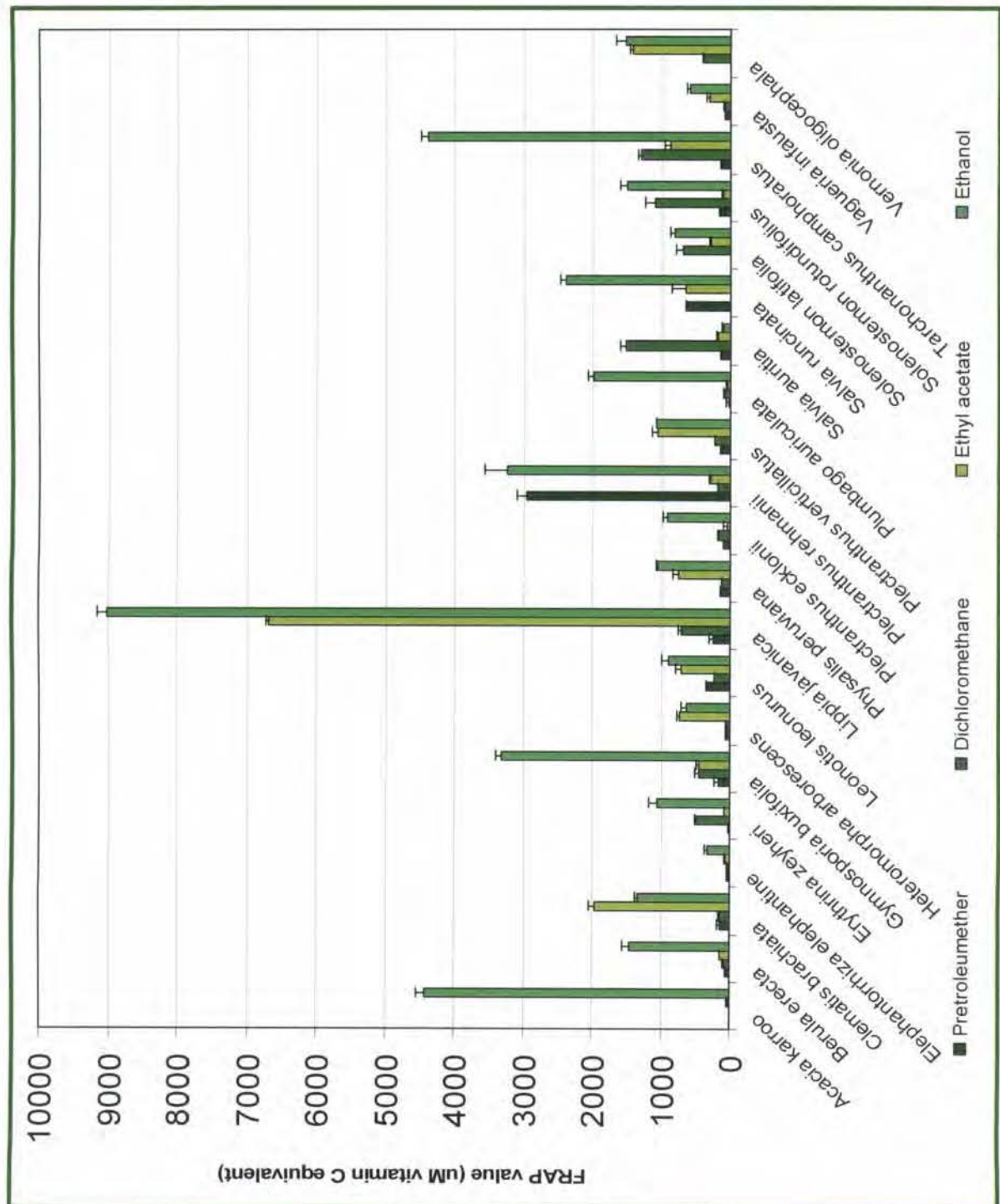


Figure 3.19: FRAP values (µM vitamin C equivalent) of the four phases of the 21 plants tested.

3.2.2. Oxygen radical absorbance capacity (ORAC)

The oxygen radical absorbance capacity (ORAC) assay is largely based on the work reported by Glazer's laboratories which depends on the unique properties of B- or R-phycoerythrin. This method of Glazer measures the decrease in fluorescence of B- or R-phycoerythrin in the presence of reactive oxygen species (peroxyl radical) and relates a lag phase or rate constant of phycoerythrin fluorescence decay to antioxidant capacity of an added antioxidant sample (Cao and Prior, 1999; Ou *et al.*; 2001). The B-phycoerythrin (B-PE) is a protein isolated from *Porphyridium cruentum* and was the chosen fluorescent probe. The major limitation of the ORAC_{PE} assay is the use of B-PE as the probe. The B-PE produces inconsistency from lot to lot, which results in variable reactivity to peroxyl radical. Another negative is that the B-PE is not photostable, and after exposure to excitation light for a certain time, it can be photobleached. The third limitation of B-PE is that it interacts with polyphenols due to the nonspecific protein binding. These disadvantages prompted the utilisation and validation of a stable fluorescent probe to replace B-PE. Fluorescein replaced the original fluorescent B-PE as fluorescent probe (Bank and Schauss, 2007; Perez-Jimenez and Saura-Calixto, 2006; Ou *et al.*, 2001). The fluorescein also allows the direct measurement of hydrophilic chain-breaking antioxidants capacity against peroxyl radicals (Aruoma, 2003; Ou *et al.*, 2001).

The ORAC assay is to date the only method that take reactive species reactions to completion and uses an "area under curve" technique for quantitation, thus combining both inhibition time and inhibition percentage of the reactive species action by antioxidant into a single quantity (Cao and Prior, 1999; Perez-Jimenez and Saura-Calixto, 2006; Ou *et al.*, 2001).

Principle: The ORAC assay depends on the detection of chemical damage to B- or R-phycoerythrin through the decrease in its fluorescence emission. B- and R-phycoerythrin fluorescence is highly sensitive to the conformation and chemical integrity of the protein. The loss of fluorescence under appropriate conditions in the presence of reactive oxygen species is an index of oxidative damage of the protein. The protection against the loss of phycoerythrin fluorescence in the ORAC assay is a reflection of the inhibition of the reactive species action by the antioxidant and is a measure of its antioxidant capacity against the reactive species (Cao and Prior, 1999).

In measuring the inhibition of reactive species action by an added antioxidant sample, two elements need to be considered: the time that the inhibition lasts and the percentage that the inhibition displays at different times. A lag phase can be produced when a specific

antioxidant may display 100 % inhibition of a specific reactive species action over a period of time. This lag phase is however not a general condition with all antioxidants. Some antioxidants, such as reduced glutathione and melatonin, do not show lag phase in inhibiting the oxidation of phycoerythrin by peroxy radicals. In addition, the loss of phycoerythrin fluorescence in the presence of reactive species, which includes peroxy radical generation from 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH), does not follow zero-order kinetics. Any methods based on the assumption of zero-order kinetics will inevitably have technical difficulties in measuring the lag phase in the loss of phycoerythrin fluorescence. Another aspect to consider is the percentage inhibition observed at a specific time point to the antioxidant capacity of an antioxidant which is not ideal. Two compounds having the same inhibition percentage at one time point may exhibit different inhibition percentage at another time point. Because of all of this the area under curve technique was developed for quantitation of the results in the ORAC assay. The ORAC assay successfully overcomes all related problems in quantitation of the antioxidant capacity of a biological sample by integrating inhibition percentage over the whole inhibition period (Cao and Prior, 1999).

3.2.2.1. Experimental

3.2.2.1.1. Chemicals

Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) and fluorescein were purchased from Sigma Chemical Co. All chemicals were of analytical grade.

3.2.2.1.2. Preparation of reagents

- Phosphate buffer, 75 mM, pH 7.4.
(Mix 1 M KH_2PO_4 and NaH_2PO_4 (61.6:38.9) and dilute to 75 mM – check pH)
kept at 4 °C
- Fluorescein.
A main stock solution of 265 mM (10% w/v in water, pH 7.4) of Fluorescein (disodium salt) was made. Before each assay dilutions was made in phosphate buffer as follows:
1 μl (265 mM) + 999 μl buffer D1 (265 mM)
(For 50 reactions) 2 μl D1 + 9998 μl buffer D2 (56 nM)
kept at 4 °C
- 2,2'-Azobis(2-amidino-propane) dihydrochloride (AAPH).
240 mM (86.8 mg/ml in buffer) prepare fresh before use, keep on ice.

- Trolox.

500 μ M (122.5 μ g/ml in buffer) aliquoted in vials and kept at -70 °C for 4 months. Prepare from newly thawed vial each day.

3.2.2.1.3. Preparation of samples

10 mg of each extract of the plant extract was used for this experiment. It was dissolved in its respective solvents and extracted with 2 x 1 ml hexane followed by centrifugation and removal of the hexane layer. The hexane fractions were combined. Residual hexane was evaporated, and the residue was extracted with 1 ml of acetone/water/acetic acid (70:29.5:0.5, v/v/v). After adding solvent the tube was vortexed for 30 s, followed by sonication at 37 °C for 5 minutes. The tube was inverted once in the middle of the sonication step to suspend the sample. Then the tube remained at room temperature for 10 minutes with occasional shaking. The tube was centrifuged at 2200 x g for 15 minutes. The supernatant was removed and solvent was stored in a freezer (Prior *et al.*, 2003).

3.2.2.1.4. Reaction

The Bio Tek FI 600 plate reader was used in this experiment. The standards (Trolox) were placed in the first row of the plate. The samples were diluted 500 times with buffer and 20 μ l of the samples were placed, in triplicate, into the wells. 80 μ l of fluorescein solution (D2) were added to each well. The plate was then pre incubated at 37 °C to check the sensitivity. The reaction was then started by adding 100 μ l AAPH to each well. The reader was initiated and left to read every 5 minutes (excitation 485 nm, emission 520 nm) for 3 hours (Thaipong *et al.*, 2006; Silva *et al.*, 2007; Cao and Prior, 1999).

3.2.2.1.5. Results

The results were calculated as follows using Excel:

$$S = (0.5 + f_5/f_0 + f_{10}/f_0 + f_{15}/f_0 + \dots f_{65}/f_0 + f_{70}/f_0) \times 5$$

(where f_0 is initial fluorescence and f_i is fluorescence value at time i)

Standards were used to draw a graph with a 2nd order polynomial slope ($y = ax^2 + bx + c$). The equation is shown on graph and a , b , c and y (S) is determined. The ORAC value (μ M) is then calculated. $X = (-b + \sqrt{(b^2 - 4(c-y)a})/2a) \times \text{dilution factor}$

(Cao and Prior, 1999; 2006; Ou *et al.*, 2001; Prior *et al.*, 2003)

The area under curve is measured and is interpolated in a Trolox curve and the results are expressed as Trolox equivalents (Perez-Jimenez and Saura-Calixto, 2006; Cao and Prior, 1999; Chanjirakul *et al.*, 2006; Silva *et al.*, 2007; Ou *et al.*, 2001; Prior *et al.*, 2003).

The relative ORAC values obtained in this assay are presented in Table 3.4 and Figure 3.20.

The total ORAC values measured in terms of Trolox equivalent ranges from as low as $-1491.8 \pm 227 \mu\text{M}$ for *Solenostemon rotundifolius* petroleum ether phase to as high as 75908.1 ± 1336 for *Lippia javanica* ethyl acetate phase.

In this study the plant of interest is the one with the highest oxygen radical absorbance capacity. The higher the trolox equivalent the better the results because the higher the antioxidant ability to scavenge free radicals.

Table 3.4: ORAC value of the different phases of the 21 tested plants. ORAC values is in terms of μM trolox equivalent $\pm$ standard deviation (Stdev) and samples were done in triplicate (n=3)

Compounds	μM trolox equivalent per litre (n= 3) + stdev			
	Petroleum ether	Dichloromethane	Ethyl acetate	Ethanol
<i>Acacia karroo</i>	4732.4 $\pm$ 228	3837.9 $\pm$ 378	4753.9 $\pm$ 686	23382.7 $\pm$ 198
<i>Berula erecta</i>	4371.2 $\pm$ 474	6804.4 $\pm$ 862	8404.8 $\pm$ 1677	20768.6 $\pm$ 798
<i>Clematis brachiata</i>	7814.5 $\pm$ 996	12276.4 $\pm$ 2120	27462.3 $\pm$ 1037	19368.8 $\pm$ 1561
<i>Elephantorrhiza elephantina</i>	11388.7 $\pm$ 2753	9923.4 $\pm$ 1452	10413.5 $\pm$ 570	11111.1 $\pm$ 419
<i>Erythrina zeyheri</i>	5729.4 $\pm$ 602	26486.6 $\pm$ 737	11144.7 $\pm$ 2378	67362.9 $\pm$ 8936
<i>Gymnosporia buxifolia</i>	11045.2 $\pm$ 1911	21091.8 $\pm$ 969	26974.7 $\pm$ 898	64318.8 $\pm$ 355
<i>Heteromorpha arborescens</i>	4745.8 $\pm$ 833	6433.8 $\pm$ 914	13246.7 $\pm$ 975	11698.7 $\pm$ 94
<i>Leonotis leonurus</i>	4480.4 $\pm$ 20	9504.5 $\pm$ 3085	13742.8 $\pm$ 2229	13750.6 $\pm$ 173
<i>Lippia javanica</i>	14189.2 $\pm$ 671	49147.8 $\pm$ 14472	75908.1 $\pm$ 11336	NUM ^(a)
<i>Physalis peruviana</i>	3048.3 $\pm$ 341	2208.6 $\pm$ 3520	13271.2 $\pm$ 160	14423.5 $\pm$ 125
<i>Plectranthus ecklonii</i>	5003.9 $\pm$ 194	7079.8 $\pm$ 717	9818.1 $\pm$ 773	11863.1 $\pm$ 1124
<i>Plectranthus rehmanii</i>	15534.1 $\pm$ 169	7302 $\pm$ 1541	8293.7 $\pm$ 834	15288.5 $\pm$ 1559
<i>Plectranthus verticillatus</i>	8268.1 $\pm$ 6613	13539.5 $\pm$ 4326	13068.3 $\pm$ 1943	8438.7 $\pm$ 177
<i>Plumbago auriculata</i>	3185.3 $\pm$ 1703	6801.9 $\pm$ 2105	5406.8 $\pm$ 1509	35586.7 $\pm$ 5580
<i>Salvia auritia</i>	-442.3 $\pm$ 111	8834.7 $\pm$ 1225	1757.6 $\pm$ 291	5902.1 $\pm$ 459
<i>Salvia runcinata</i>	31944.5 $\pm$ 1188	14914.9 $\pm$ 12599	12415.5 $\pm$ 528	28229.6 $\pm$ 4153
<i>Solenostemon latifolia</i>	5352.4 $\pm$ 427	9853.7 $\pm$ 1159	7014.9 $\pm$ 405	10141.4 $\pm$ 38
<i>Solenostemon rotundifolius</i>	-1491.8 $\pm$ 227	-444.8 $\pm$ 72	4305.5 $\pm$ 330	14542.5 $\pm$ 731
<i>Tarchonanthus camphoratus</i>	6285.4 $\pm$ 1015	25822.6 $\pm$ 2552	18347.8 $\pm$ 2834	32077 $\pm$ 2026
<i>Vageria infausta</i>	6614.9 $\pm$ 1542	10469.2 $\pm$ 2002	11581.2 $\pm$ 699	13629.4 $\pm$ 2044
<i>Vernonia oligocephala</i>	6513.6 $\pm$ 1222	19838.9 $\pm$ 2826	24994 $\pm$ 1232	19601.1 $\pm$ 1056

(a) NUM means it was too high for the machine used to calculate a result.

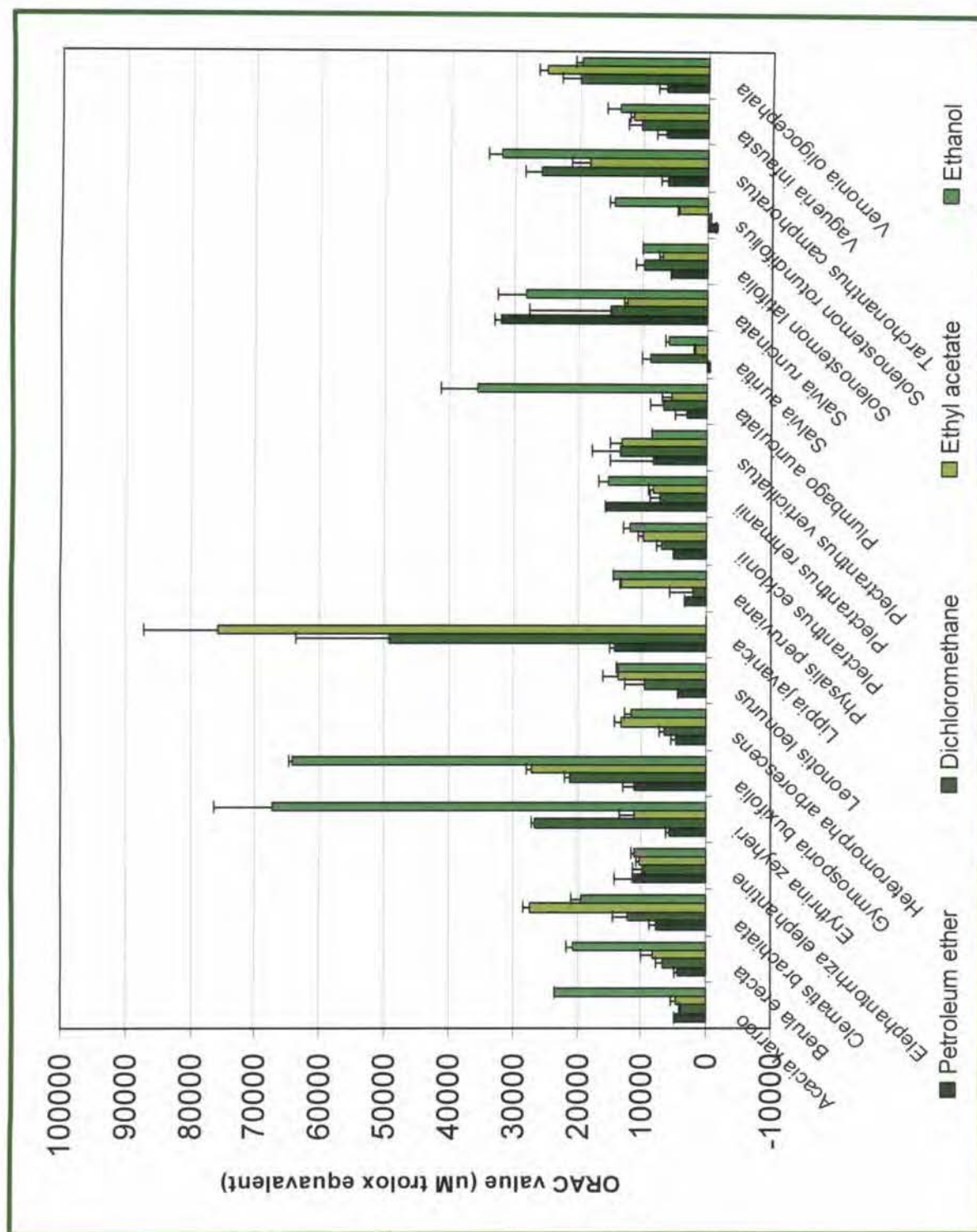


Figure 3.20: ORAC values (μM Trolox equivalent) of the four phases of the 21 plants tested.

CHAPTER 4: *IN VITRO* BIOLOGICAL TESTS

Gymnosporia buxifolia was chosen as the plant with most promise and availability after the result of the ORAC and FRAP tests, were it showed the ability (chemical test) to directly act as an antioxidant by reducing peroxy radicals. It was then decided to test the radical scavenging ability of this promising extracts of *Gymnosporia buxifolia* by doing biological tests.

4.1. *Gymnosporia buxifolia*

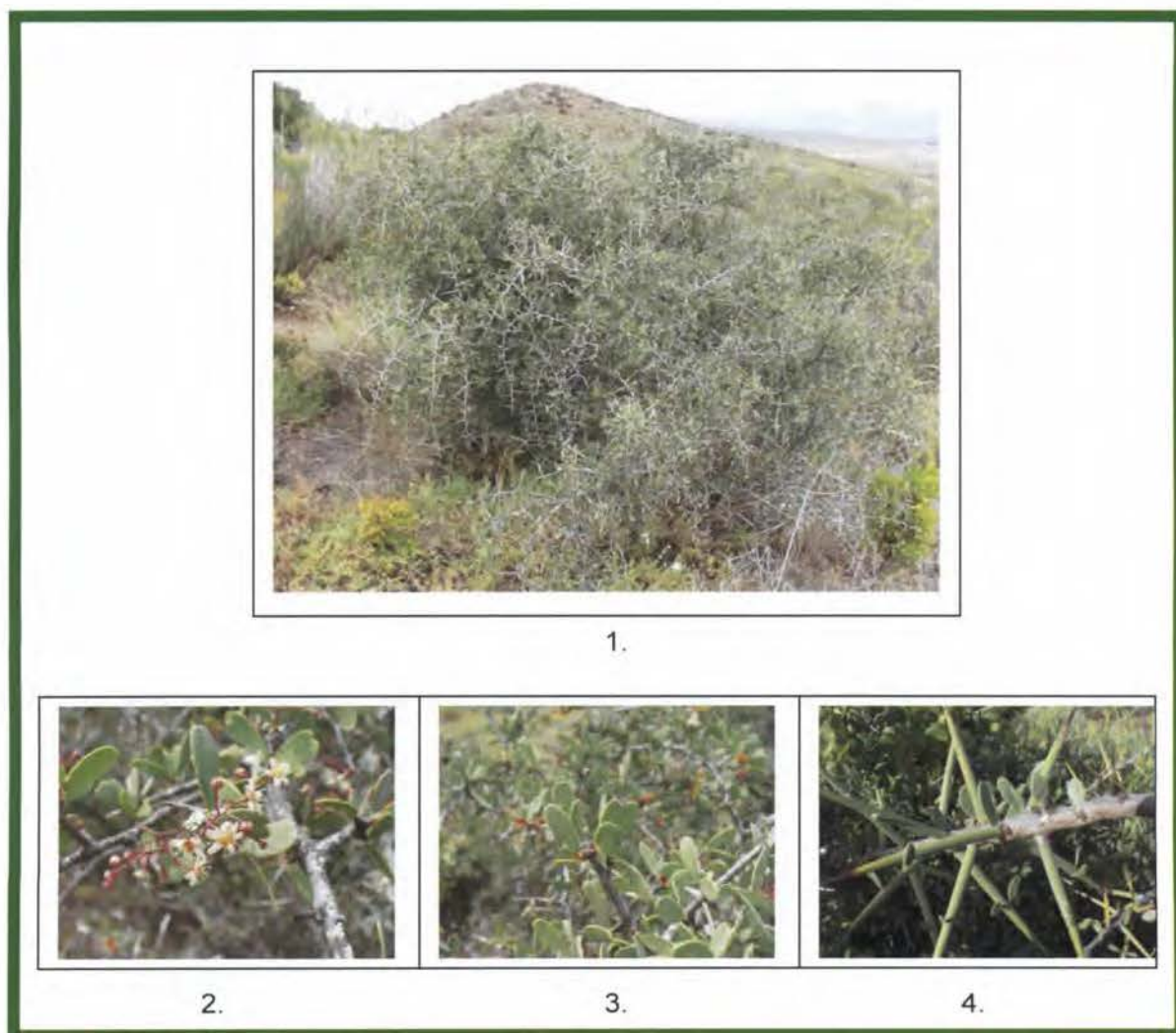


Figure 4.1: *Gymnosporia buxifolia* plant (1), flower (2), leaves (3), thorns (4) (Seiler, 2003).

Authority: (L) Szyszyl

Family: Celastraceae (Namibian Biodiversity database, 2003)

Synonyms: *Maytenus heterophylla* (Hyde and Wursten, 2007)

Common name: Common spike thorn (Namibian Biodiversity database, 2003)

Description: A very spiny, evergreen shrub or small tree with terminal branches drooping (Namibian Biodiversity database, 2003; Hyde and Wursten, 2007).

Spines up to 100 mm long and straight, they rarely carry leaf clusters (Namibian Biodiversity database, 2003; Hyde and Wursten, 2007).

Leaves are clustered on branch tips, 25-80 mm long, smooth, papery to parchment-like with a short petiole (up to 5 mm long). It has a dull green colour, midrib sunken above, margin irregularly toothed and the apex rounded to slightly notched (Namibian Biodiversity database, 2003).

Flowers are small, white to cream. Flowers are produced in profusion and are thus conspicuous. The flower is said to have an unpleasant smell (Namibian Biodiversity database, 2003; Hyde and Wursten, 2007).

The fruit has a rough-textured capsule which usually splits into three parts; it is 2-5 mm in diameter. The fruit seeds have a yellow aril. Fruits may be overlooked when green (Namibian Biodiversity database, 2003).

Habitat: Various habitats, but mainly on plains and hill slopes. It is also often a pioneer in disturbed places (Namibian Biodiversity database, 2003; Hyde and Wursten, 2007).

Growth form: A shrub up to 3 m high, occasionally a tree of over 3 m (Namibian Biodiversity database, 2003).

Annual cycle: Although flowering and fruit records are sparse flowering mainly take place in March and April, with single records from August to February. The fruiting takes place in January and February, but with single records intermittently in other months. The leaves are evergreen (Namibian Biodiversity database, 2003; Hyde and Wursten, 2007).

Traditional medicinal uses: The spines of this plant are used as needles and for extracting thorns of other plants from the skin. It is said to have an irritant and toxic effect (Schmidt, 2007)

Previous biological testing on *Gymnosporia buxifolia*: The ethanol extract of the leaves showed antimicrobial activity and the compounds isolated in that study include dihydroagarofuran alkaloid (1), maytenfolic acid, 3 α -hydroxy-2-oxofriedelane-20 α -carboxylic acid (2) and lup-20(29)-ene-1 β ,3 β -diol (Orabi *et al.*, 2001). Other compounds that have been previously isolated are β -amyrin, (-)-4'-methylepigallocatechin, (-)-epicatechin and dulcitol (Bosch, 2004).

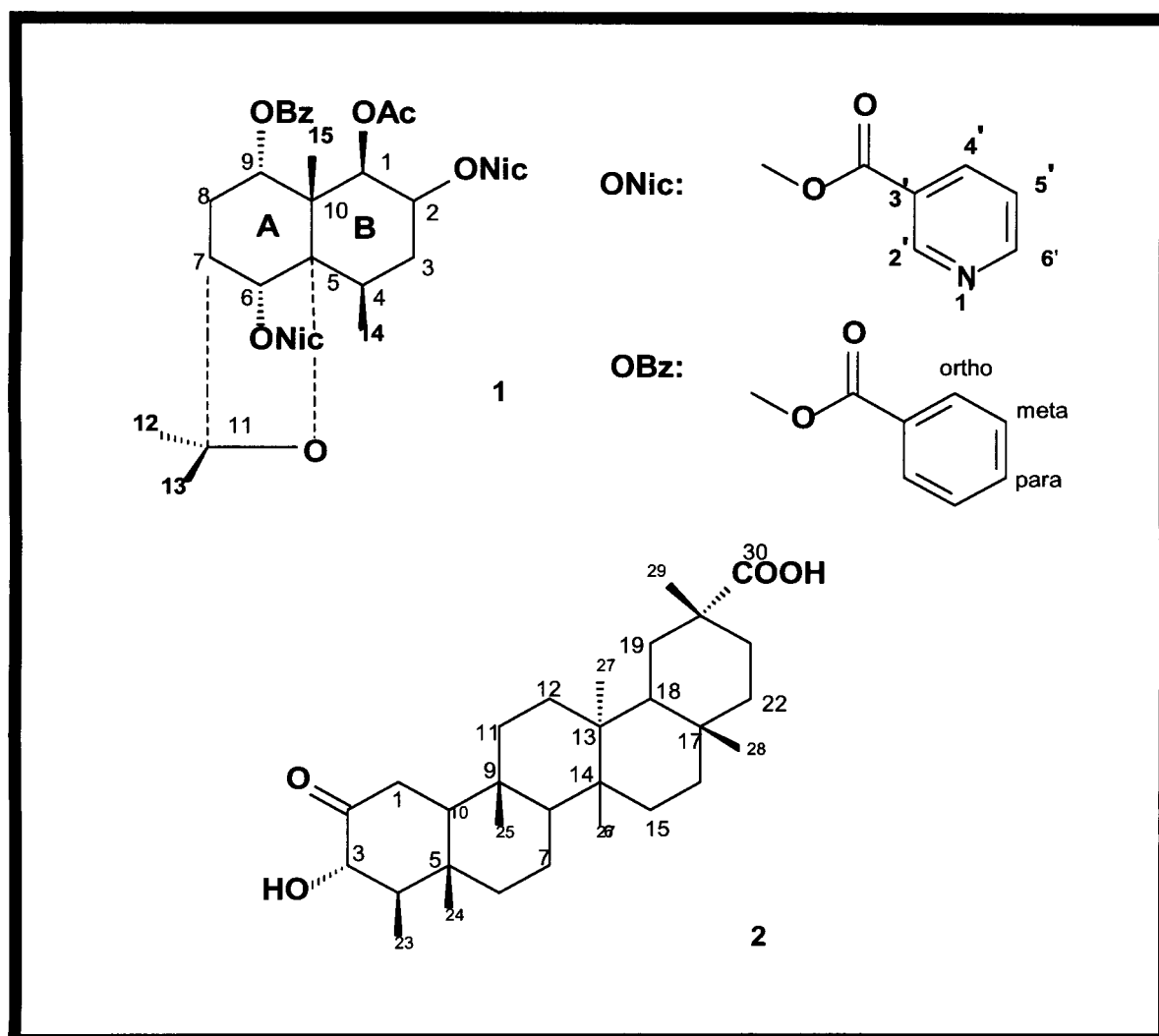


Figure 4.2: Compounds isolated from *Gymnosporia buxifolia* during antimicrobial studies (Orabi *et al.*, 2001).

4.2. In vitro biological tests

Two *in vitro* tests that work on different mechanisms were done on the crude extract from *Gymnosporia buxifolia*. First, the nitroblue tetrazolium (NBT) assay, which determines

superoxide scavenging by the reduction of NBT was used (Sabu and Kuttan, 2003). The second test is lipid peroxidation; this test evaluates the oxidative stress by measuring malondialdehyde (MDA), the last product of lipid breakdown caused by oxidative stress (Kiruthiga *et al.*, 2007). Both tests utilise colour to determine the results.

4.2.1. Nitroblue tetrazolium assay

This is a simple reliable technique used for assaying superoxide and other free radicals. The principle of this assay is based on the ability of free radicals to reduce nitroblue tetrazolium (NBT) to the insoluble nitroblue diformazan (NBD). When the superoxide reacts with NBT it produces a colour change from light yellow to dark purple. This coloured product (NBD) can be extracted with glacial acetic acid and is measured by spectrophotometer at 560 nm (Ottino and Duncan, 1997a; Ottino and Duncan, 1997b; Hanson *et al.*, 2006).

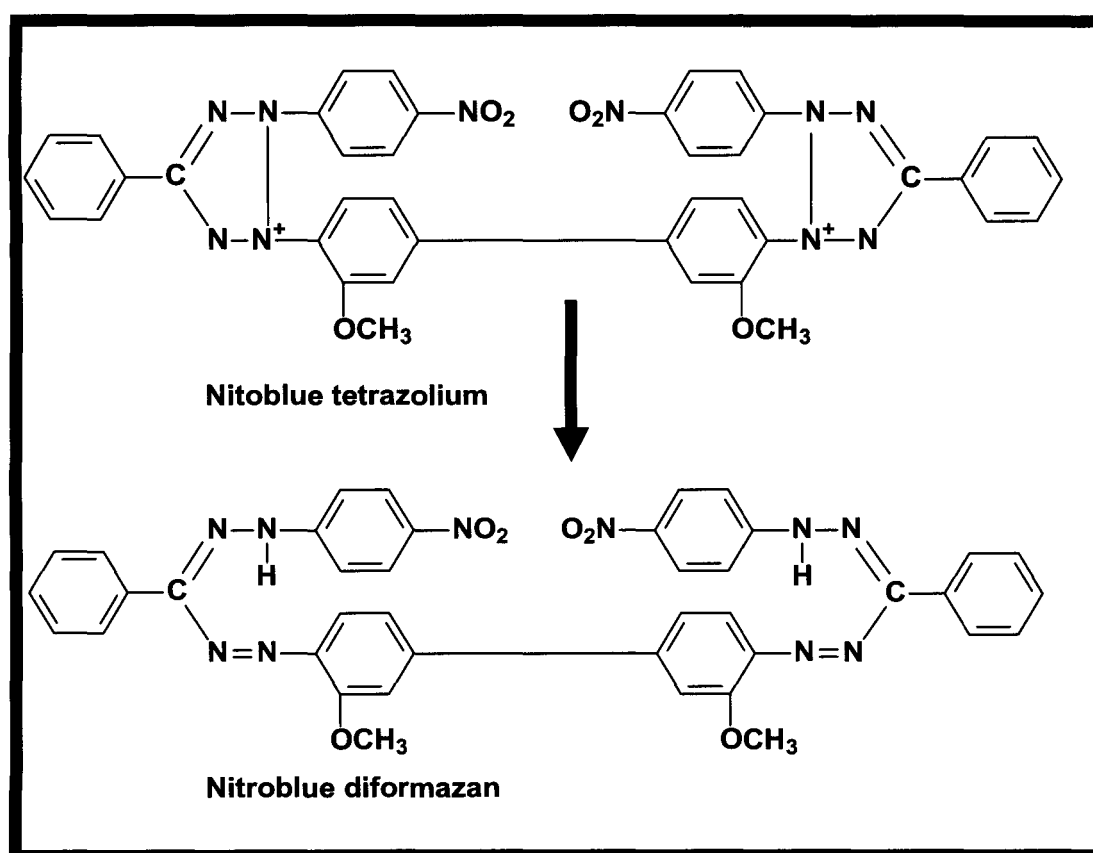


Figure 4.3: The nitroblue tetrazolium reduces to nitroblue diformazan (Stain files, 2005).

Any factor which would enhance oxidative species generation by dopamine would likely hasten the onset of cell injury and subsequent death. The well known mitochondrial poison, cyanide, increases dopamine-induced formation of intracellular oxidants (Jones *et al.*, 2003). Potassium cyanide was selected as the toxin in this assay, for its neurotoxic effect.

4.2.1.1. Experimental

4.2.1.1.1. Chemicals

Bovine serum albumin (BSA), nitroblue tetrazolium (NBT), nitroblue diformazan (NBD) and chemicals used in the protein determination: Folin & Ciocalteu's reagents, were purchased from Sigma-Aldrich chemie GmbH, Steinheim, Germany. Glacial acetic acid and potassium cyanide (KCN) were purchased from Saarchem, Wadeville, Gauteng, South Africa. All other chemicals and reagents were obtained locally and were of the highest available purity.

4.2.1.1.2. Animals

Male Sprague-dawley rats weighing 200-250 g were used for the experiment. The animals were housed in a windowless, well-ventilated constant environment room under a diurnal lighting cycle: 12 hours light; 12 hours darkness. Artificial lighting was provided by standard cool-white fluorescent bulbs (75 W). Ambient temperature of the animal room was maintained at $21\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$ and the humidity is kept at 55%. Cage cleaning and feeding is done ad libitum and is only done in the photo phase, to avoid induction of secondary exogenous rhythms. The protocol for the experiment was approved by the North-West University Animal Ethics Committee.

4.2.1.1.3. Reagents

- Phosphate Buffer solution (PBS)

Take 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4 and 0.24 g KH_2PO_4 and dissolve in 800 ml of water. Adjust pH to 7.4. When pH is correct add water till 1 l. Store in fridge.

- Nitroblue tetrazolium (NBT)

Dissolve 0.002 g of NBT in 0.4 ml ethanol and add 19.6 ml water. NBT is light sensitive and should be made up fresh every day of use.

- Potassium cyanide.

Make a 4 mM KCN by dissolving 0.0026 g of KCN in 100 ml of Milli-Q water.

- Copper reagent.

Take 1 ml of a 1% CuSO_4 (1 g in 100 ml water), 1 ml of a 2 % Na tartrate (2 g in 100 ml water) and 98 ml of 2% Disodium carbonate (2 g in 100 ml 0,1 M NaOH).

4.2.1.1.4. Sample preparation

Concentrations of crude extract used were 5 mg, 2.5 mg and 1.25 mg crude extract in 0.25 ml solution. Each of the four crude extracts of *Gymnosporia buxifolia* was used at all three concentrations in this assay. The crude plant extract was dissolved in 40 % ethanol.

4.2.1.1.5. Preparations of standards

4.2.1.1.5.1. Bovine Serum Albumin (BSA) standard curve

An estimate of the protein content of each brain was determined prior to the NBT assay in order to express the scavenging of superoxide anions in terms of μmoles diformazan/mg protein.

BSA in increasing concentrations was used as a standard for determining the protein content of each brain. Protein standards containing 0-300 $\mu\text{g/ml}$ of BSA at intervals of 60 $\mu\text{g/ml}$ were used to generate a standard curve.

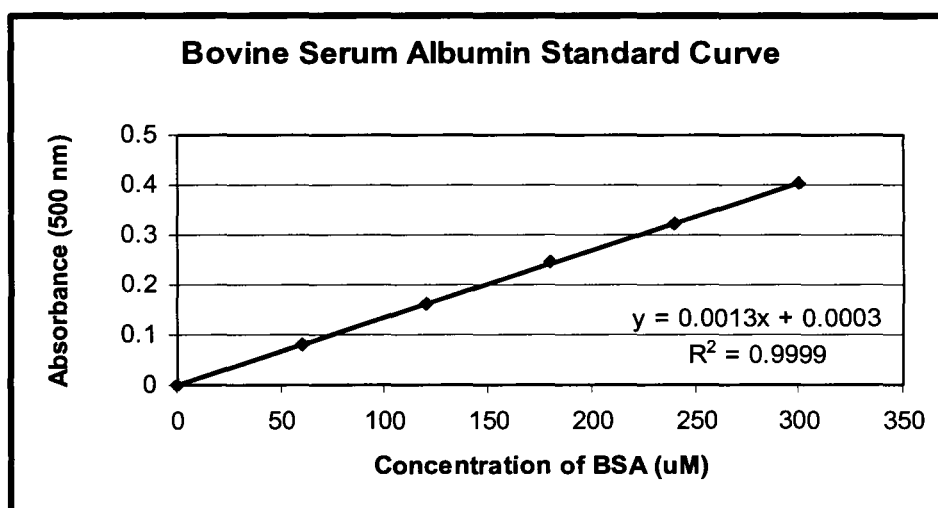


Figure 4.4: Protein standard curve generated from bovine serum albumin.

4.2.1.1.5.2. Nitroblue diformazan (NBD) standard curve

NBD was used as a standard measuring the level of induced superoxide anions with the NBT assay. A series of reaction tubes, each containing appropriate aliquots of NBD dissolved in glacial acetic acid was prepared. A calibration curve was generated by measuring the absorbance at 560 nm in 100 μM increments of concentration. The absorbance was read at 560 nm using an ultraviolet visible spectrophotometer.

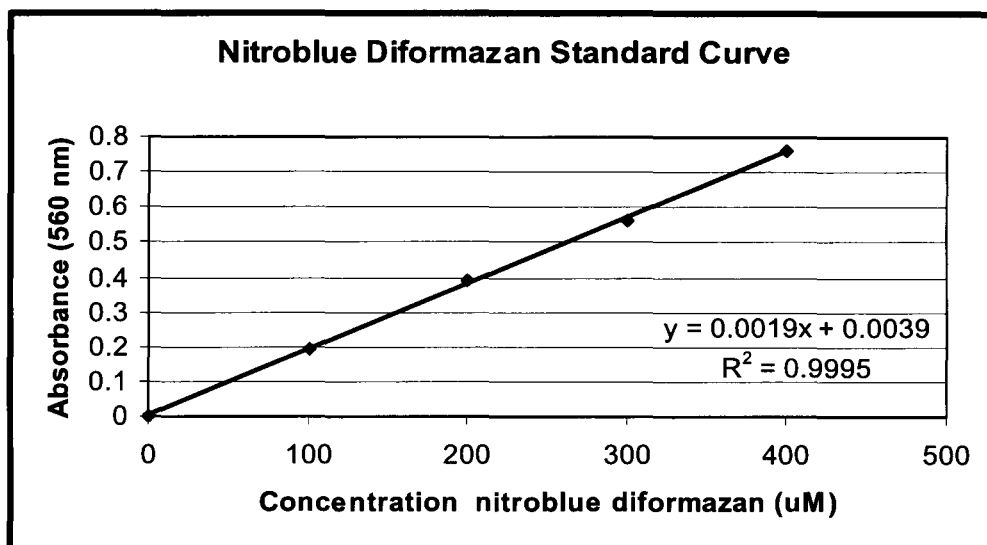


Figure 4.5: Nitroblue diformazan standard curve

4.2.1.1.6. Method

On the morning of the experiment the rats were sacrificed by rapid decapitation and the whole brain was removed. An incision was made through the bone on either side of the parietal suture from the foramen magnum to near the orbit. The calvarium was lifted and removed by using the forceps. All adhering tissue and visible blood was removed by washing the tissue in PBS buffer. The whole brain was homogenised in 0.1 M phosphate buffered saline (PBS), pH 7.4, so as to give a final concentration of 10 % (w/v). A glass Teflon homogenizer was used. This was done to prevent lysosomal damage of the tissue. The PBS buffer was used as it has been shown not to scavenge free radicals.

Potassium cyanide (KCN) was used as the toxin to provide neurotoxic effects. In preliminary studies to determine the effective concentrations, varying concentrations of KCN (0; 0.25; 0.5; 1 mM) was used.

The NBT assay was performed by using 0.5 ml samples of brain homogenate, 0.25 ml KCN (1 mM), 0.25 ml of the relevant crude extract being tested (1.25; 2.5; 5 mg/ml) and 0.4 ml 0.1% NBT solution and incubating it at 37 °C for 1 hour in a oscillating water bath. Termination of the assay and extraction of reduced NBT was carried out by centrifugation of the suspensions at 2000 x g for 10 minutes. The supernatant was decanted and the pellet was resuspended with 2 ml glacial acetic acid, into which the reduced NBT dye was extracted. The absorbance of the glacial acetic acid fraction was measured at 560 nm.

The protein estimation for each brain was performed prior to the NBT assay. 0.1 ml of homogenate was added to 4.9 ml PBS. 1 ml of this mixture was added to 6 ml alkaline copper reagent solution, it was vortexed and left to stand at room temperature for 10 minutes. 0.3 ml Folin & Ciocalteu's reagent was then added to the tubes and vortexed and then left to stand in the dark at room temperature for 30 minutes. The absorbance was then measured at 500 nm.

The statistical analysis was done using the Student-Newman-Keuls multiple range tests.

4.2.1.1.7. Results

Absorbance values of the protein assay were converted to mg protein, using the calibration curve generated from increasing concentrations of bovine serum albumine (figure 4.4). The values were used in expressing the superoxide anion scavenging results.

Absorbance values of each NBT assay was converted to μ moles diformazan produced using the standard curve generated from increasing concentrations of NBD (figure 4.5). Final results are expressed as μ moles diformazan/mg protein.

Table 4.1: The *in vitro* effects of selected extracts on KCN-induced superoxide anion formation in rat brain homogenate.

Test compounds	Concentration mg/ml	Diformazan (μ M/mg protein)	$\pm$ Stdev
Control		54.056	$\pm$ 5.075
KCN	1 mM	88.791	$\pm$ 6.34
Petroleum ether extract	1.25 mg/ml	51.061	$\pm$ 5.99
	2.5 mg/ml	52.374	$\pm$ 6.96
	5 mg/ml	35.965	$\pm$ 14.729
Dichloromethane extract	1.25 mg/ml	69.45	$\pm$ 11.264
	2.5 mg/ml	66.532	$\pm$ 4.154
	5 mg/ml	61.823	$\pm$ 10.81
Ethyl acetate extract	1.25 mg/ml	52.212	$\pm$ 6.045
	2.5 mg/ml	28.453	$\pm$ 6.399
	5 mg/ml	36.662	$\pm$ 3.394
Ethanol extract	1.25 mg/ml	54.346	$\pm$ 2.316
	2.5 mg/ml	44.477	$\pm$ 4.271
	5 mg/ml	24.273	$\pm$ 5.29

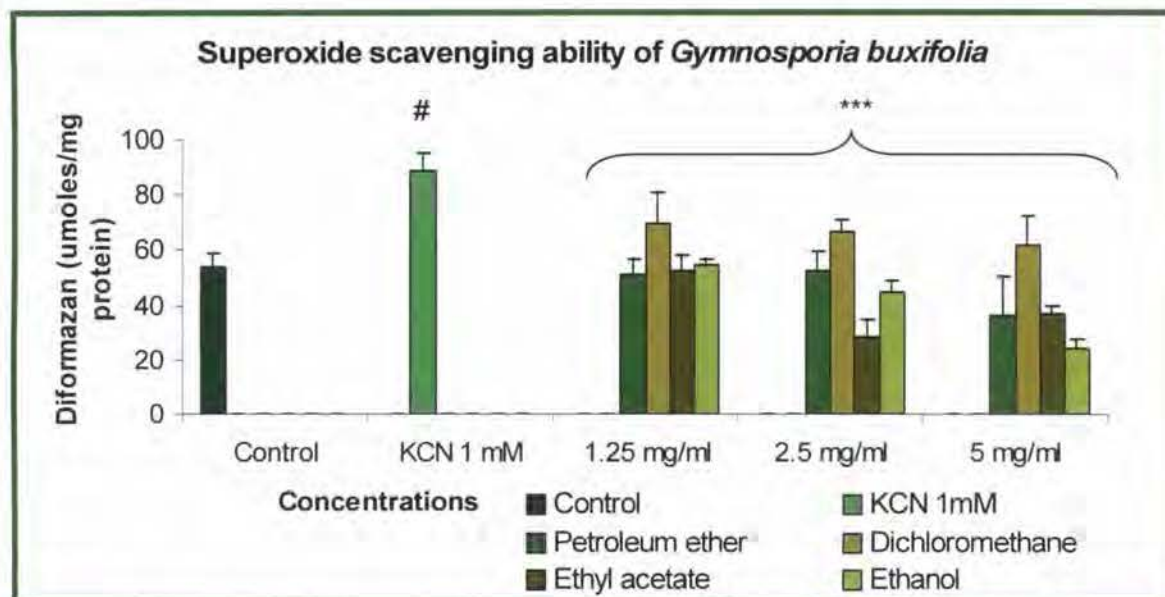


Figure 4.6: The superoxide scavenging properties of increasing concentrations of plant extract in the presence of 1 mM KCN in rat brain homogenate. Each bar represents the mean $\pm$ Stdev. $n=10$ # $p<0.001$ vs. Control; *** $p<0.001$ vs. 1 mM KCN. The Student-Newman-Keuls multiple range test was used.

The *in vitro* exposure of the whole rat brain homogenate showed that KCN caused a significant concentration dependent rise in superoxide anion generation. As can be seen from the results, all the crude plant extracts of *Gymnosporia buxifolia* were able to significantly reduce superoxide generation compared to KCN. The ethanol extract showed the most promise in reducing the superoxide anions at 5 mg/ml but the ethyl acetate showed better reduction at lower concentrations than the ethanol extract.

4.2.2. Lipid peroxidation

Lipid peroxidation was measured using the thiobarbituric acid (TBA) assay, utilising the modified methods of Ottino & Duncan (1997a; 1997b) and Nandita & Rajini (2004). This assay is one of the most widely used methods for determination of lipid peroxidation in biological samples. The principle of this assay is based on the reaction of malondialdehyde (MDA) equivalents with TBA to form a pink coloured complex which can be extracted with butanol and read at 532 nm. MDA, produced by the oxidation of polyunsaturated fatty acids, reacts with two molecules of TBA yielding a pinkish red chromogen with an absorbance maximum at 532 nm which was measured (Dasgupta and De, 2006; Manhanom *et al.*, 2007; Nandita and Rajini, 2004; Ottino and Duncan, 1997a; Ottino and Duncan, 1997b).

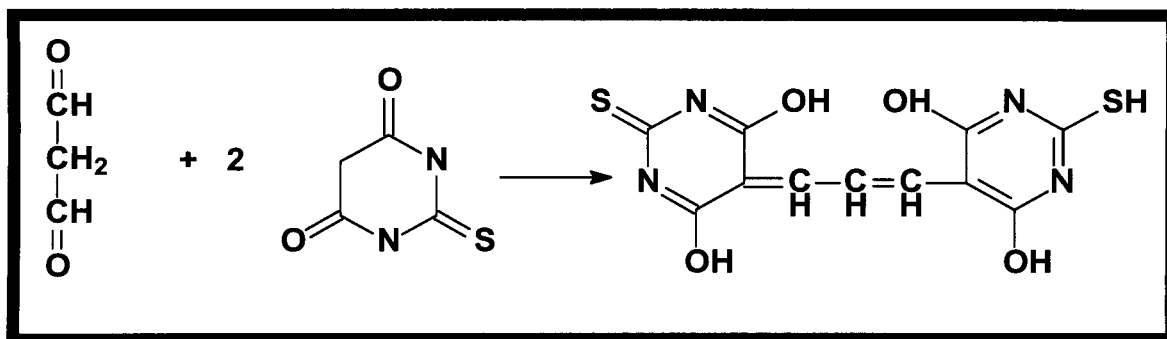


Figure 4.7: MDA reacts with two molecules of TBA to form MDA/TBA-complex (Northwest life science specialities, 2007).

Hydrogen peroxide is used as a source of hydroxyl radicals in a purely nonenzymatic chemical reaction. The reaction system is based on a coupled reaction in which ferrous ions, in the presence of ascorbic acid, react with hydrogen peroxide to give rise to hydroxyl radicals (Cui *et al.*, 2004; Halliwell, 2001). Ascorbate aid in the reduction of inorganic iron from the insoluble Fe^{3+} to the soluble Fe^{2+} (Halliwell, 2001). The ferrous ion then produced the hydroxyl radicals from hydrogen peroxide by the Fenton reaction (2.2.2.1.3.). The ferrous ions are constantly regenerated in a coupled reaction in which ferric ions are reduced by ascorbic acid. Although ascorbic acid can act as a scavenging antioxidant, it acts as a pro-oxidant in the presence of free iron (Cui *et al.*, 2004).

4.2.2.1. Experimental

4.2.2.1.1. Chemicals

The ascorbic acid (Vitamin C), Iron (III) Chloride (FeCl_3) and Dimethyl Sulfoxide (DMSO) was purchased from SAARCHEM (merck), Wadeville, Gauteng, South Africa. The Butylated Hydroxytoluene (BHT), Thiobarbituric acid (TBA), Trichloroacetic acid (TCA) and 1,1,3,3-Tetramethoxypropane (TEP)/ Malonaldehyde (MDA) was purchased from Sigma-Aldrich Chemie GmbH, Steinheim, Germany. All other chemicals and reagents were obtained locally and were of the highest available purity.

4.2.2.1.2. Animals

Male Sprague-dawley rats weighing 200-250 g were used for the experiment. The rats were maintained in separate cages in a controlled environment as described in section 4.2.1.1.2. The protocol for the experiment was approved by the North-West University Animal Ethics Committee.

4.2.2.1.3. Reagents

- Phosphate Buffer solution (PBS)

Take 8 g NaCl, 0.2 g KCl, 1.44 g Na₂HPO₄ and 0.24 g KH₂PO₄ and dissolve in 800 ml of water. Adjust pH to 7.4. When pH is correct add water till 1 L. Store in fridge.

- Butylated hydroxytoluene (BHT) (0.5 g/L) was dissolved in methanol.
- Trichloroacetic acid (TCA) (10 %) was prepared in Milli-Q water.
- Thiobarbituric acid (TBA) (0.33 %) was prepared in Milli-Q water. The TBA is light sensitive and was therefore always prepared fresh and protected from light by covering the container with aluminium foil.

4.2.2.1.4. Sample preparation

Concentrations of crude extract used were 5 mg, 2.5 mg and 1.25 mg crude extract in 0.1 ml solution. Each of the four crude extracts of *Gymnosporia buxifolia* were used at all three concentrations in this assay. The crude extracts were dissolved in DMSO.

4.2.2.1.5. Preparation of standards

1,1,3,3-Tetramethoxypropane was used as a standard. A series of reaction tubes, each containing appropriate aliquots of water and standard solution were prepared with Milli-Q water to a final volume of 0.5 ml. A calibration curve was generated by measuring the absorbance at 5 nmole/ml increments. The absorbance was read at 532 nm using an ultraviolet-visible spectrophotometer. The absorbance of the TBA/MDA-complex was plotted against the known concentration of malondialdehyde

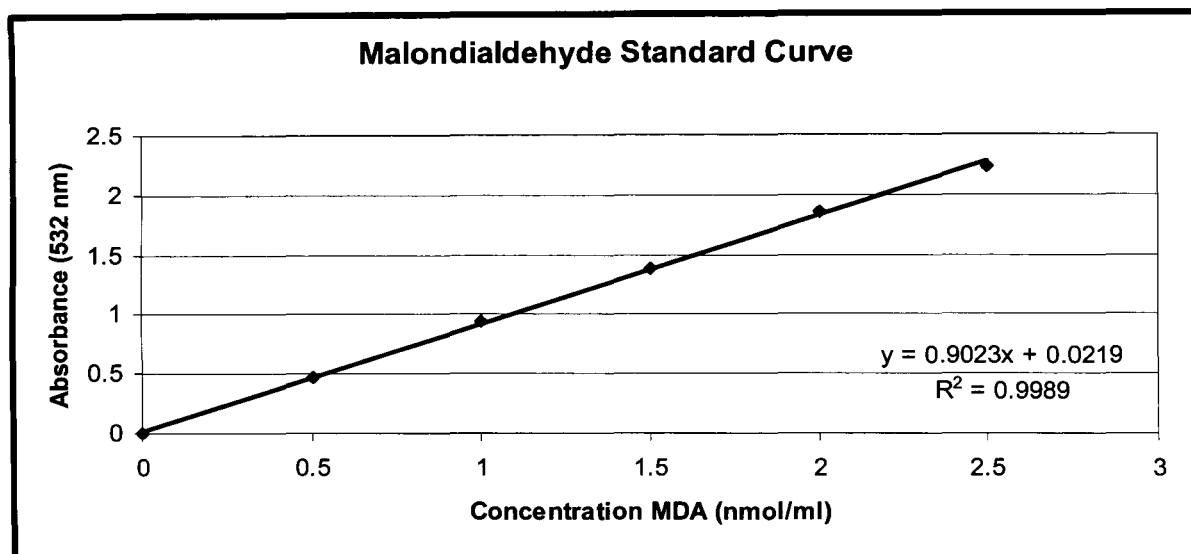


Figure 4.8: Malondialdehyde standard curve generated from 1,1,3,3-tetramethoxypropane.

4.2.2.1.6. Method

On the morning of the experiment, the brains were removed and weighed. The brains were homogenised in ice cold 0.1 M PBS in a glass homogeniser to produce a 10 % (w/v) homogenate (4.2.1.1.6).

The toxin used is hydrogen peroxide, a source of hydroxyl radical in a purely nonenzymatic chemical reaction with the help of FeCl_3 and ascorbic acid as described in section 4.2.2.

The assay was preformed by using 0.8 ml samples of brain homogenate, 0.05 ml H_2O_2 (5 mM), 0.025 ml FeCl_3 (4.88 mM), 0.025 ml Vitamin C (1.4 mM) and 0.1 ml of the relevant crude extract being tested (1.25; 2.5; 5 mg/ml). The reaction mixture was incubated in an oscillating water bath for 1 hour at 37 °C. At the end of the incubation period the tubes were centrifuged at 2000 x g for 20 minutes. Following the centrifugation the supernatant was removed from each tube and 0.5 ml BHT, 1 ml TCA and 0.5 ml TBA were added to the mixture. All tubes were then heated in a water bath for 1 hour at 60 °C. After cooling, 2 ml butanol was added to the TBA/MDA complexes, which was then vortexed and centrifuged for 10 minutes at 2000 x g. The absorbance of the complex was read at 532 nm and malondialdehyde levels were determined from the generated curve.

The statistical analysis was done using the Student-Newman-Keuls multiple range tests.

4.2.2.1.7. Results

The absorbance value obtained at 532 nm was converted to malodialdehyde levels from the standard curve generated from 1,1,3,3-tetramethoxypropane. The extent to which lipid peroxidation occurred was expressed as nmole MDA equivalents/mg tissue.

Table 4.2: The *in vitro* effects of selected extracts on the toxin induced lipid peroxidation in rat brain homogenate.

Test compounds	Concentration mg/ml	nmol MDA/ mg tissue	± Stdev
Control		0.002014	± 0.000385
Toxin	1 mM	0.009921	± 0.000999
Petroleum ether extract	1.25 mg/ml	0.005021	± 0.000223
	2.5 mg/ml	0.004659	± 0.000421
	5 mg/ml	0.004537	± 0.000186
Dichloromethane extract	1.25 mg/ml	0.004725	± 0.000453
	2.5 mg/ml	0.003997	± 0.0002
	5 mg/ml	0.004178	± 0.000225
Ethyl acetate extract	1.25 mg/ml	0.005348	± 0.0015
	2.5 mg/ml	0.005168	± 0.001097
	5 mg/ml	0.003772	± 0.000511
Ethanol extract	1.25 mg/ml	0.000596	± 0.000221
	2.5 mg/ml	0.000786	± 0.0000873
	5 mg/ml	0.001096	± 0.0000388

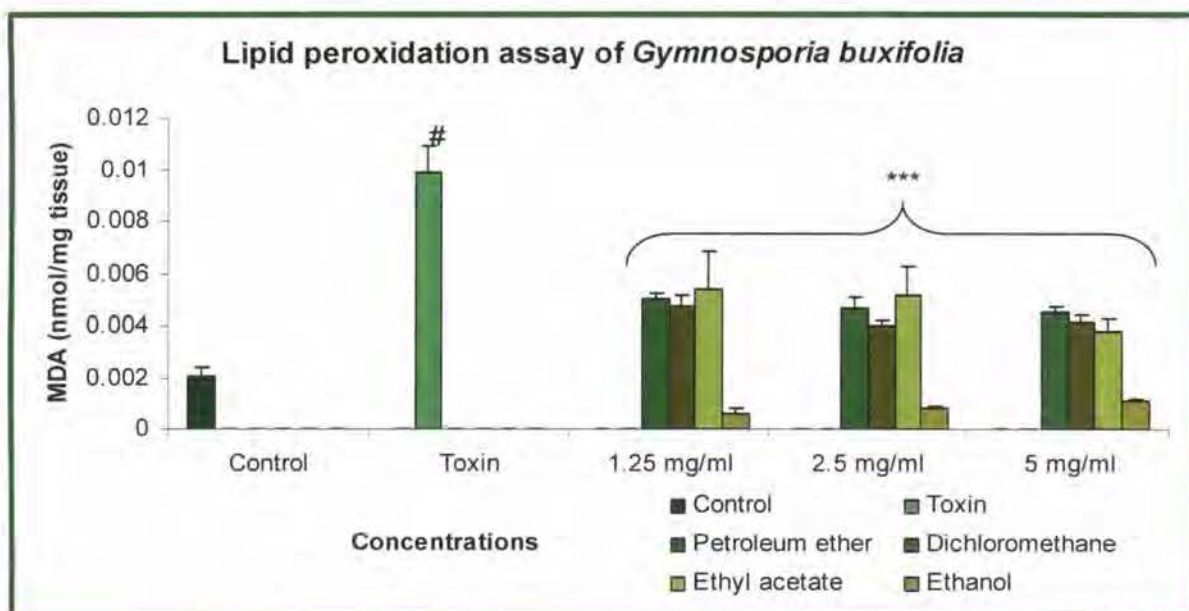


Figure 4.9: The effects of the selected crude plant extract on Toxin-induced lipid peroxidation in rat brain homogenate. Each bar represents the mean $\pm$ Stdev; $n=5$; # $p<0.001$ vs. Control, *** $p<0.001$ vs. Toxin. The Student-Newman-Keuls multiple range test was used.

The *in vitro* exposure of the whole rat brain homogenate showed that the toxin caused a significant concentration dependent rise in malondialdehyde generation. The ethanol was by far the most significant crude extract in reducing the lipid peroxidation. All the crude plant extracts of *Gymnosporia buxifolia* however significantly lowered the lipid peroxidation produced by the toxin.

4.2.3. Discussion of NBT and lipid peroxidation results.

From the results of NBT and lipid peroxidation it is clear that all the crude plant extracts show free radical scavenging activities. While in the NBT assay the ethyl acetate and ethanol extracts showed good promise in the lipid peroxidation assay the ethanol crude extract was clearly the best and looked very promising. Therefore the ethanol extract was selected for the isolation of active compounds.

CHAPTER 5: ISOLATION

The crude ethanol extract of *Gymnosporia buxifolia* showed very promising antioxidant activity in both the screening (chapter 3) and the *in vitro* biological tests (chapter 4). Chromatographic techniques were used to isolate active compound(s) from this extract.

5.1. Separation techniques

Chromatography is defined as a broad range of physical methods used to separate and or to analyse complex mixtures (Carrier and Bordonaro, 1994).

The techniques used in this study to isolate the compound(s) from the crude ethanol extract are thin layer chromatography (TLC), column chromatography, solid phase extraction and selective precipitation.

5.1.1. Thin layer chromatography (TLC)

In this study analytical TLC was performed on 0.25 mm thick silica gel aluminium backed sheets (Merk® TLC aluminium sheet gel 60 F₂₅₄). TLC was employed in the selection of a suitable mobile phase for the isolation of compounds with column chromatography. During examination of chromatograms for detection of the individual compounds UV light (254 nm and 366 nm) and iodine vapours or a spray reagent (5% H₂SO₄ in ethanol) was used.

5.1.2. Column chromatography

In this study column chromatography was performed with glass columns of various sizes in order to isolate fractions that lead to the isolation of pure compounds. The stationary phase used was silica gel (Merk®; 0.063 – 0.2 mm).

5.1.3. Solid phase extraction (SPE)

Solid phase is usually used to clean up a sample before using a chromatographic or other analytical method to quantitate the amount of analyte(s) in the sample (The University of Adelaide, 2008).

In this study, the solid phase extraction tubes used contained a 3 ml capacity silica gel-based bonded sorbent and were made of plastic.

5.1.4. Selective precipitation

Selective precipitation can be used to remove interferences from a mixture. A chemical reagent is added to the solution and it selectively reacts with the interference to form a

precipitate. The precipitate can then be physically separated from the mixture by filtration or centrifugation (Dhont and Vaanden Berghe, 2003).

5.2. Isolation procedure of the compounds

The crude ethanol plant extract from the soxhlet extraction was dried. It was then dissolved in as little as possible ethanol to make a very concentrated solution. Selective precipitation was then performed by adding a very large amount of diethyl ether. A precipitate formed and was separated from the solution by filtration. These two mixtures were subjected to antioxidant assays. The precipitate showed the best antioxidant activity.

The precipitate was then again dissolved in ethanol. A white compound which did not dissolve with the others was filtered from the solution. This white compound only dissolved in water, showed one spot on TLC and was named compound 1.

The ethanol solution was fractionated by column chromatography with silica gel stationary phase using petroleum ether: ethanol (1:2) as a mobile phase. Eight fractions (GB1, GB2, GB3, GB4, GB5, GB5, GB7 and GB8) were collected based on similarities in TLC. Fraction GB7 showed a few spots under the UV light of which a dark purple spot was of interest and it was subjected to more TLC to separate the components even better. Fraction GB7 contained four components which were fractionated by column chromatography with silica gel stationary phase using chloroform: methanol (2:1) as a mobile phase. Seven fractions (GB7:1, GB7:2, GB7:3, GB7:4, GB7:5, GB7:6 and GB7:7) were collected based on similarities in TLC.

Fraction GB7.5 contained a single dark purple spot under the UV light. This was named compound 2. The compound was subjected to solid phase extraction tubes and was washed with acetone.

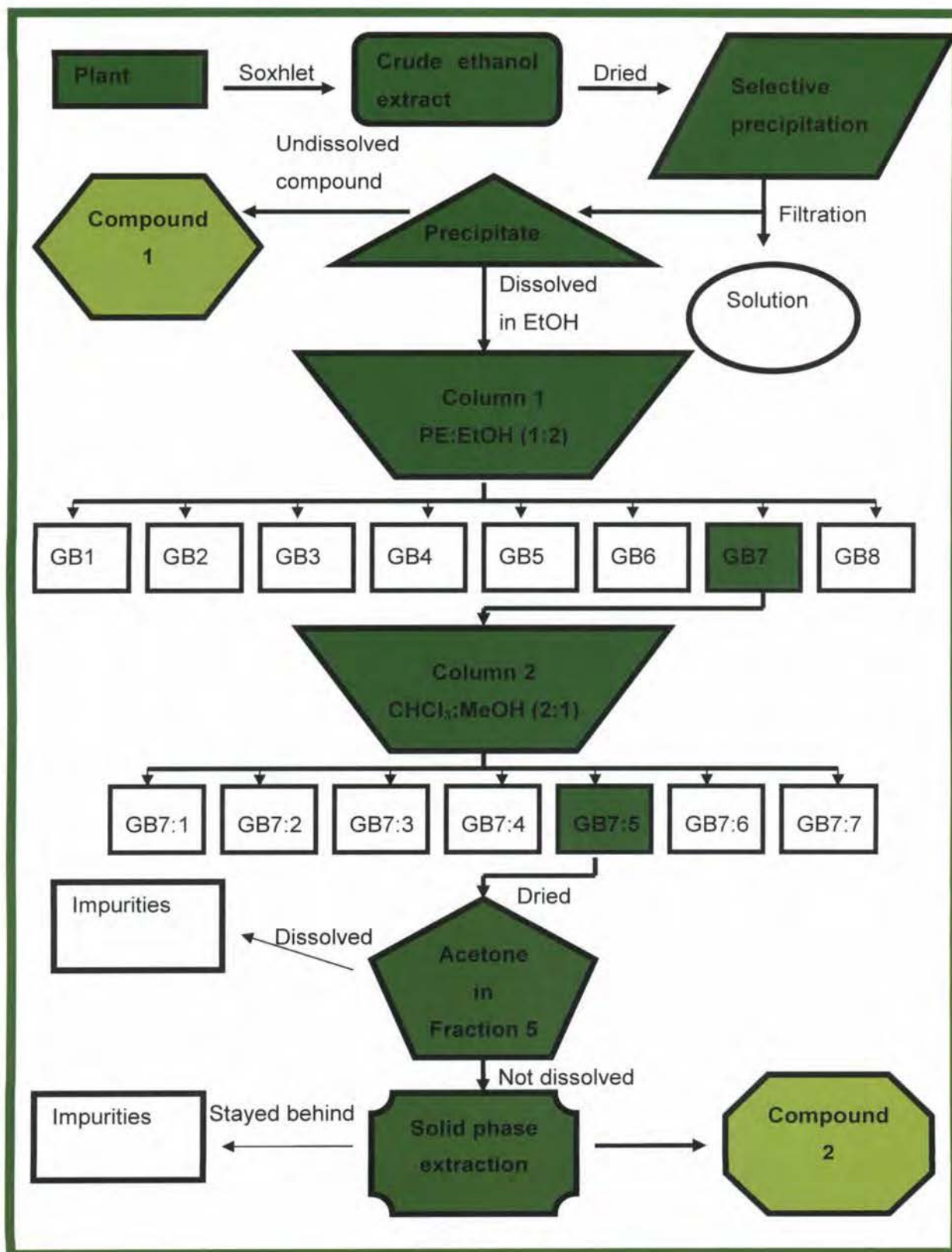


Figure 5.1: Isolation flowchart for ethanol extract of *Gymnosporia buxifolia*.

5.3. Characterisation of compounds isolated from *Gymnosporia buxifolia*

5.3.1. Instrumentation

5.3.1.1. Nuclear magnetic resonance spectroscopy (NMR)

^{13}C and ^1H nuclear magnetic resonance (NMR) spectra were recorded on a Bruker avance 600, in a 14.09 Tesla magnetic field using an ultra shield plus magnet. ^{13}C spectra were recorded using a frequency of 150.9126 MHz and ^1H spectra were recorded at a frequency of 600.1724 MHz. All chemical shifts are reported in parts per million (ppm) relative to tetramethylsilane (TMS; $\delta=0$).

5.3.1.2. Infrared spectroscopy (IR)

Infrared spectroscopy was recorded on a Nicolet Nexus 470-FT-IR spectrometer over the range 400 – 4000 cm^{-1} . Powders and dry samples were recorded in KBr with the diffuse reflectance method. Oils or solutions were put directly on a plate with a ZnSe (Zink Selenium) crystal. The method used for this is a Multi-Bounce HATR unit.

5.3.1.3. Mass spectroscopy (MS)

The mass spectra were recorded on a Micromass Autospec. It is a high resolution magnetic sector based instrument with Electron Ionisation, Chemical Ionisation and Fast Atom Bombardment Ionisation capabilities.

5.3.1.4. Melting point determination

Melting points were determined by using the Stuart® SMP10. The melting point sample was placed in a glass capillary tube which is placed in the aluminium block inside the sample chamber. The SMP10 allows the temperature to be set a few degrees below the expected melting point. This allows the SMP10 to heat to this temperature very rapidly (20 °C per minute), and hold it till operator is ready to begin. Once measuring is started the SMP10 will heat slowly (2 °C per minute) from that temperature until the melt occurs.

5.3.2. Characterisation of the proposed structure of compound 1

Compound 1 was identified as a six carbon sugar, white crystals. $\text{C}_6\text{H}_{14}\text{O}_6$; mp 181 °C; m/z % (spectrum 1): 73(100), 61(80), 43(59), 31(52), 103(48), 45(42), 133(41), 56(40), 42(32), 57(32), 28(30), 60(29), 29(21); V_{max} (spectrum 2, KBr, cm^{-1}): 668.5, 860.6, 927.8, 1030.9, 1341.1, 1541.1, 1735.1, 2918, 3567.1; δ_{H} (spectrum 3, 600.1724 MHz, DMSO): 2.5 (DMSO), 3.38, 3.45, 3.69, 4.07, 4.12, 4.43; δ_{C} (spectrum 4, 150.9126 MHz, DMSO): 63.8, 69.8, 70.8.

Chemical shifts are reported in ppm (δ) in DMSO- D_6 for ^1H and ^{13}C .

5.3.3. Characterisation of the proposed structure of compound 2

Compound 2, m/z % (spectrum 5): 256(100), 73(63), 69(56), 129(51), 57(46), 228(42), 57(42), 83(39), 60(38), 242(35), 185(34), 43(33), 97(32), 213(32); $V_{\max}$ (spectrum 6, ZnSe, cm^{-1}): 3355.4, 2924.5, 1655.1, 1600.8, 1510.5, 1453.1, 1350.9, 1259.6, 1037.0, 804.7. ; δ_{H} (spectrum 3, 600.1724 MHz, MeOH): 1.18, 1.50, 1.57, 1.58, 1.83, 1.93, 2.06, 2.15, 2.22, 2.97, 3.02, 3.09, 3.38, 3.50-3.74, 3.91, 4.10, 4.18, 4.36, 4.52, 5.00, 5.34, 5.46, 5.53, 6.037, 6.38, 6.64, 6.72, 6.87, 7.01, 7.52, 7.62, 7.72, 7.80; δ_{C} (spectrum 4, 150.9126 MHz, MeOH): 10.28, 17.77, 18.09, 22.36, 25.2, 26.50, 30.76, 35.8, 48.60, 56.3, 62.76, 62.88, 64.53, 67.12, 71.29, 71.74, 71.91, 72.98, 76.30, 77.77, 78.04, 79.48, 81.07, 82.94, 93.56, 98.22, 107.75, 113.42, 116.67, 132.02, 189.09, 193.80.

Chemical shifts are reported in ppm (δ) in methanol- D_3 for ^1H and ^{13}C .

5.4. Antioxidant activity of the compounds

Only a very small amount of these compounds was isolated from the crude ethanol plant extract of *Gymnosporia buxifolia*, and it was decided to only do one antioxidant assay. The crude plant extract showed exceptional antioxidant activity with the lipid peroxidation assay (4.2.2.1.7.). Therefore the antioxidant activity of the two compounds was assessed using the lipid peroxidation procedure (4.2.2.). They were compared to the crude ethanol plant extract of *Gymnosporia buxifolia*.

A concentration of 1.25 mg/ml was used in all the tested compounds as well as with the crude plant extract. The control group was used as 0% and the toxin was used as 100%. The 100% of the toxin represents the amount of damage it does to the rat brain homogenate compared to the 0% of the control group where no toxin was added. The samples were then tested by adding toxin to the samples and comparing it to the 100% toxin. They all showed lower percentages of lipid peroxidation than the toxin which indicate that they exhibit antioxidant ability by protecting the rat brain homogenate against the toxin. Compound 2 showed 59.8% which indicates a reduction of 40.1% from that of the toxin. Compound 1 showed 16.8% which indicates a reduction of 83.2% from that of the toxin which indicates very good antioxidant activity. The crude plant extract is an exceptional antioxidant and at -12.3% it seemed to also inhibit baseline/normal oxidative processes.

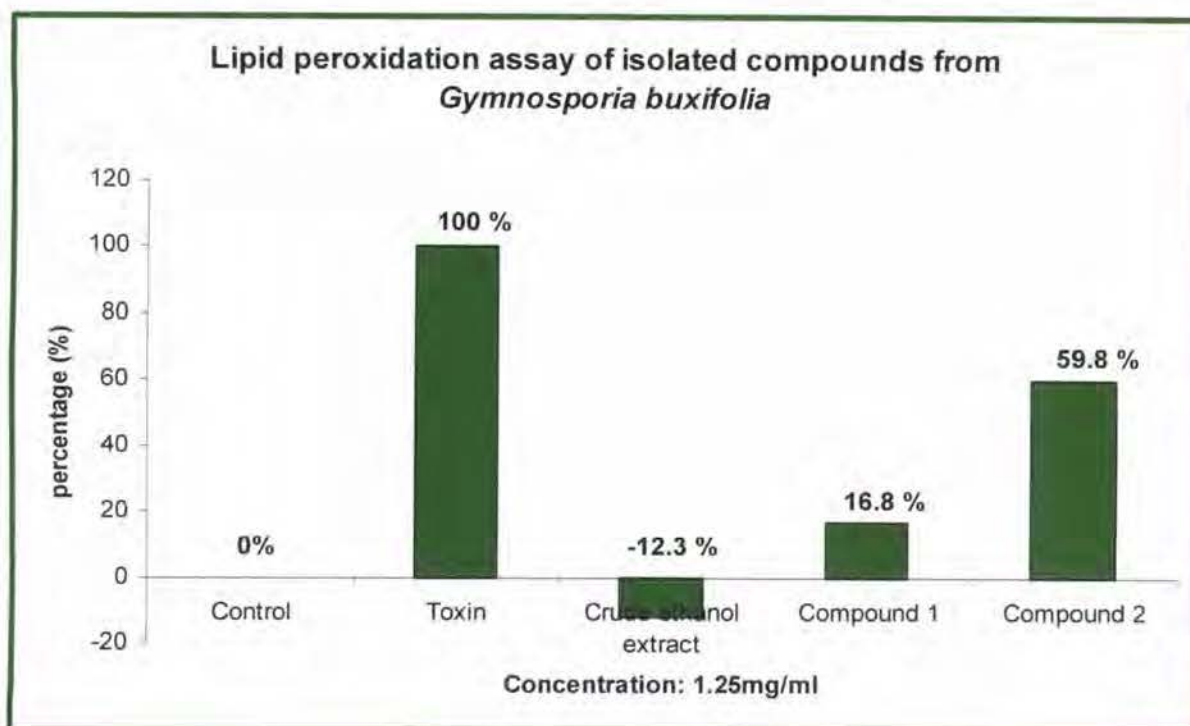


Figure 5.2: The effects of the selected compound 1, compound 2 and crude ethanol plant extract on toxin-induced lipid peroxidation in rat brain homogenate.

5.5. Discussion of the characterisation and antioxidant activity of the compounds

It was proposed through a literature survey that compound 1 could be either d-mannitol or dulcitol (galactitol) or even a combination of the two. Dulcitol has frequently been isolated from the Celastraceae family (De Oliveira *et al.*, 2006). Mannitol in plants occurs in a complex mixture with sugars and other sugar alcohols. Many of the sugars and sugar alcohols have sufficiently different characteristics to permit relatively easy separation. However, the characteristics of mannitol and dulcitol are such that the normal known methods of liquid extraction or crystallisation often used in sugar or sugar alcohol separations are not applicable (Neal, 2004). It was decided not to try and separate this mixture because dulcitol has already been isolated from *Gymnosporia buxifolia* and dulcitol and mannitol are known antioxidants (Bosch, 2004; Shen *et al.*, 1997).

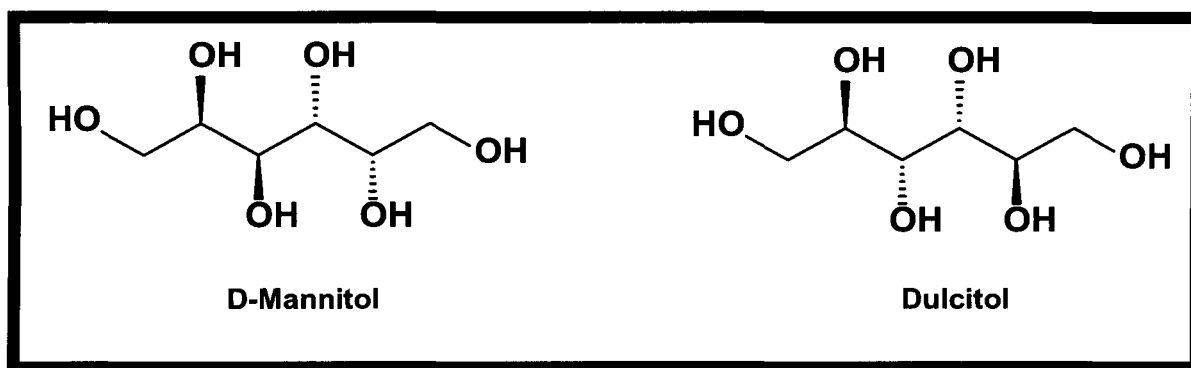


Figure 5.3: Proposed structures of compound 1

The MS data (spectrum 1) confirmed the molecular weight of compound one at m/z 183 corresponding to a molecular formula of $C_6H_{14}O_6$. It also corresponds with the MS found in the literature for d-mannitol and dulcitol (SDBS, 2004a; SDBS, 2004b). The IR spectrum showed an OH peak from $3200\text{--}3500\text{ cm}^{-1}$. It also shows a C-O peak at 1030.9 cm^{-1} . This corresponds with the IR found in the literature for d-mannitol and dulcitol (SDBS, 2004a; Keller, 1986). The structures were proposed by observing the ^1H -NMR (spectrum 3) and the ^{13}C -NMR (spectrum 4) in tables 5.1 and 5.2.

Chemical shifts are reported in ppm (δ) in $\text{DMSO-}D_6$ for ^1H and ^{13}C and also in ppm (δ) in $\text{DMSO-}D_6$ for ^1H and ^{13}C in the literature for d-mannitol and ^{13}C for ducitol. The chemical shifts are reported in ppm (δ) D_2O for ^1H in literature for dulcitol, the solvent peaks suppressed the OH peaks.

Table 5.1: ^1H spectral assignment of compound 1 in comparison to data found in the literature (SDBS, 2004a; SDBS, 2004b).

Position	Compound 1	D-Mannitol	Ducitol
	^1H	^1H	^1H
1, 6	3.69/ 3.38	3.61/ 3.38	3.684
2, 5	3.45	3.45	3.700
3, 4	3.38	3.54	3.978
1, 6-OH	4.07	4.35	-
2, 5-OH	4.43	4.42	-
3, 4-OH	4.12	4.15	-

Table 5.2: ^{13}C spectral assignment of compound 1 in comparison to data found in the literature (Karsten *et al.*, 1995; Gaidamauskas *et al.*, 2005).

Position	Compound 1	D-Mannitol	Ducititol
	^{13}C	^{13}C	^{13}C
1, 6	63.8	63.9	64.1
2, 5	70.8	71.46	71
3, 4	69.8	69.86	70.3

The only difference between the two is their melting points. In literature the melting point d-mannitol is 164 - 170 °C and ducititol is 188 – 191 °C indicating that compound 1 might be dulcitol.

The compound showed very good antioxidant activity when it was tested for lipid peroxidation. It lowered the toxin levels from 100% to 16.8% which indicate very good antioxidant activity.

The amount of compound 2 yielded after isolation was very small and because of this small amount left to work with it was decided not to purify it further. This made it difficult to identify the structure of compound 2. It was also determined after the spectra were examined that it could still be a mixture of compounds. It was therefore decided to only identify the class of the compound because identifying a specific structure from this data would not be possible. It is proposed through literature that compound 2 is a type of dihydro- β -agarofuran sesquiterpenoid.

The family of Celatraceae comprising of approximately 88 genera and 1300 plant species, is the major source of dihydro- β -agarofuran sesquiterpenoids (Gao *et al.*, 2007). Sesquiterpenoids are variously polyoxygenated tricyclic polyols with a dihydro- β -agarofuran core, bearing from as few as two ester groups, to as many as nine ester groups. Hydroxyl groups can be present on almost any carbon atom of this core, with various stereochemistries. The esterifying residues are mostly aliphatic acids such as acetic acid, iso-butyric acid and 2-methylbutyric acid, and the aromatic or heterocyclic acids such as benzoic acid, cinnamic acid, furic acid, nicotinic acid and elaborated pyridine-containing dicarboxylic acids (Figure 5.4). The relative position, number and configuration of these residues distinguish each sesquiterpene and create a large structural diversity of sesquiterpenes (Gao *et al.*, 2007).

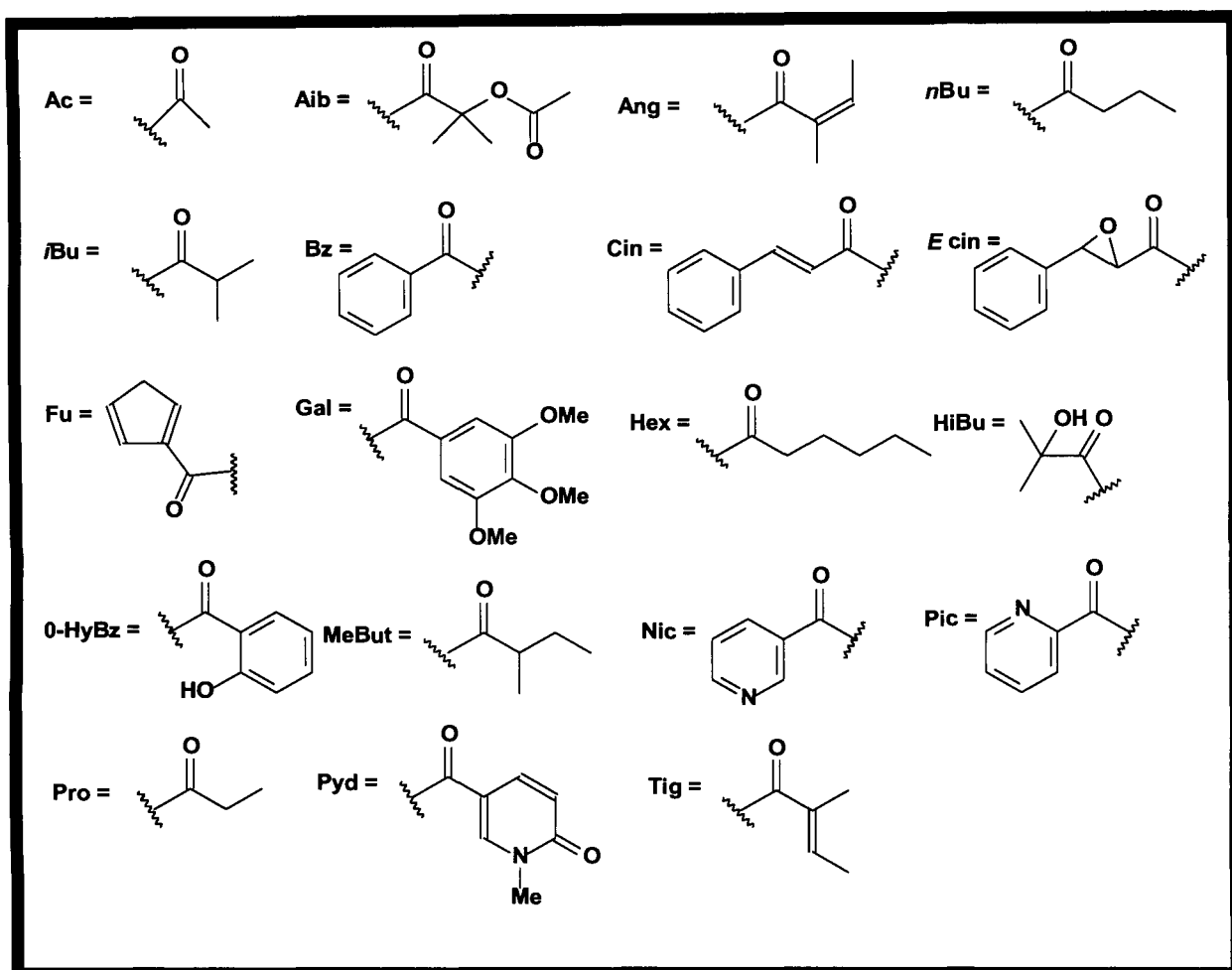


Figure 5.4: Structural abbreviations of esterifying substituents used in sesquiterpenoids (Gao *et al.*, 2007).

The sesquiterpenoids are classified in eight groups based on their structural similarities and the number of hydroxyl groups. The eight groups are: dihydroxylated sesquiterpene polyesters, trihydroxylated sesquiterpene polyesters, tetrahydroxylated sesquiterpene polyesters, pentahydroxylated sesquiterpene polyesters, hexahydroxylated sesquiterpene polyesters, heptahydroxylated sesquiterpene polyesters, octahydroxylated sesquiterpene polyesters and nonahydroxylated sesquiterpene polyesters (Gao *et al.*, 2007).

Based on comparisons between literature and data from compound 2 it is proposed that compound 2 could be tetrahydroxylated sesquiterpene polyester. This group is then divided into six subgroups based on the position of binding of the hydroxyl groups to the core structure and then based on stereochemistry it is further divided into 17 subgroups.

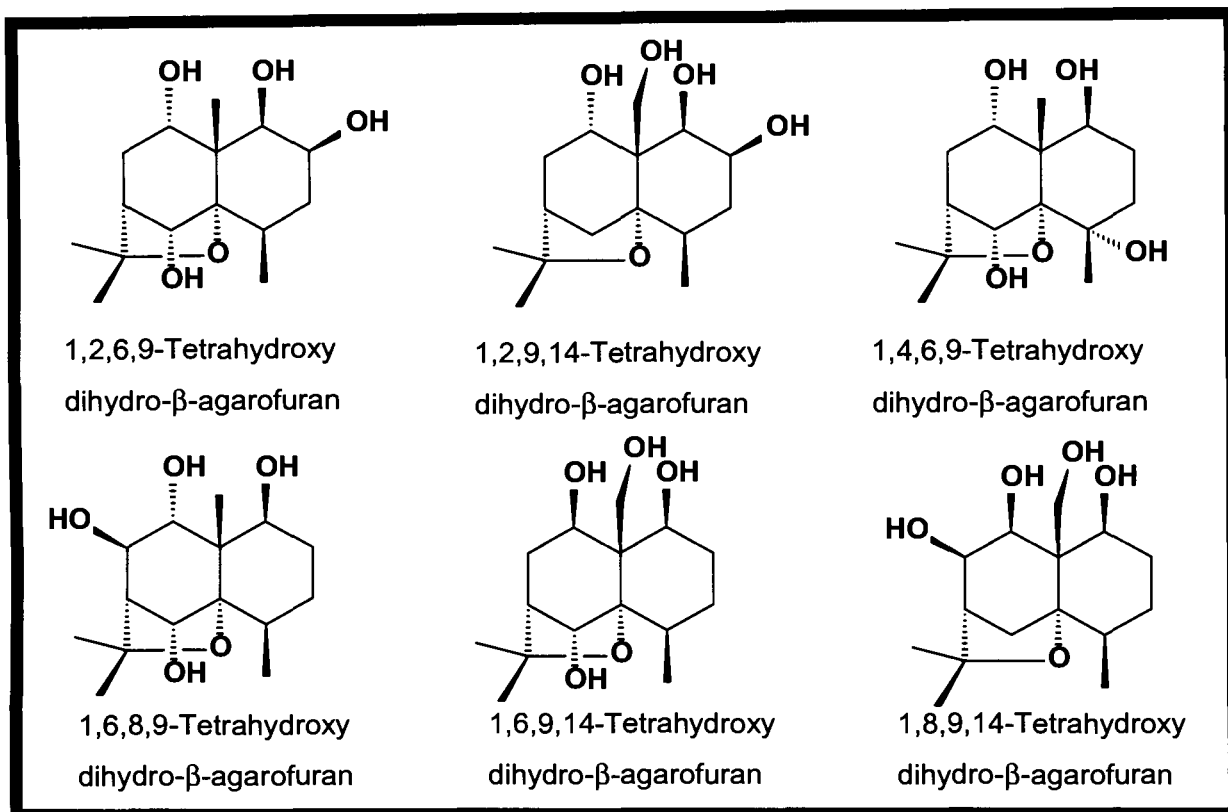


Figure 5.5: The six subgroups of tetrahydroxylated sesquiterpenes based on position.

After a literature review it was proposed that compound 2 has a core structure of tetrahydroxyl dihydro-β-agarofuran. Any of the four hydroxylated carbons can be substituted with any of the esterifying substituents, which leaves endless possibilities for this structure.

A comparison was made between compound 2 and three different sesquiterpenoids with the tetrahydroxylated dihydro-β-agarofuran core skeleton.

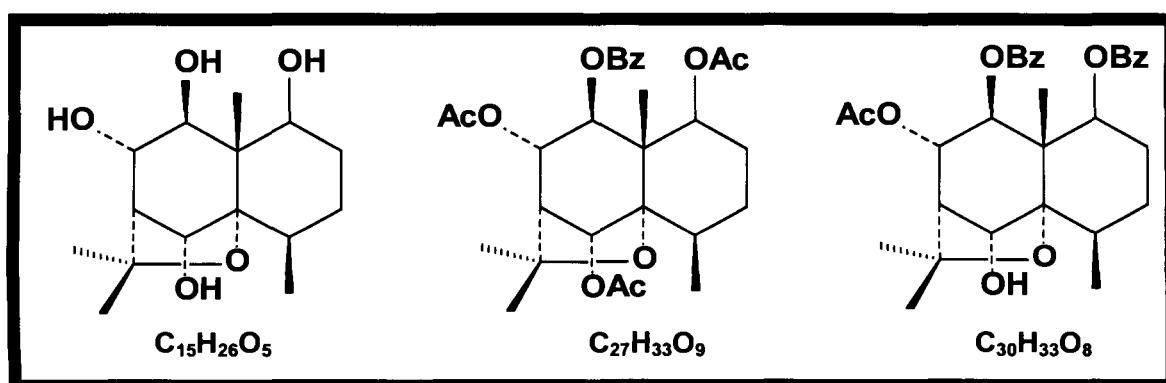


Figure 5.6: Three structures compound 2 was compared to (Tu *et al.*, 1991).

The IR spectrum showed an OH peak at 3355.4 cm^{-1} . It also shows C-O peak at 1037.0 cm^{-1} , C=O and C=C peaks at 1600.9 cm^{-1} and 1655.1 cm^{-1} . These peaks correspond with peaks found in the literature for structures with a core skeleton of tetrahydroxylated dihydro- β -agarofuran (Tu *et al.*, 1991).

The core structure was further proposed by observing the ^1H -NMR (spectrum 7) and the ^{13}C -NMR (spectrum 8) and comparing them to table 5.3 and 5.4. Chemical shifts are reported in ppm (δ) in methanol- D_3 for ^1H and ^{13}C and in ppm (δ) in CDCl_3 for ^1H and ^{13}C for the literature except for $\text{C}_{15}\text{H}_{26}\text{O}_5$ that were obtained in pyridine- D_5 .

The peaks showed good correlation to those found in literature. The differences can be contributed to the fact that different solvents were used, for compound 2 methanol- D_3 was used and in literature CDCl_3 and pyridine- D_5 was used. The peaks that are not accounted for in the core structure is due to the fact that several substitutions can take place with esterifying substituents (figure 5.4). These substitutions can account for chemical shifts and other peaks. The difference could also be because of the impurities still present in the compound. The MS data (spectrum 5) was not used as definitive data for the proposed structure, because of all the impurities and therefore only a core structure was proposed. The MS data only showed us that the structure was similar in mass to the proposed structures.

Table 5.3: ^1H spectral assignment of three structures found in the literature for comparison to compound 2 (Tu *et al.*, 1991).

Position	Compound 2	$\text{C}_{15}\text{H}_{26}\text{O}_5$	$\text{C}_{27}\text{H}_{33}\text{O}_9$	$\text{C}_{30}\text{H}_{33}\text{O}_8$
	^1H	^1H	^1H	^1H
1	5.34 dd	4.59 dd	5.22 dd	5.50 dd
6	5.00 s	4.73 d	5.50 s	4.95 s
7	2.22 d	2.75 d	2.54 d	2.35 d
8	5.46 dd	4.26 dd	5.32 dd	5.57 dd
9	5.53 d	4.88 d	5.93 d	5.63 d
Me-12	1.32 d	1.43 d	1.02 d	1.26 d
Me-13	1.5 s	1.48 s	1.39 s	1.55 s
Me-14	1.57 s	1.93 s	1.41 s	1.57 s
Me-15	1.58 s	1.94 s	1.50s	1.58 s
	2.06 s		1.61 s	2.00 s
Ac	1.83 s		1.86 s	
	2.15 s		2.15 s	

Table 5.4: ^{13}C spectral assignment of three structures found in the literature for comparison to compound 2 (Tu *et al.*, 1991).

Position	Compound 2	$\text{C}_{25}\text{H}_{26}\text{O}_5$	$\text{C}_{27}\text{H}_{33}\text{O}_9$	$\text{C}_{30}\text{H}_{33}\text{O}_8$
	^{13}C	^{13}C	^{13}C	^{13}C
1	71.7	74.8	73.8	71.5
2	26.5	28.1	26.5	26.8
3	22.3	26.3	22	22
4	35.8	34.8	33.9	33.7
5	93.5	93.3	91.2	92.6
6	72.9	75.9	76.2	72.8
7	56.3	57.7	52.1	54.5
8	75.2	78.1	76.8	74.7
9	79.4	80.9	79	79.6
10	48.3	46.8	46.7	48.7
11	81.0	82.7	82.5	81.8
12	17.7	18	16.9	17.4
13	10.2	11.5	12.5	12.3
14	25.2	26.7	25.7	24.5
15	30.7	32.4	30.8	31.1

The dihydro- β -agarofuran sesquiterpenoids possess a broad spectrum of biological activities such as immunosuppressive, cytotoxic, insect antifeedant, insecticidal, anti-HIV, reversing multi-drug resistance phenotype and antitumor activities (Gao *et al.*, 2007).

The results of the antioxidant assay that was done on compound 2 showed antioxidant activity when it was tested with lipid peroxidation. It lowered the toxin levels from 100% to 59.8% which indicate low antioxidant activity.

The results for the crude ethanol extracts are still much better than the values for these two isolated compounds. It is speculated that compounds with better activity might thus still be present in the crude extract or synergism may play a role.

CHAPTER 6: CONCLUSION

Plants play a significant role in maintaining human health and improving the quality of human life. They serve as components of food, such as seasoning and beverages, as well as cosmetics, dyes and medicines. It is estimated by the World Health Organisation that almost 80% of the earth's inhabitants rely on traditional medicine for their primary health care needs and most of this therapy involves the use of plant extracts and their active components (Tripathi *et al.*, 2007).

The studies on "oxidative stress" and its adverse effects on humans have become a subject of considerable interest (Tripathi *et al.*, 2007). Oxidative stress is defined as excess formation and/or incomplete removal of highly reactive molecules such as reactive oxygen species (ROS). The ROS include free radicals such as superoxide, hydroxyl radical, peroxy radical and nonradical species such as hydrogen peroxide. ROS are capable of damaging a wide range of essential biomolecules such as proteins, DNA and lipids (Ardestani and Yazdanparast, 2007).

Oxidative stress is recognised as a contributing factor in neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease. It also promotes the progression of a number of chronic diseases such as cancer, cardiovascular diseases, cataract, inflammation and the normal aging process (Kamath *et al.*, 2004).

Antioxidants reduce oxidative damage to biomolecules by modulating the effect of reactive oxygen (Kamath *et al.*, 2004). Antioxidant defence mechanisms that help to prevent the destructive effects of ROS includes antioxidative enzymes, such as superoxide dismutase, catalase and glutathione peroxidase. It also includes small molecules such as glutathione and vitamins C and E. The efficiency of the antioxidant defence is altered under pathological conditions and therefore the ineffective scavenging and/or overproduction of free radicals may play a crucial role in determining tissue damage (Ardestani and Yazdanparast, 2007).

Natural antioxidants are in high demand, because of their potential in health promotion and disease prevention and their improved safety and consumer acceptability (Su *et al.*, 2007). In addition to the long term safety of the natural antioxidants, it also improves the quality and stability of food. It also acts as nutraceuticals to terminate free radical chain reactions in biological systems and may thus provide additional health benefits to consumers (Kamath *et al.*, 2004).

The aim of this study was to identify and screen specific plants with possible free radical scavenging effects and then to isolate and characterise the active compounds responsible for this activity.

After an initial literature survey 21 plants were selected for the screening of antioxidant activity. These plants were: *Acacia karroo*, *Berula erecta*, *Clematis brachiata*, *Elephantorrhiza elephantine*, *Erythrina zeyheri*, *Gymnosporia buxifolia*, *Heteromorpha arborescens*, *Leonotis leonurus*, *Lippia javanica*, *Physalis peruviana*, *Plectranthus ecklonii*, *Plectranthus rehmanii*, *Plectranthus venticullatus*, *Plumbago auriculata*, *Salvia auritia*, *Salvia runcinata*, *Solenostemon latifolia*, *Solenostemon rotundifolius*, *Tarchonanthus camphorates*, *Vangueria infausta* and *Vernonia oligocephala*. They were all available in the Potchefstroom area.

The leaves of all the plants were dried and then extracted with four solvents (petroleum ether, dichloromethane, ethyl acetate and ethanol) using soxhlet extraction. Therefore each of the plants had four crude plant extracts. The FRAP and ORAC assays were used to determine the total antioxidant activity of all these crude plant extracts. Some plants had higher antioxidant activity than other. In the FRAP assay the top six in order from highest to lowest were: *Lippia javanica*, *Acacia karroo*, *Tarchonanthus camphorates*, *Gymnosporia buxifolia*, *Plectranthus rehmanii* and *Salvia runcinata*. In the ORAC assay the top six in order from highest to lowest were: *Lippia javanica*, *Erythrina zeyherii*, *Gymnosporia buxifolia*, *Plumbago auriculata*, *Tarchonanthus camphorates*, *Salvia runcinata*. The ORAC and FRAP assays have different mechanisms of action and cannot be compared but the plants that showed high ferric reducing absorbance power also showed high oxygen radical absorbance capacity. *Gymnosporia buxifolia* was selected because of its good test results and its availability.

After selecting the promising plant the biological tests were done to determine the most promising crude extract from that plant. The *in vitro* biological tests done were NBT and lipid peroxidation. From the results of NBT and lipid peroxidation it was clear that all the crude plant extracts showed free radical scavenging activities. While in the NBT assay the ethyl acetate and ethanol extracts showed good promise, in the lipid peroxidation assay the crude ethanol extract was clearly the best and looked very promising. Therefore the ethanol extract was chosen for the isolation of active compounds.

To isolate the active compound(s) in the ethanol extract of the plant, column chromatography, thin layer chromatography, selective precipitation and solid phase

extraction tubes was used. Two compounds were isolated. The isolated compounds were characterised using NMR, MS and IR.

The proposed structures for the isolated compound 1 were d-mannitol or dulcitol or a combination of the two. The proposed structure for isolated compound 2 was a structure with a dihydro- β -agarofuran sesquiterpenoid core skeleton. Both the compounds were tested using the lipid peroxidation assay and both showed antioxidant activity. The six carbon sugar had higher antioxidant activity than the dihydro- β -agarofuran sesquiterpenoid but they still did not show the high antioxidant activity of the ethanol crude extract.

This leads to the assumption that there is still a very good antioxidant in the crude ethanol plant extract that has not yet been isolated. Further isolation is proposed for future study. It is also proposed that the toxicity of the plant be tested and that *in vivo* studies are to be done.

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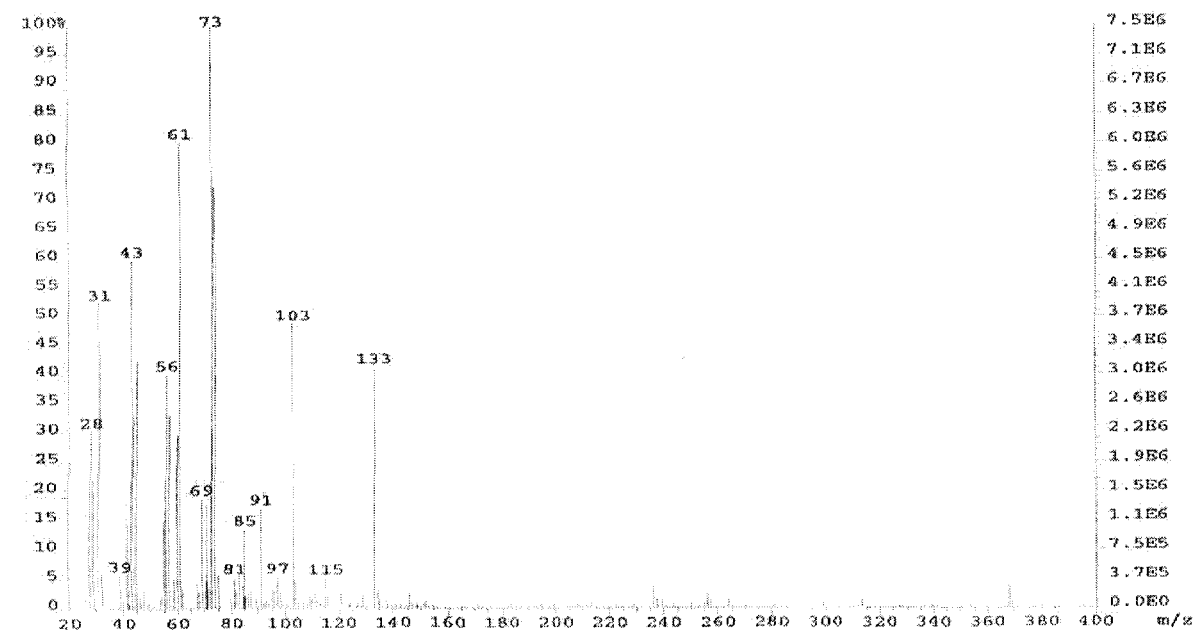
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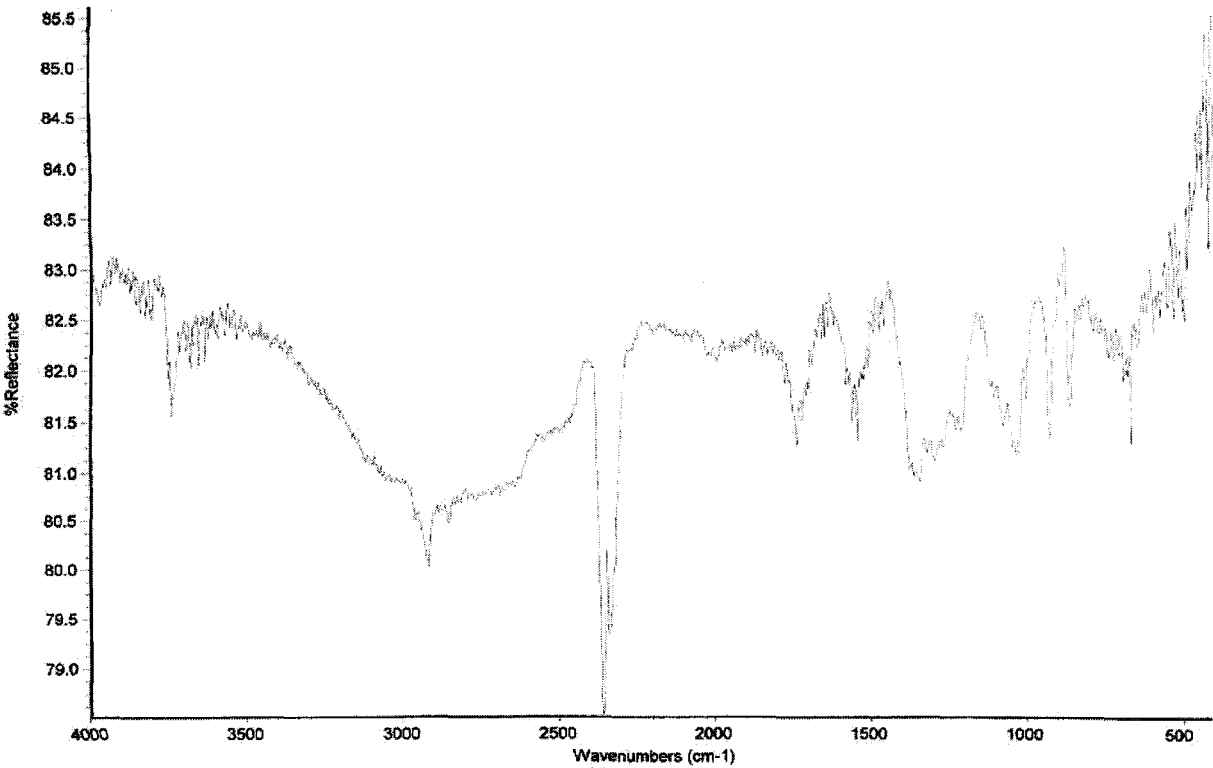
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SPECTRA

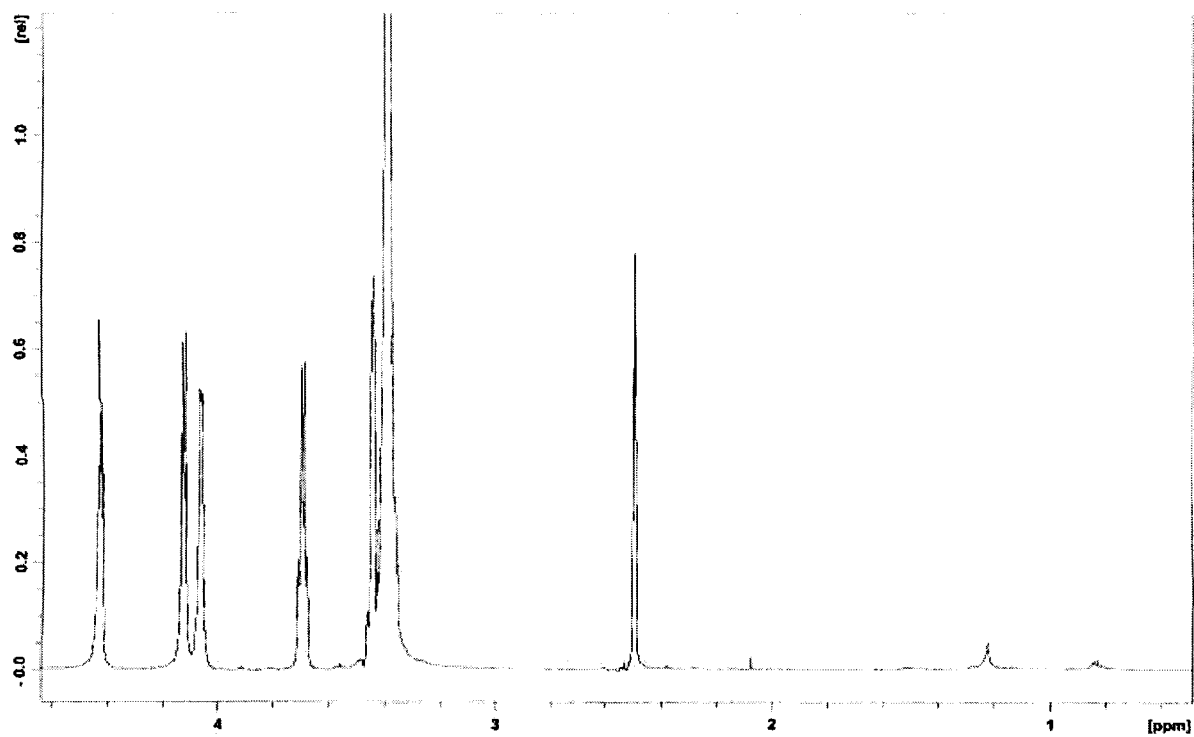
Spectrum 1: MS compound 1



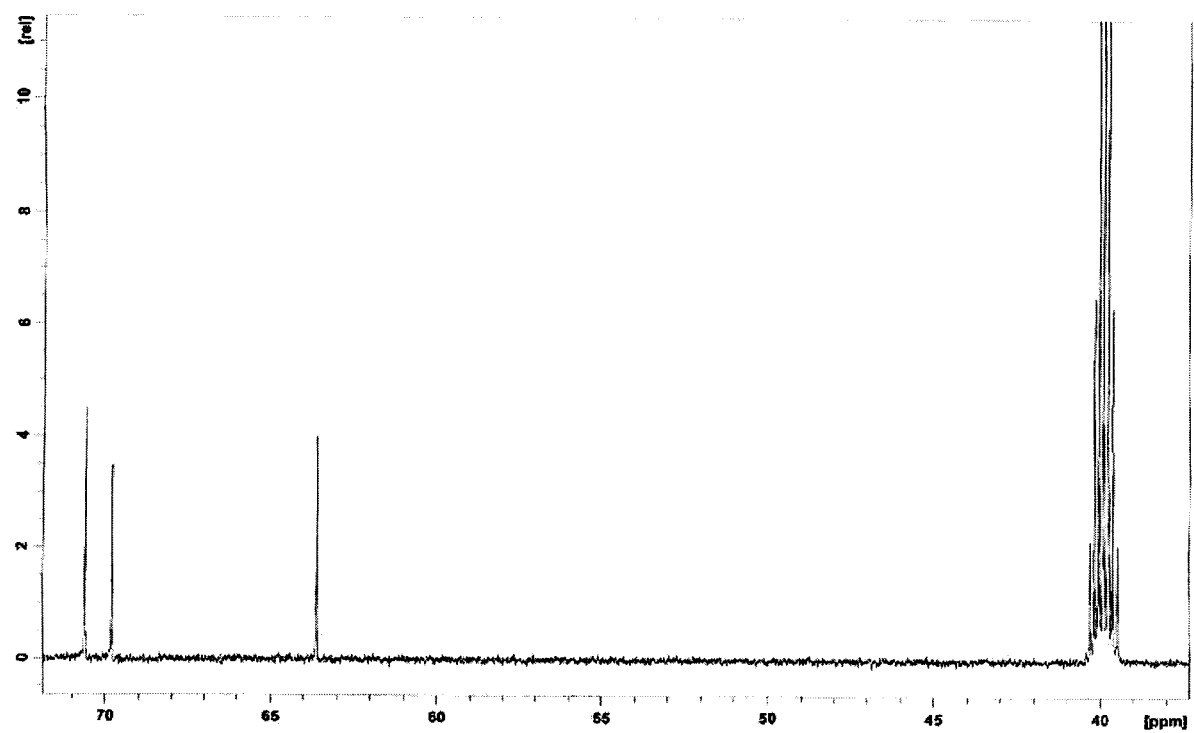
Spectrum 2: IR compound 1



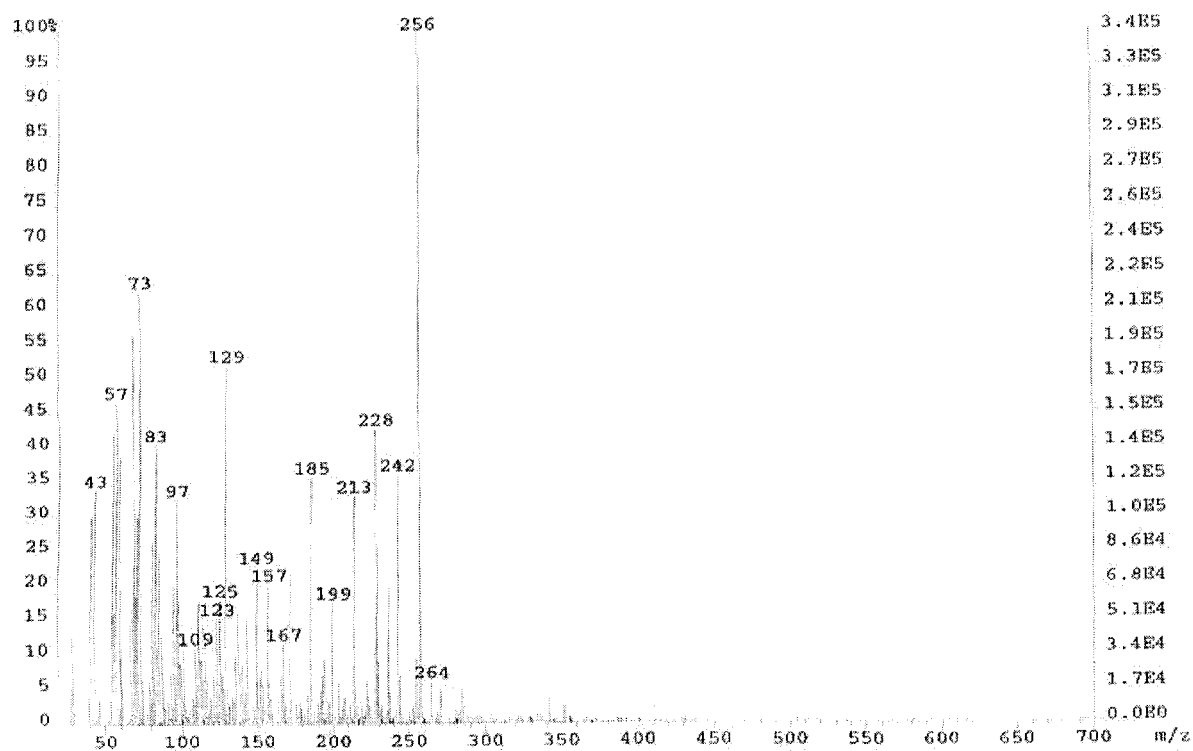
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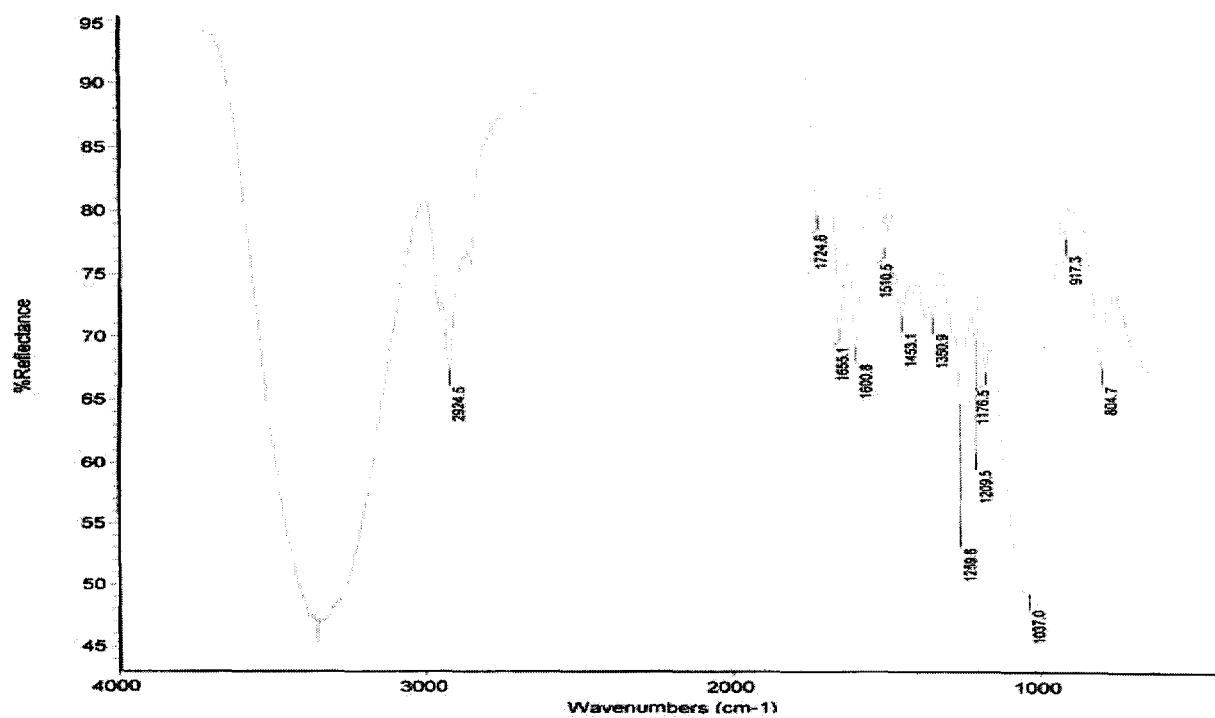
Spectrum 4: ^{13}C -NMR compound 1



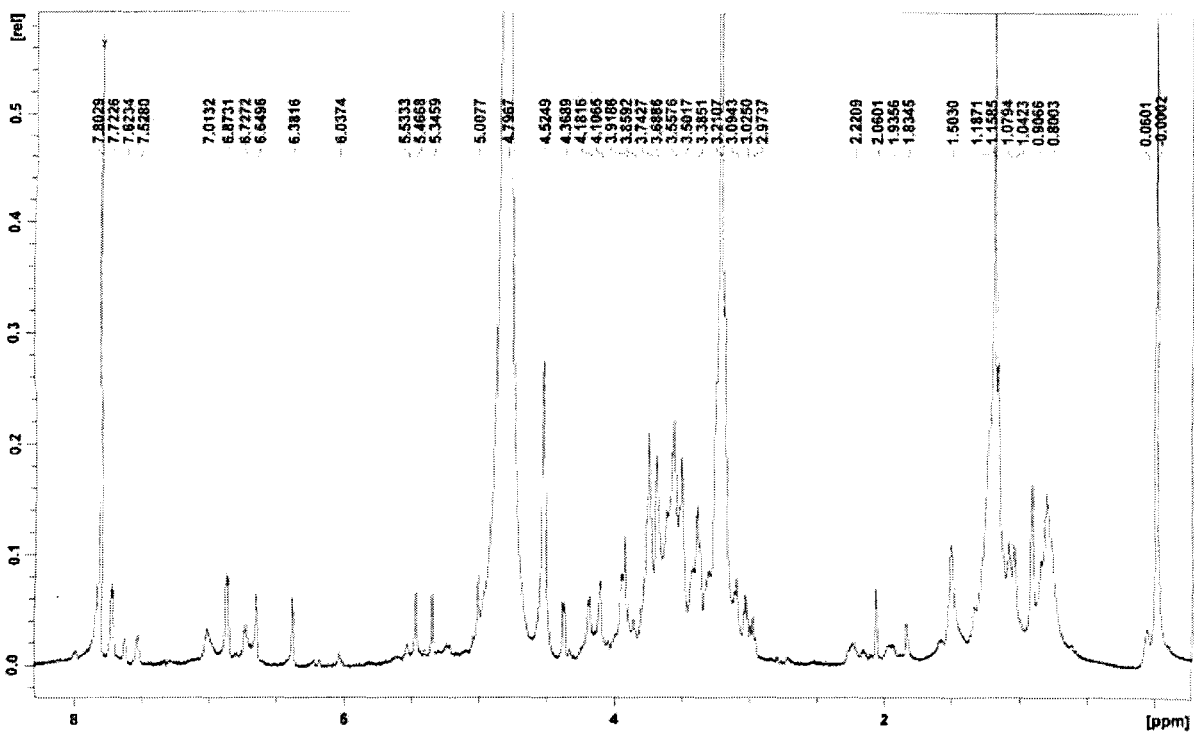
Spectrum 5: MS compound 2



Spectrum 6: IR compound 2



Spectrum 7: ¹H-NMR compound 2



Spectrum 8: ¹³C-NMR compound 2

