Antioxidant properties of South African plants

Melanie van Heerden

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Supervisor: Prof. S. van Dyk
Co-Supervisor: Prof. S.F. Malan

"Don't be afraid of hard work. Nothing worthwhile comes easily. Don't let others discourage you or tell you that you can't do it. In my day, I was told women didn't go into chemistry. I saw no reason why we couldn't." – Gertrude B. Elion

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ABSTRACT

South Africa with its vast biodiversity and use of plants in traditional medicine provides a platform for natural drug research and development through the discovery of new chemical entities. The present study was based on the identification of plants as a natural resource for compounds conveying potential antioxidant properties. Free radicals and related molecules, collectively termed reactive oxygen/nitrogen species (ROS/RNS), in excess, promotes oxidative stress within cells and ultimately neurodegeneration. The role of the antioxidant defences is to eliminate the excess in ROS/RNS and therefore the interest in prevention and/or treatment of neurodegenerative diseases. These compounds have the ability to scavenge free radicals and prevent oxidative stress and related diseases such as neurodegenerative diseases; Parkinson's disease, Alzheimer's disease and amyotrophic lateral sclerosis.

For this study, *Plumbago auriculata* and *Tarchonanthus camphoratus* were selected from an initial screening (ORAC/FRAP) of a selection of 21 plant species.

P. auriculata and *T. camphoratus* were extracted by Soxhlet extraction, using four solvents, petroleum ether, dichloromethane, ethyl acetate and ethanol, in order of increasing polarity. The extracts were bleached using an ultra-violet irradiation apparatus. Each extract of both plants were then subjected to *in vitro* assays to determine the antioxidant properties thereof.

Both, the thiobarbituric acid (TBA) and nitro-blue tetrazolium (NBT) assays of Ottino & Duncan (1996) were used for antioxidant activity screening of the extracts in rat brain homogenates. The TBA assay relies on the assessment of lipid peroxidation, via hydroxyl anions ($OH^{\bullet}$), on the basis of the complex formation between malondialdehyde (MDA) and TBA, generating a pink colour to be measured spectrophotometrically at 532 nm. The principal of the NBT assay is the reduction of NBT to nitro-blue diformazan (NBD), signified as a purple colour formation, in the presence of the superoxide anions ($O_2^{\bullet-}$). The intensity of the purple colour is then measured spectrophotometrically at 650 nm.

The experimental data showed that all of the extracts of *P. auriculata* and *T. camphoratus* were able to scavenge for $OH^{\bullet}$ and $O_2^{\bullet-}$, which were then correlated to the antioxidant activity of the extracts. The best results were obtained with the EtOAc and EtOH-extracts of *T. camphoratus.* In comparison to the toxin (H_2O_2), 0.0270 ± 0.00045 nmole MDA/mg tissue, the extracts showed significant decrease in MDA formation (TBA assay). The EtOH-extract of *T. camphoratus* attenuated the lipid peroxidation to 0.00386 ± 0.00015 nmole MDA/mg tissue. The extracts were also compared to the toxin (KCN), 30.5006 ± 0.7812 μmole/mg

proteins (NBT assay) and showed significant reduction of NBD formation and $O_2^{\bullet-}$ scavenging ability. The EtOAc-extract of *T. camphoratus* obtained the best $O_2^{\bullet-}$ scavenging results, 3.5891 ± 0.5029 µmole/mg protein reduction in NBD formation. However, based on the correlation of TBA, FRAP and ORAC results the EtOH-extract was chosen for further research. Plant extracts were also subjected to toxicity testing via the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. The extracts showing toxicity were; PE (10 mg/ml) of both plants and DCM (10 mg/ml) of *T. camphoratus*. All other extracts showed non-significant toxicity against HeLa cell growth.

The EtOH-extracts of *T. camphoratus* was submitted to bioassay-guided fractionation to isolate the compound responsible for these properties. Compound MVHS9 was isolated through column chromatography and purified by solid phase extraction. ^{1}H, ^{13}C, COSY NMR and IR spectral data were used for structure elucidation and a phthalate ester was proposed for MVHS9.

OPSOMMING

Suid Afrika met sy biodiversiteit en gebruik van plante in tradisionele medisyne verskaf 'n platform vir natuurlike geneesmiddelnavorsing en –ontwikkeling d.m.v. die ontdekking van nuwe chemiese entiteite. Die doel van hierdie studie was die identifikasie van natuurlike verbinding(s) met potensiële antioksidant eienskappe. Vryradikale en verwante molekules, gesamentlik benaam as reaktiewe suurstofstowwe/stikstofstowwe (RSS), veroorsaak oksidatiewe stres en ooreenkomstig neurodegenerasie. Antioksidante vervul die rol om die oormaat RSS te elimineer, oksidatiewe stres te beperk en neurodegeneratiewe siektes te voorkom en/of behandel, dit sluit in siektes soos Parkinson se siekte, Alsheimer se siekte en amiotrofiese laterale sklerose. Vir die studie was *Plumbago auriculata* en *Tarchonanthus camphoratus* geselekteer uit siftingstoetse (FRAP/ORAC) van 21 plant spesies.

Soxhlet ekstraksies met die volgende oplosmiddels, in orde van toenemende polariteit, petroleumeter, dichloormetaan, etielasetaat en etanol is van *P. auriculata* en *T. camphoratus* voorberei. Ekstrakte is voor die aanvang van *in vitro* toetse gebleik m.b.v. UV-bestraling.

Beide die tiobarbituursuur en nitro-blou tetrasolium (NBT) toetse van Ottino & Duncan (1996) is aangewend om die antioksidantaktiwiteit van ekstrakte te bepaal. Die tiobarbituursuurtoets is gebasseer op die bepaling van lipiedperoksidasie d.m.v. kompleksvorming tussen malondialdehied (MDA) en tiobarbituursuur, die intensiteit van die pienk kompleks word dan by 532 nm gelees. Lipiedperoksidasie is geïnduseer d.m.v. hidroksielradikale ($OH^{\bullet}$) in rotbreinhomogenaat met die toksien, waterstofperoksied (H_2O_2). Die NBT-toets meet die reduksie van NBT na nitro-blou diformasan (NBD) (pers), in die teenwoordigheid van superoksied ($O_2^{\bullet-}$) radikale in rotbreinhomogenaat. Die $O_2^{\bullet-}$ is gegenereer met die toksien, kaliumsianied. Die intensiteit van die pers kleur, afhangende van die NBD vlakke, word dan by 650 nm gelees.

Die eksperimentele data het gewys dat die ekstrakte van, *P. auriculata* en *T. camphoratus,* beide $OH^{\bullet}$ en $O_2^{\bullet-}$ opruim. Die resultate verkry is herlei na die antioksidantaktiwiteit van die ekstrakte. Die beste resultate, vir beide toetse, is verkry met die etielastetaat –en etanolekstrakte van *T. camphoratus.* Al die ekstrakte het 'n merkwaardige afname in lipiedperoksidasie geïnduseer in vergelyking met die toksien (H_2O_2), 0.027 ± 0.0004 nmol MDA/mg weefsel. Die etanolekstrak het lipiedperoksidasie laat afneem na 0.004 ± 0.0001 nmol MDA/mg weefsel. Die ekstrakte is ook vergelyk met die toksien, kaliumsianied (NBT toets) en het 'n merkwaardige afname in NBD-vorming veroorsaak. Die toksien (kaliumsianied), 30.5 ± 0.78 µmol/mg proteïen, is deur die etielastetaatekstrak verminder na 3.59 ± 0.5 µmol/mg proteïne. Die ekstrakte is ook onderwerp aan toksisiteit toetsing, d.m.v.

die MTT (3-(4,5-dimetielthiasol-2-yl)-2,5-difenieltetrasolium bromied) toets. Die ekstrakte wat toksisiteit getoon het was die petroleumeter (10 mg/ml) van albei plante en die dichloormetaan (10 mg/ml) van *T. camphoratus*. Die res van die ekstrakte het geen merkwaardige toksisiteit getoon teen die groei van HeLa selle nie.

Die etanol ekstrak van *T. camphoratus* is geselekteer vir isolering en karakterisering van die moontlike verbinding(s) verantwoordelik vir die antioksidantaktiwiteit. Verbinding MVHS9 is geissoleer d.m.v. kolomchromatografie en finale suiwering d.m.v. soliede fase ekstraksie. ^{1}H, ^{13}C KMR and IR spektra data is gebruik vir struktuuropklaring en 'n phthalaatester is voorgestel vir MVHS9.

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LIST OF ABBREVIATIONS

4-HNE	-	4-Hydroxynonenal
6-OHDA	-	6-Hydroxydopamine
α-TOH	-	α-Tocopherol
ADP	-	Adenosine diphosphate
AIDS	-	Acquired immunity defect syndrome
ANOVA	-	One way analysis of variance
ATP	-	Adenosine triphosphate
BHA	-	Butylated hydroxyanisole
BHT	-	Butylated hydroxytoluene
BSA	-	Bovine serum albumin
CD	-	Cell density
CoQ_{10}	-	Co enzyme Q_{10}
COSY	-	Correlation NMR spectrometry
COX	-	Cyclo-oxygenase
CuSOD	-	Copper superoxide dismutase
$CuSO_4.5H_2O$	-	Aqueous coppersulphate solution
CuZnSOD	-	Copper zinc superoxide dismutase
CYP	-	Cytochrome *P*
DCM	-	Dichloromethane
DMEM	-	Dulbeccos's modified eagle's medium
DMSO	-	Dimethyl sulphoxide
*e*NOS	-	Endothelial nitric oxide synthase
EtOAc	-	Ethyl acetate
EtOH	-	Ethanol
Fe^{2+}	-	Ferrous (iron II)
Fe^{3+}	-	Ferric (iron III)
FBS	-	Foetal bovine serum

$FeCl_3$	-	Iron(III)chloride
FRAP	-	Ferric reducing antioxidant power
Fe^{2+}-TPTZ	-	Ferrous tripyridyltriazine
Fe^{3+}-TPTZ	-	Ferric tripyridyltriazine
FVSS	-	Final volume of single cell
GAA	-	Glacial acetic acid
GPx	-	Glutathione peroxidase enzyme
GSH	-	Glutathione (reduced)
GSSG	-	Oxidised glutathione
HeLa	-	Human epithelial cells
H_2O_2	-	Hydrogen peroxide
HOCl	-	Hypochlorous acid
*i*NOS	-	Inducible nitric oxide synthase
KCl	-	Potassium chloride
KCN	-	Potassium cyanide
KH_2PO_4	-	Potassium dihydrogen orthophosphate
$L^{\bullet}$	-	Lipid radical
$LOO^{\bullet}$	-	Peroxyl radical
LOOH	-	Hydroperoxides
LOX	-	Lipoxygenase
MDA	-	Malondialdehyde
MAO-A/B	-	Monoamine oxidase A & B
MnSOD	-	Manganese superoxide dismutase
$MPDP^+$	-	1-Methyl-4-phenyl-2,3-dihydropyridinium
MPP^+	-	1-Methyl-4-phenylpyridinium
MPPP	-	1-Methyl-4-phenyl-4-propionoxypiperidine
MPTP	-	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MTT	-	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NaCl	-	Sodium chloride
Na_2CO_3	-	Disodiumcarbonate solution
NADH	-	Nicotinamide adenine dinucleotide
NADPH	-	Nicotinamide adenine dinucleotide phosphate (reduced)
Na_2HPO_4	-	di-Sodium hydrogen orthophosphate anhydrous
NaOH	-	Sodium hydroxide
NBD	-	Nitro-blue diformazan
NBT	-	Nitro-blue tetrazolium
NCSC	-	Number of counted single cells available
$NO^{\bullet}$	-	Nitric oxide
*n*NOS	-	Neuronal nitric oxide synthase
NOS	-	Nitric oxide synthase
$^1O_2$	-	Singlet oxygen
$O_2^{\bullet-}$	-	Superoxide anion/radical
O_3	-	Ozone
$OH^{\bullet}$	-	Hydroxyl anion/radical
ONOOH	-	Peroxynitrous acid
$ONOO^-$	-	Peroxynitrite anion/radical
ORAC	-	Oxygen radical absorbance capacity
PBS	-	Phosphate buffered saline
PE	-	Petroleum ether
PUFAs	-	Polyunsaturated fatty acid(s)
RNS	-	Reactive nitrogen species
ROS	-	Reactive oxygen species
SNpc	-	Substantia nigra pars compacta
SOD	-	Superoxide dismutase
TBA	-	Thiobarbituric acid
TCA	-	Trichloroacetic acid

TEP	-	1,1,3,3-Tetramethoxypropane
TNf	-	Tumour necrosis factor
VCR	-	Volume of cells required

CHAPTER 1. INTRODUCTION AND AIM OF STUDY

1.1 Introduction

Research into neurodegenerative diseases has increasingly targeted the evaluation of the effectiveness of various antioxidants. The rationale being supported by many years of basic science generally showing that reactive oxygen species and oxidative damage are important factors for degenerative neurological disorders (Floyd, 1999).

Oxygen supports life but the utilisation of oxygen by cells in many physiological processes result in the formation of highly reactive free radical products. Reactive oxygen/nitrogen species (ROS/RNS) is a collective term that includes all reactive forms of oxygen and nitrogen, including both the radical and non-radical species that participate in the initiation and/or propagation of radical chain reactions. ROS/RNS include free radicals such as the hydroxyl radical ($OH^{\bullet}$), superoxide radical ($O_2^{\bullet -}$), nitric oxide ($NO^{\bullet}$) and molecules with the ability to generate free radicals through chemical reactions such as, hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^{\bullet}$) (Cui *et al.*, 2004).

Biological systems have evolved with endogenous defence mechanisms to help protect against free radical induced cell damage (Singh *et al.*, 2004). The antioxidant defence mechanisms should keep a balance between ROS production and ROS elimination, therefore keeping a pro-oxidant/antioxidant equilibrium (Singh *et al.*, 2004; Gilgun-Sherki *et al.*, 2001). A disruption in the equilibrium due to an inadequate defence mechanism causes an excess of ROS and leads to the oxidative degradation of lipids, proteins and DNA in cells and tissues. This imbalance between cellular production of ROS and the ability of cells to efficiently defend against them is called oxidative stress (Gilgun-Sherki *et al.*, 2001). Oxidative stress has been implicated in the process of ageing and in the etiology of several diseases (Devasagayam *et al.*, 2004) such as atherosclerosis, diabetes, ischemia/reperfusion (I/R) injury, cancer, neurodegenerative diseases, hypertension, pulmonary diseases and haematological diseases (Cui *et al.*, 2004), of which neurodegenerative diseases was the most important to this particular study. The brain is especially susceptible to oxidative damage leading to neuropathologies such as Parkinson's disease, Alzheimer's disease and amyotrophic lateral sclerosis (Floyd, 1999).

Clearly, strategies aimed at limiting free radical production, oxidative stress and damage may slow the progression of neurodegenerative diseases. Therefore, the interest in antioxidants is to combat oxidative stress by working to neutralise excess free radicals and stopping them from starting the chain reactions that contribute to various diseases and

aging (Singh *et al.*, 2004). A number of synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) have been developed but their uses have declined because of their toxicity. Vitamin E is an effective natural antioxidant but has a limited use. As a result, there is considerable interest in preventative medicine as antioxidants from natural sources (Haraguchi *et al.*, 1997).

Plants have formed the basis of sophisticated traditional medicine systems such as Ayurvedic, Chinese and African among others. African (this includes South Africa) traditional medicine is the oldest and perhaps the most diverse of all medicine systems. These systems of medicine have given rise to some important drugs still in use today (Gurib-Fakim, 2006). According to Balunas and Kinghorn (2005) research on natural products accounts for approximately 48 % of the new chemical entities reported from 1981 – 2002 (Balunas & Kinghorn, 2005).

Recent health concerns drew much attention to the use of natural antioxidants (Karthikumar *et al.*, 2007). The plant-based antioxidants are believed to have an important role in the maintenance of human health because our endogenous antioxidants provide insufficient protection against the constant and unavoidable challenges of ROS (Benzie, 2003). Numerous studies in the past 10 years have shown that polyphenols have *in vitro* and *in vivo* activity in preventing or reducing the deleterious effects of oxygen derived free radicals associated with several diseases. The flavonoids are the largest group of polyphenols in plants (Weinreb, 2004) and the antioxidant activities thereof have been recognised for decades (Harborne & Williams, 2000). For the interest of this study, it was important to look for compounds other than the flavonoids with proposed antioxidant activity. Among the other compounds with antioxidant properties are anthocyanidins (Einbond *et al.* 2004), quinones, coumarins and tannins (pharmaceutical effects similar to those of flavonoids) (Van Wyk *et al.*, 2002) and carotenoids (Gilgun-Sherki *et al.*, 2001).

Examples of plants that have been examined for their antioxidant activity include *Foeniculum vulgare* (Choi & Hwang, 2004) and *Cichorium intybus* (Gazzani *et al.*, 1999). Compounds already isolated from plants include a wide range of polyphenolic structures such as the naphtoquinone plumbagin from the *Plumbago* species and resveratrol, a stilbene, found in the skin of grapes (Urquiga & Leighton, 2000 and Hwang *et al.*, 2004) These compounds are all reported as having potential chain-breaking antioxidant properties (Halliwell, 2001).

Even with all the challenges facing drug discovery from medicinal plants, natural products isolated form medicinal plants can be predicted to remain an essential component in the search for new medicines (Balunas & Kinghorn, 2005).

1.2 Aim and Objectives

In view of the above considerations, the role of ROS in degenerative diseases of the brain and the protective effects of exogenous antioxidants against oxidative stress, the aim and objectives of this study were set. Therefore, the purpose of this study was to subject selected plants to *in vitro* assays to determine the antioxidant properties thereof for further investigation and bioassay-guided fractionation to obtain the compound(s) responsible for these properties.

Several plant species were already screened for antioxidant properties with the oxygen radical absorbance capacity (ORAC) and ferric reducing antioxidant power (FRAP) assays.

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

- To select two plant species based on their ORAC and FRAP values from a list of 21 plants species.
- To screen extracts of chosen plants for *in vitro* antioxidant properties using appropriate assays.
- To determine the toxicity of the chosen plants with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.
- To choose the plant extract with the most promise of antioxidant activity for the isolation of active compound(s).
- To isolate active compound(s) by chromatographic techniques.
- To characterise the compound(s) responsible for antioxidant activity from active extract by spectrometric methods.

CHAPTER 2. LITERATURE REVIEW

2.1 Free Radicals, Reactive Oxygen Species and Reactive Nitrogen Species

The explosion of the atom bombs on 6th August 1945 at Hiroshima and on 9th August at Nagasaki, during World War II, marked the beginning of free radical biochemistry. The bombs accounted for massive deaths and shortened the life span of the survivors. It was thereafter that Gershman and Gilbert, in 1954, speculated that the lethal effects of ionising radiation might be ascribed to formation of reactive oxygen species (ROS). Since then free radicals such as ROS and reactive nitrogen species (RNS) have gained notoriety (Devasagayam *et al.*, 2004).

Free radicals represent a class of chemical entities with the capacity to bring about the oxidation of target molecules, either directly by abstraction of electrons or indirectly through the production of highly reactive intermediates. Their reactivity is due to the key factor of an unpaired electron in their atomic structure (Cui *et al.*, 2004). This includes the hydrogen atom (one unpaired electron), most transition metals and the oxygen molecule itself (two unpaired electrons) (Halliwell *et al.*, 1984). They are capable of independent existence for very brief intervals of time (Cui *et al.*, 2004). Free radicals react with their surroundings in order to gain stability, thus "stealing" an electron from a nearby stable molecule and rendering this molecule a free radical itself. This starts a chain reaction that cascades into the disruption of living cells (Fouad, 2008b).

Oxygen is necessary for life but paradoxically as a by-product of its metabolism, it produces ROS that are highly toxic to cells (Andersen, 2004). Free radicals and related species are mainly derived from oxygen (reactive oxygen species/ROS) but also from nitrogen (reactive nitrogen species/RNS) (Devasagayam *et al.*, 2004). ROS/RNS include both the radical and non-radical species that participate in the initiation and/or propagation of radical chain reactions (Cui *et al.*, 2004). The most common cellular free radicals are hydroxyl radicals, superoxide radicals and nitric oxide. Other molecules such as hydrogen peroxide and peroxynitrite are not free radicals, but can lead to their generation through various chemical reactions. Free radicals and related molecules are often classified together as ROS to signify their ability to promote oxidative changes within the cell (Gilgun-Sherki *et al.*, 2001).

Free radicals are constantly produced *in vivo* by all body tissue and generated by various endogenous systems, exposure to different physicochemical conditions or pathophysiological states (Devasagayam *et al.*, 2004).

2.1.1 Types of free radicals and related species

2.1.1.1 Ozone (O_3), singlet oxygen ($^1O_2$), and molecular oxygen

Ozone is a toxic form of oxygen that oxidises proteins, nucleic acids and lipids (Cui *et al.*, 2004).

Singlet oxygen is very reactive although it does not contain unpaired electrons and therefore is not a free radical. $^1O_2$ is an electronically excited form of oxygen. It is formed *in vivo* by enzymatic activation of oxygen through peroxidases or lipooxygenase activity during prostaglandin biosynthesis. It can also be produced by physicochemical reactions such as energy transfer due to type II photosensitisation, thermal decomposition of endoperoxides and dioxetanes, reaction of ozone (O_3) with human body fluids and interactions between hydrogen peroxide and peroxynitrite or during the respiratory burst of phagocytes. It induces various genotoxic, carcinogenic and mutagenic effects through its action on polyunsaturated fatty acids (PUFAs) and DNA (Cui *et al.*, 2004; Fouad, 2008b).

Molecular oxygen may be considered a diradical (dioxygen) since it contains two unpaired electrons. When molecular oxygen is reduced to water in the electron transport chain there is a stepwise addition of four electrons resulting in the formation of several hydrogen containing ROS such as hydroperoxyl radical, superoxide radical, hydrogen peroxide and the hydroxyl radical (table 2.1) (Cui *et al.*, 2004).

Tabel 2.1 The formation of ROS by reduction of molecular oxygen in the electron transport chain (Cui et al., 2004).

i	$O_2 + e + H^+ \rightarrow HO_2$	Hydroperoxyl radical
ii	$HO_2 \rightarrow H^+ + O_2^{\bullet-}$	Superoxide radical
iii	$O_2^{\bullet-} + 2H^+ + e \rightarrow H_2O_2$	Hydrogen peroxide
v	$OH + e + H^+ \rightarrow H_2O$	
vi	$O_2^{\bullet-} + H_2O_2 \rightarrow OH^{\bullet} + OH + O_2$	Haber-Weiss reaction
vii	$Fe^{2+} + H_2O_2 \rightarrow OH^{\bullet} + OH + Fe^{3+}$	Fenton reaction

2.1.1.2 Superoxide anion ($O_2^{\bullet-}$)

It is well known that superoxide anions are the starting point in the chain production of free radicals (Menvielle-Bourg, 2005). $O_2^{\bullet-}$ is an anionic radical formed by the single electron reduction of oxygen (Fouad, 2008b). If a single electron is accepted by the ground-state O_2, $O_2^{\bullet-}$ is formed. This happens in almost all aerobic cells. The hydroperoxyl radical, which is unstable at physiological pH, dissociates to $O_2^{\bullet-}$. The dissociation can occur *in vivo* by autoxidation reactions and leakage of electrons from mitochondrial electron transport chains, a phenomenon that increases with an increase in oxygen utilisation and is especially important in brain tissue that uses elevated quantities of oxygen for its metabolism. $O_2^{\bullet-}$ are also formed by metal ion-dependant oxidation of epinephrine and norepinephrine and by the action of enzymes such as tryptophane hydroxylase, indoleamine dioxygenase and xanthine oxygenase. Another important source is the respiratory burst of phagocytic cells when they contact foreign particles or immune complexes in response to bacterial infection. Phagocytic cells known to produce $O_2^{\bullet-}$ include neutrophils, monocytes, macrophages and eosinophils (Halliwell *et al.*, 1984). The $O_2^{\bullet-}$ radical does not cross cell membranes on its own or directly attack DNA, proteins or lipid and is therefore not particularly damaging. However, the $O_2^{\bullet-}$ appears to play a central role as other reactive intermediates are formed from it. The dismutation of $O_2^{\bullet-}$ yields hydrogen peroxide (Cui *et al.*, 2004).

$O_2^{\bullet-}$ can also be a reductant of transition metals that are precursors to the formation of the lethal hydroxyl radical (Fouad, 2008b). $O_2^{\bullet-}$ also mobilises small amounts of iron from the iron storage protein ferritin (Halliwell, 2001).

$$O_2^{\bullet-} + \text{ferritin } (Fe^{3+})_n \rightarrow \text{ferritin } (Fe^{3+})_{n-1} + Fe^{2+} + O_2$$

2.1.1.3 Hydrogen peroxide (H_2O_2)

Hydrogen peroxide is not a free radical but falls in the category of ROS. It is an oxidising agent that is not particularly reactive with most biomolecules and appears unable to directly oxidise DNA, lipids and proteins, except for a few proteins that have hyper-reactive thiol groups or menthionine residues (Halliwell, 2001). In biological systems H_2O_2 can be generated from the two electron reduction of oxygen or the dismutation of $O_2^{\bullet-}$, two $O_2^{\bullet-}$ molecules can react to form H_2O_2 and oxygen. This reaction is called a dismutation reaction as the radical reactants produce non-radical products. The enzymes aiding in this process is the superoxide dismutate family (SOD) (Fouad, 2008b; Contestabile, 2001).

$$2\ O_2^{\bullet-} + 2\ H^+ \rightarrow H_2O_2 + O_2$$

Apart from the dismutation of $O_2^{\bullet-}$, H_2O_2 is also produced by enzymes such as *L*-amino acid oxidase, glycolate oxidase and xanthine oxidase. It is also produced by the action of monoamine oxidase B on dopamine, a phenomenon associated with Parkinson's disease. H_2O_2 is also a comparatively inactive molecule but unlike $O_2^{\bullet-}$, it can easily cross cell membranes (Cui *et al.*, 2004). H_2O_2 is not very toxic *per se*, unless it reaches concentrations above physiological levels and its toxicity is thought to be mediated by the hydroxyl radical (Contestabile, 2001). Therefore, the main significance of H_2O_2 lies in that it is the main source of hydroxyl radicals in the presence of transition metal ions (iron and copper). Millimolar levels of H_2O_2 can cause iron ion release from haem proteins such as myoglobin and cytochromes. Iron is required by the human body and its adequate supply in early life is essential for normal brain development. However, iron is dangerous; its ability to transfer single electrons as it oscillates between the ferrous (Fe^{2+}) and ferric (Fe^{3+}) states makes it a powerful catalyst of free radical reactions. Fe^{2+} converts H_2O_2 into hydroxyl radicals, probably the most reactive free radical found *in vivo* (Halliwell, 2001). This conversion occurs according to the Haber-Weiss and Fenton reactions (review table 2.1) (Cui *et al.*, 2004). The reaction can be perpetuated by any reducing agent capable of recycling Fe^{3+} back to Fe^{2+}. Suitable reductants include $O_2^{\bullet-}$ (Halliwell, 2001).

$$Fe^{3+} + O_2^{\bullet-} \rightarrow Fe^{2+} + O_2$$

The ability of H_2O_2 to produce hydroxyl radicals, combined with the membrane permeability of H_2O_2, gives $O_2^{\bullet-}$ and H_2O_2 the ability to affect the integrity of distant molecules within the cell (Cui *et al.*, 2004).

The main source of $O_2^{\bullet-}$ and H_2O_2 generation is the enzyme xanthine oxidase; xanthine + O_2 –xanthine oxidase→ uric acid + $O_2^{\bullet-}$ and H_2O_2 (Gilgun-Sherki *et al.*, 2001). H_2O_2 and $O_2^{\bullet-}$ can then readily be converted to the dangerous hydroxyl radical in the presence of iron or other transition metals (Contestabile, 2001).

2.1.1.4 Hydroxyl radical ($OH^{\bullet}$)

Hydroxyl radicals are extremely reactive oxidising radicals that will react immediately with all biomolecules and bring about damage to lipids, proteins and DNA. Around 1933, Fritz Harber and Joseph Weiss first proposed that $OH^{\bullet}$ was produced when $O_2^{\bullet-}$ and H_2O_2 reacts, this formula was coined the Haber-Weiss reaction (Halliwell *et al.*, 1984):

$$O_2^{\bullet-} + H_2O_2 \rightarrow O_2 + OH^{\bullet} + OH^-$$

About 100 years ago, Henry Fenton observed that the reducing agent, ferrous iron (Fe^{2+}), together with H_2O_2 could oxidise some organic compounds. The mechanism is now known to involve $OH^{\bullet}$, with a key step analogous to the previous reaction but with the electron donor, $O_2^{\bullet-}$ replaced by Fe^{2+}. This reaction is most commonly referred to as the iron catalysed Haber-Weiss reaction or the $O_2^{\bullet-}$-driven Fenton reaction: iron-salt-decomposition of dihydrogen peroxide, generating the highly reactive $O_2^{\bullet-}$, possibly *via* an oxoiron (IV) intermediate (Halliwell *et al.*, 1984).

$$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^{\bullet} + OH^{-}$$

Traces of Fe^{3+} can react further with H_2O_2;

$$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + O_2^{\bullet-} + H^{+}$$

and more reactions are possible;

$$OH^{\bullet} + H_2O_2 \rightarrow H_2O + H^{+} + O_2^{\bullet-}$$

$$O_2^{\bullet-} + Fe^{3+} \rightarrow Fe2^{+} + O_2$$

$$OH^{\bullet} + Fe^{2+} \rightarrow Fe^{3+} + OH^{-}$$

thus provoking a whole series of radical reactions (Halliwell *et al.*, 1984).

In the body the pool of free iron that is available to catalyse the reaction from H_2O_2 to the $OH^{\bullet}$ is, under normal conditions, extremely limited. Red blood cells contain much of the iron in the body. Fortunately, several iron transporters are available to prevent inadvertent release and therefore limit the availability of free iron to catalyse the Haber-Weiss reaction (Fouad, 2008b). Copper also reacts with H_2O_2 to produce $OH^{\bullet}$ with a greater rate constant (Halliwell *et al.*, 1984).

Ascorbic acid can also act as a reductant. Although, ascorbate normally acts as a powerful scavenger of reactive species, in the presence of iron or copper ions it can become a pro-oxidant, stimulating damage to biomolecules by promoting the formation of $OH^{\bullet}$ (Halliwell, 2001). Because of its low half-life (10^{-9} s at 37°C) the direct action of the $OH^{\bullet}$ is confined to regions immediately in the vicinity of its formation. In DNA, the $OH^{\bullet}$ can induce several effects including base and sugar modifications, cross-linking between bases, cross-linking between DNA and protein, strand breaks and formation of adducts. The action of $OH^{\bullet}$ on proteins leads to extensive protein-protein cross-linking. This may be further aggravated in metalloproteins and metalloenzymes by transition metals that act as foyers for the resulting

in site-specific destruction of the critical regions of the molecule. The most extensive studies on the oxidative properties of $OH^{\bullet}$ have been carried out on membrane lipids in which poli-unsaturated fatty acids (PUFAs) are particularly vulnerable to oxidation. The peroxidation of PUFAs by $OH^{\bullet}$ constitutes one of the most severe attacks on cellular integrity (Cui *et al.*, 2004).

2.1.1.5 Nitric oxide ($NO^{\bullet}$)

Several forms of ROS contain nitrogen or chlorine, in addition to oxygen. $NO^{\bullet}$, a nitrogen-based free radical is a gaseous, poorly reactive free radical. Vascular endothelium produces $NO^{\bullet}$ as do neutrophils and macrophages form arginine using the enzyme nitric oxide synthase (NOS). This event can be stimulated by cytokines, tumour necrosis factor (TNf) or interleukins. Inhibition of production is known to reduce microbicidal and tumouricidal activities of macrophages (Fouad, 2008b). $NO^{\bullet}$ also acts as an important signal transducer and effects metabolic processes in vascular smooth muscles, neurons and other cell types (Cui *et al.*, 2004), particularly in the brain where the constitutive, neuronal nitric oxide synthase (*n*NOS), endothelial (*e*NOS) and the inducible (*i*NOS) isoforms are present in different cellular components. Therefore, $NO^{\bullet}$ plays an essential role in brain vasoregulation and neurotransmission (Fouad, 2008b). When escaping controlled production $NO^{\bullet}$ may damage brain tissue through nitrosylation of proteins, DNA breaking and other molecular interactions. At physiological pH $NO^{\bullet}$ reacts with $O_2^{\bullet-}$ giving rise to the non-radical peroxynitrite (Contestabile, 2001).

2.1.1.6 Peroxynitrite ($ONOO^-$)

Peroxynitrite is a powerful oxidant and can be produced by the reaction of $NO^{\bullet}$ with $O_2^{\bullet-}$, a radical-radical reaction in which $ONOO^-$ is formed (Cui *et al.*, 2004).

$$O_2^{\bullet-} + NO^{\bullet} \rightarrow ONOO^-$$

At physiological pH $ONOO^-$ rapidly protonates and the resulting peroxynitrous acid (ONOOH) leads to the formation of a wide range of cytotoxic agents with nitrating, oxidising and hydroxylating abilities. Thus, generation of $ONOO^-$ *in vivo* can lead to oxidation and nitration of lipids, DNA and the amino acid residues on proteins through the formation of the highly reactive $OH^{\bullet}$ (Halliwell, 2001).

$$ONOO^- + H^+ \rightarrow ONOOH$$

$$ONOOH \rightarrow OH^{\bullet} + NO_2$$

2.1.1.7 Hypochlorous acid (HOCl)

Hypochlorous acid, which is a powerful oxidant, is produced by activated polymorph nuclear cells as a major bactericidal agent (Fouad, 2008b). It is generated by activated neutrophils from H_2O_2 by the haem containing enzyme, myeloperoxidase (Cui *et al.*, 2004). This reaction occurs in the neutrophills phagocytic vacuole after fusion with the myeloperoxidase containing lysosomal vesicles (Fouad, 2008b).

$$H_2O_2 + Cl^- \rightarrow HOCl + OH^\bullet$$

HOCl can cross cell membranes and in the presence of transitional metal ions or through reaction with reductants that are one-electron donors generate $OH^\bullet$. Important examples include $O_2^{\bullet-}$ and ferrous iron (Fe^{2+}) (Fouad, 2008b):

$$HOCl + O_2^{\bullet-} \rightarrow OH^\bullet + Cl^- + O_2$$

$$HOCl + Fe^{2+} \rightarrow OH^\bullet + Cl^- + Fe^{3+}$$

Hypochlorous acid has been shown to be capable of initiating lipid peroxidation, and combined with H_2O_2, to damage DNA altering intracellular free Ca^{2+} and pH. It may contribute to tissue damage during the inflammation process. This latter event may result from the activation of collagenases or the inactivation α-1 antiproteinase (Fouad, 2008b).

2.1.2 Sources of free radicals and related species

Essentially all of the cells in the brain can produce oxidants (Ischiropoulos & Beckman, 2003).

2.1.2.1 Endogenous sources

(a) Autoxidation

Molecules that undergo autoxidation, a by-product of the aerobic internal milieu, include catecholamine, haemoglobin, myoglobin, reduced cytochrome *c* and thiol. The autoxidation of any of the above molecules in a reaction results in the reduction of the oxygen diradical (O_2) and the formation of ROS. $O_2^{\bullet-}$ is the primary radical formed. Ferrous ion (Fe^{2+}) can have its electron "stolen" from it by oxygen to produce $O_2^{\bullet-}$ and ferric ion (Fe^{3+}) by the process of autoxidation (Fouad, 2008b).

(b) Enzymatic oxidation

A variety of enzyme systems are capable of generating significant amounts of free radicals, including xanthine oxidase (activated in ischemia-reperfusion), prostaglandin synthase, lipoxygenase, aldehyde oxidase and amino acid oxidase. The enzyme myeloperoxidase produced in activated neutrophils utilises H_2O_2 to oxidise chloride ions into the powerful oxidant HOCl (Fouad, 2008b).

(c) Respiratory burst

Phagocytic cells destroy bacteria or virus infected cells with an oxidative burst of $NO^{\bullet}$, $O_2^{\bullet-}$, H_2O_2 and OCl^- (Ames *et al.*, 1993). Phagocytic cells consume large amounts of oxygen during phagocytosis, the most of it accounted for in terms of $O_2^{\bullet-}$ production. These phagocytic cells possess a membrane bound flavoprotein, cytochrome-*b-245* NADPH oxidase system. Cell membrane enzymes such as the NADPH-oxidase exist in an inactive form and exposure to immunoglobulin-coated bacteria, immune complexes, complement *5a* or leukotriene activates the enzyme. This activation initiates a respiratory burst at the cell membrane to produce $O_2^{\bullet-}$, in turn H_2O_2 is then formed from $O_2^{\bullet-}$ with subsequent generation of $OH^{\bullet}$ and HOCl (Fouad, 2008b).

(d) Subcellular organelles

Organelles such as mitochondria, chloroplasts, microsomes, peroxisomes and nuclei have been shown to generate $O_2^{\bullet-}$. Mitochondria are the main cellular organelle for cellular oxidation reactions and the main source of reduced oxygen species in the cell. The leaks in the mitochondrial electron transport system allow O_2 to accept a single electron forming $O_2^{\bullet-}$. It has been shown that $O_2^{\bullet-}$ production by the mitochondria increases in two conditions; either when the oxygen concentration is greatly increased or when the respiratory chain becomes fully reduced (as happens during ischemia). Microsomes are responsible for 80 % of the H_2O_2 produced *in vivo* at 100 % hyperoxia sites (Fouad, 2008b). Peroxisomes, which are organelles responsible for degrading fatty acids and other molecules, produce H_2O_2 as a by-product, but not $O_2^{\bullet-}$ under physiologic conditions (Ames *et al.*, 1993). Although, the liver is the primary organ where peroxisomal contribution to the overall H_2O_2 production is significant, other organs that contain peroxisomes are also exposed to these H_2O_2 generating mechanisms (Fouad, 2008b).

(e) Transition metals ions

Iron and copper play a major role in the generation of free radical injury and the facilitation of lipid peroxidation. Transition metal ions participate in the Haber-Weiss reaction that generates $OH^{\bullet}$ from $O_2^{\bullet-}$ and H_2O_2. The Haber-Weiss reaction accelerates the non-enzymatic oxidation of molecules such as epinephrine and glutathione that generates $O_2^{\bullet-}$ and H_2O_2 and subsequently $OH^{\bullet}$ (Fouad, 2008b; Ames, *et al.*, 1993).

2.1.2.2 Exogenous sources

(a) Xenobiotics

Xenobiotics are chemicals present in organisms that are not normally produced or expected to be present. The body removes xenobiotics by metabolism, deactivation and secretion via various pathways. This is catalysed by a wide variety of enzymes resulting in the production of xenobiotic free radical products capable of redox cycling with oxygen. The redox cycling of xenobiotics results in a cascade in which various radicals capable of causing cellular damage can arise (Metosh-Dickey *et al.*, 1998). Various xenobiotics including, paraquat, paracetamol and bleomycin, cause tissue damage as consequence of free radical generation (Young & Woodside, 2001).

(b) Radiation

Radiotherapy may cause tissue injury mediated by free radicals. Electromagnetic radiation (x rays, γ rays) and particulate radiation (electrons, photons, neutrons, α and β particles) generate primary radicals by transferring their energy to cellular components such as H_2O. These primary radicals can undergo secondary reactions with O_2 or with cellular solutes (Fouad, 2008b).

(c) Tobacco smoking

It has been shown that tobacco smoke oxidants severely deplete intracellular antioxidants in the lung cells *in vivo* by a mechanism that is related to oxidant stress. These include aldehydes, epoxides, peroxides and other free radicals that may be sufficiently long lived to survive till they cause damage to the alveoli. The oxides of nitrogen ($NO^{\bullet}$) in cigarette smoke cause oxidation of macromolecules and deplete antioxidant levels. Peroxyl radicals and carbon centred radicals are also present. These radicals are likely to contribute significantly to the pathology of smoking (Ames *et al.*, 1993).

(d) Inorganic particles

Inhalation of inorganic particles (e.g. asbestos, quartz, silica) can lead to lung injury that seems at least in part to be mediated by free radical production (Fouad, 2008b).

2.1.3 Targets of free radicals and related species

2.1.3.1 Lipids

Lipids are the most susceptible biomolecules to free radical attack, especially $OH^{\bullet}$ (Fouad, 2008b). Cell membranes are a rich resource of PUFAs making membrane lipids highly susceptible to attack by oxidising agents; a process that is called lipid peroxidation (Devasagayam *et al.*, 2004). The damage caused by lipid peroxidation is highly detrimental to the functioning of the cell. A primary effect of lipid peroxidation is decreased membrane fluidity that alters membrane properties and can significantly disrupt membrane-bound proteins (Beckman *et al.*, 1998). The oxidation of lipids by ROS generally consists of three steps:

(1) Initiation

Initiation of lipid peroxidation is due to the attack of any species that has sufficient reactivity to abstract a hydrogen atom from PUFAs forming a lipid radical ($L^{\bullet}$). Since, a hydrogen atom has only one electron this leaves behind an unpaired electron on the carbon atom. The carbon radical in a PUFA tends to be stabilised by a molecular rearrangement to produce a conjugated diene which rapidly reacts with O_2 to give a hydroperoxy radical ($LOO^{\bullet}$, peroxyl radical) (Cui *et al.*, 2004; Halliwell *et al.*, 1984).

(2) Propagation

The propagation step involves the hydroperoxy radical, in its turn, starting a self-perpetuating chain reaction in which most of the membrane lipids are converted to a variety of lipid hydroperoxides (LOOH) and cyclic peroxides. The lipid hydroperoxides can be further degraded to hydrocarbons, alcohols, ether, epoxides and aldehydes, these toxic by-products formed can have effects at a site away form the area of generation behaving as second messengers. Of these products, malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) have the additional ability to inactivate phospholipids, proteins and DNA by bringing about cross-linking between these molecules (Cui *et al.*, 2004; Halliwell *et al.*, 1984).

(3) Termination

Termination happens when the chain reaction is stopped by interactions between the radicals themselves or between the radicals and antioxidants giving rise to non-radical products or unreactive radicals. The major termination reaction involves the reaction of hydroperoxy radicals or lipid radicals with α-tocopherol (α-TOH) forming more stable tocopherol phenoxyl radicals that are not involved in further chain reactions (Cui *et al.*, 2004; Halliwell *et al.*, 1984). The process of lipid peroxidation is as follows (Devasagayam *et al.*, 2004);

$LH + R^{\bullet}(OH^{\bullet}) \rightarrow L^{\bullet} + RH(H_2O)$ ---------- **Initiation** by a free radical

$L^{\bullet} + O_2 \rightarrow LOO^{\bullet}$

$LOO^{\bullet} + LH \rightarrow L^{\bullet} + LOOH$ } **Propagation**

$LOO^{\bullet} + \alpha\text{-}TO^{\bullet} \rightarrow LOOH + \alpha\text{-}TO^{\bullet}$ ---------- **Termination** by an antioxidant

$LOOH \rightarrow LO^{\bullet}, LOO^{\bullet}$, aldehydes. ---------- Continuing the chain reaction.

2.1.3.2 DNA

Oxidative damage to nucleic acids of the DNA structure is a result of interaction with ROS. Free radicals such as $OH^{\bullet}$ react with nucleic acids by addition to bases or abstractions of hydrogen atoms from the sugar moiety, single- and double-strand breaks in the backbone and cross-links to other molecules. This ultimately leads to significant oxidative damage to DNA. Damage caused to DNA can result in mutagenesis and carcinogenesis (Devasagayam *et al.*, 2004; Beckman *et al.*, 1998). DNA damage is counteracted in cells by DNA repair that is a basic and universal process to protect the genetic integrity of organisms. If left unrepaired, oxidative DNA damage can lead to detrimental biological consequences in organisms including cell death, mutations and transformation of cells to malignant cells (Dizdar & Jaruga, 2005).

2.1.3.3 Proteins

Proteins and nucleic acids seem to be less susceptible than PUFAs to free radicals in that there seems to be less possibility in the formation of rapidly progressing chain reactions. Random attacks of radicals on proteins are unlikely unless extensive. This happens only if radicals are allowed to accumulate (which is not likely in normal cells) or if the damage is focused on a particular site of the protein. One way that the damage is focused is if the

protein binds a transition metal ion (Fouad, 2008b). This interaction with transition metal ions can generate a range of stable as well as reactive products such as protein hydroperoxides that can generate additional radicals. Free radical damage to protein can result in loss of enzyme activity. Although most oxidised proteins that are functionally inactive are rapidly removed, some can gradually accumulate with ageing as well as various diseases (Devasagayam *et al.*, 2004).

2.1.4 Useful purposes of free radicals and related species

It has to be emphasised that ROS and RNS are both produced in a well-regulated manner, to help maintain homeostasis at the cellular level, and in normal healthy tissues, play an important role as inter and intra-cellular signalling molecules and defence against intrusion of foreign organisms. Signal transduction may be generally described as the process through which cellular components (i.e. contractile elements or the transcription machinery) receive information from each other and from outside the cell and transmit that information intracellularly to elicit a response (Cui *et al.*, 2004). Most cells can produce $O_2^{\bullet-}$, H_2O_2 and $NO^{\bullet}$ on demand. Hence, it is worth emphasising the important beneficial role of free radicals that include;

- detoxification of xenobiotics by cytochrome *P450*,
- apoptosis of affected or defective cells,
- killing of micro-organisms and cancer cells by macrophages and cytotoxic lymphocytes,
- oxygenases (e.g. cyclo-oxygenases/COX, lipoxygenase/LOX) for the generation of prostaglandins and leukotrienes which have many regulatory functions and
- generation of ATP (universal energy currency) from ADP in the mitochondria via oxidative phosphorylation (Devasagayam *et al.*, 2004).

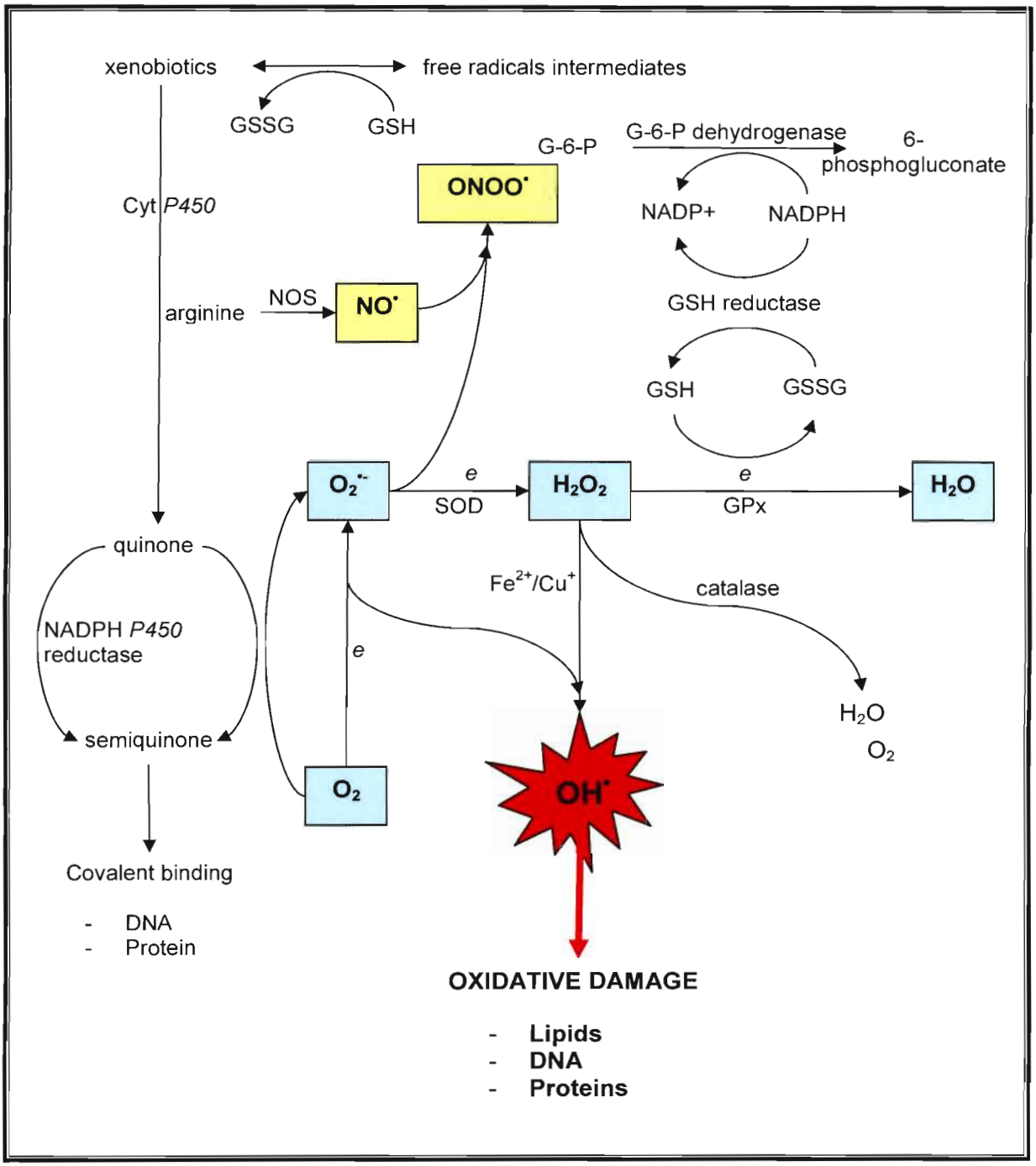


Figure 2.1 ROS/RNS the causative factors in the occurrence of oxidative stress in living cells (Andersen, 2004; Sigma-Aldrich, 2008a).

2.2 Antioxidants

Antioxidants are substances that neutralise free radicals or their actions hindering the process of oxidation (Devasagayam *et al.*, 2004; Fouad, 2008b). The body has developed several endogenous antioxidant systems to deal with the production of reactive species. Endogenous or exogenous antioxidant molecules that have the ability, even at low concentrations, to delay or inhibit the oxidation of a substrate, generally inactivate the excess of ROS (Cui et al., 2004). These systems can be divided into 2 groups; enzymatic and non-enzymatic (see figure 2.4 and section 2.2.2) (Fouad, 2008b). Thus, cells are equipped with an impressive repertoire of antioxidant enzymes as well as small antioxidant molecules mostly derived from dietary fruits and vegetables. These antioxidant defences include;

- enzymatic scavengers such as SOD, catalase and glutathione peroxidase (GPx),
- hydrophilic radical scavengers such as ascorbate, urate and glutathione (GSH),
- lipophillic radical scavengers such as tocopherols, flavonoids, carotenoids and ubiquinol,
- enzymes involved in the reduction of oxidised forms of small molecular antioxidants (GSH reductase, dehydroascorbate reductase) or responsible for the maintenance of protein thiols (thioredoxin reductase) and
- the cellular machinery that maintains a reducing environment to maintain a pro-oxidant/antioxidant equilibrium (e.g. glucose-6-phosphate dehydrogenase which regenerates NADPH) (Beckman *et al.*, 1998).

The actions of reactive species are opposed *in vivo* by this balanced and coordinated system of antioxidant defences (Halliwell, 2001). The enzymatic and non-enzymatic antioxidant systems are intimately linked to one another and appear to interact with one another (Fouad, 2008b). Critical structures in the cell are protected not only by the availability of several types of antioxidants but also by other mechanisms such as the repair or elimination of damaged molecules by appropriate enzymes (Cui *et al.*, 2004). Despite this complex defence system, there are no known endogenous enzymatic antioxidant systems for $OH^{\bullet}$ (Fouad, 2008b).

2.2.1 Mode of actions of antioxidants

Antioxidants, capable of neutralising free radicals or their actions, act at different stages of the free radical chain reaction. They act at the levels of prevention, interception and repair. Preventative antioxidants attempt to stop the formation of ROS and include, SOD and

catalase. Interception of free radicals is mainly by radical scavenging while at the secondary level scavenging of peroxyl radicals are affected. The effectors include various antioxidants like vitamin C and E, glutathione, other thiol compounds, carotenoids and flavonoids. At the repair and reconstitution level, mainly repair enzymes are involved (Devasagayam *et al.*, 2004). Thus, antioxidants may intervene at any of the three major steps: initiation, propagation or termination of the oxidative process (Cui *et al.*, 2004). Antioxidant defence mechanisms include:

- Chain-breaking reactions e.g. α-tocopherol which acts in the lipid phase to trap radicals that spread oxidation to neighbouring molecules (Foaud, 2008a).

- Reducing the concentration of ROS e.g. glutathione (Foaud, 2008a). They may do so by removing or lowering the local concentrations of one or more of the participants in this reaction such as oxygen, ROS or metal ions which catalyse oxidation (Fe^{3+}, Cu^{+2}) (Gilgun-Sherki *et al.*, 2001).

- Scavenging initiating radicals e.g. SOD which acts in the aqueous phase to trap $O_2^{\bullet-}$ free radicals and the removal of O_2, scavenging ROS/RNS or their precursors (Foaud, 2008a).

- Chelating the transition metal catalysts by the binding of metal ions needed for the catalysis of ROS. A group of compounds serves an antioxidant function by sequestration of transition metals that are well-established pro-oxidants. In this way, transferring, lactoferrin and ferritin function to keep iron induced oxidant stress in check and ceruloplasmin and albumin as copper sequestrants (Foaud, 2008a).

- Antioxidants may also act by enhancing the antioxidant defences of the cell by generation and up-regulation of endogenous antioxidant defences (Cui *et al.*, 2004).

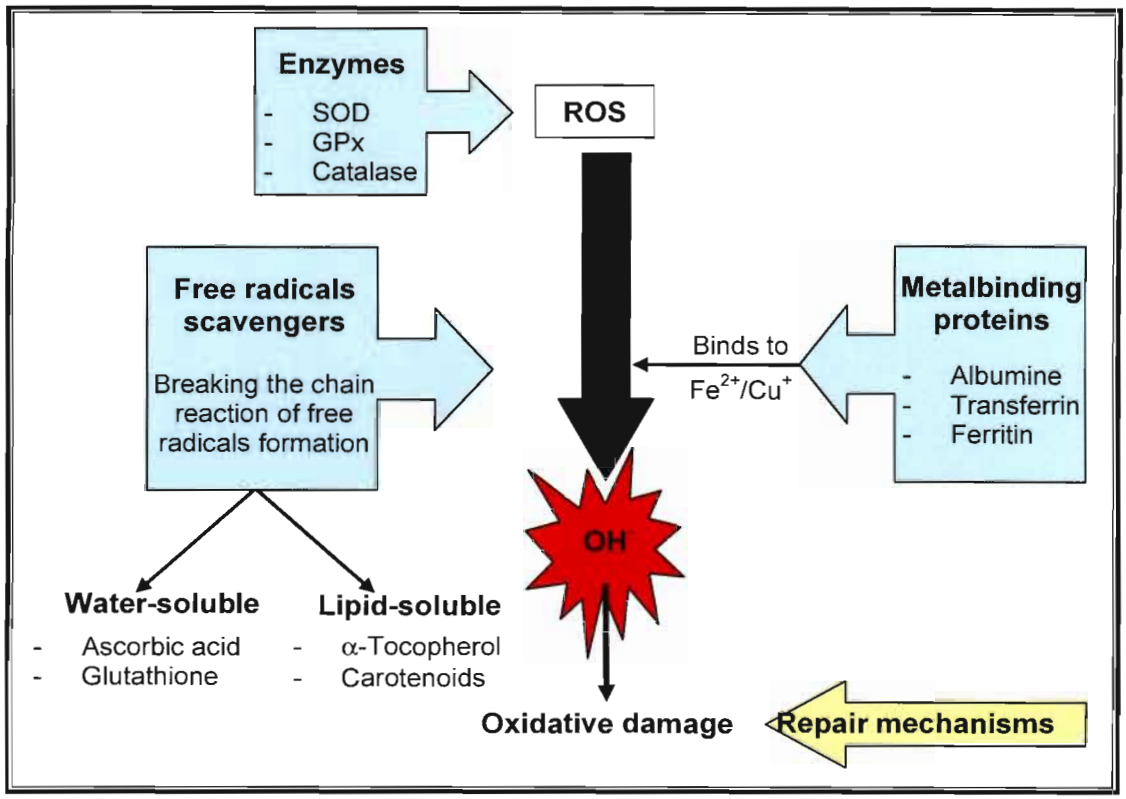


Figure 2.2 Antioxidant defence against ROS (Young & Woodside, 2001).

2.2.2 Antioxidant defences

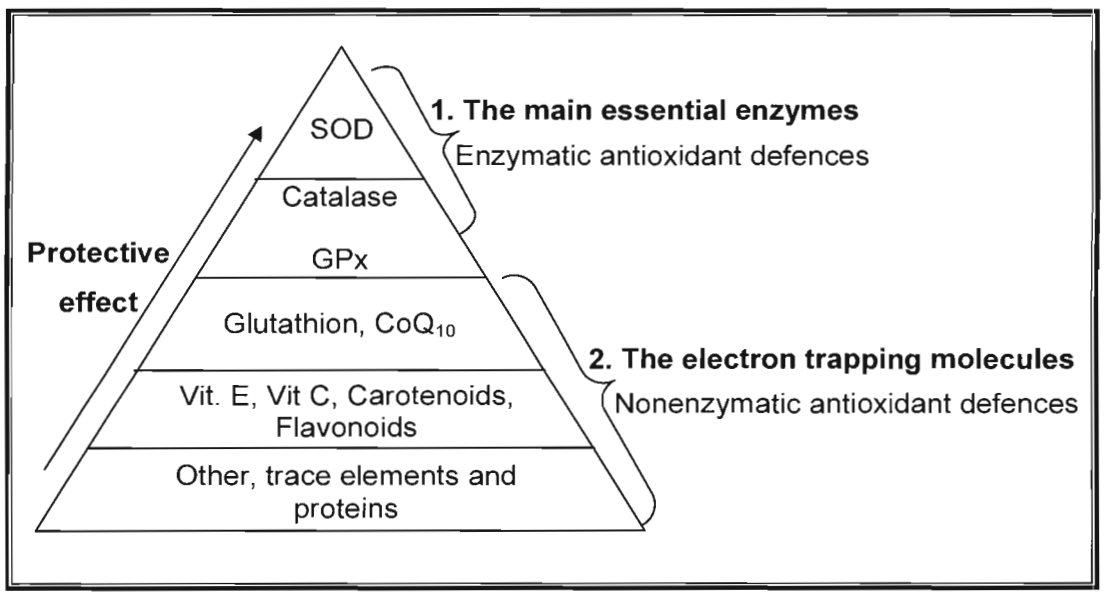


Figure 2.3 The two main groups of antioxidant defences in the body (Menvielle-Bourg, 2005).

2.2.2.1 Nonenzymatic antioxidant defences

(a) α-Tocopherol (vitamin E)

α-Tocopherol is the most potent lipophyllic antioxidant that can break the propagation of the free radical chain reaction in the lipid part of the biological membrane (Gilgun-Sherki *et al.*, 2001). The main function of α-tocopherol is to prevent the peroxidation of membrane phospholipids and avoid cell membrane damage (Fouad, 2008a). α-Tocopherol deficiency influences the activities of SOD, catalase and GPx and is thus very important for the enzymatic antioxidant defence of the body. It has shown some promise in the treatment of Alzheimer's disease, Parkinson's disease and Huntington's disease (Gilgun-Sherki *et al.*, 2001).

(d) Ascorbic acid (Vitamin C)

Humans cannot synthesise this water-soluble chain breaking antioxidant. Vitamin C has a variety of roles, one of which is the regeneration of vitamin E. It also inhibits peroxidation of membrane phospholipids and act as a scavenger of free radicals. Vitamin C in combination with other vitamins has been shown to have some benefit for treatment/prevention of oxidative stress related diseases (Gilgun-Sherki *et al.*, 2001).

2.2.2.2 Enzymatic antioxidant defences

(a) Superoxide dismutase (SOD)

Superoxide dismutase, a large protein molecule, is an endogenously produced intracellular enzyme present in essentially every cell in the body. A group of metalloenzymes with various prosthetic groups represents cellular SOD. SOD appears in three forms;

(1) CuZnSOD in the cytoplasm containing copper and zinc at its active site, with two isoforms,

(2) MnSOD in the mitochondria, containing manganese at its active site and

(3) CuSOD, extracellular SOD recently described as containing copper (Gilgun-Sherki *et al.*, 2001; Fouad, 2008a).

Trace metals such as copper, zinc and manganese are essential for maintaining the antioxidant activity of SOD (Gilgun-Sherki *et* al., 2001). SOD is considered fundamental in the process of eliminating ROS by reducing $O_2^{\bullet-}$ to form H_2O_2. SOD enzymes accelerate the dismutation of $O_2^{\bullet-}$ to H_2O_2; they thus diminish damage by $O_2^{\bullet-}$ and related reactions. SOD reduces $O_2^{\bullet-}$ that would otherwise lead to the reduction of Fe^{3+} to Fe^{2+} and thereby promote $OH^{\bullet}$ formation (Fouad, 2008a). The dismutation of $O_2^{\bullet-}$ is called the Farber reaction (Gilgun-Sherki *et al.*, 2001).

(b) Glutathione peroxidase enzyme (GPx)

GPx is an enzyme found in the cytoplasm and mitochondria (Andersen, 2004). The glutathione redox cycle is a central mechanism for reduction of intracellular hydroperoxides. GPx reduces H_2O_2 to H_2O and in the processes oxidises glutathione (GHS) which is converted to reduced gluthatione (GSSG). Reduction of the oxidised form of glutathione (GSSG) is then catalysed by glutathione reductase (Fouad, 2008a).The concentrations of GSH and GSSG and their ratio, reflect the redox state of the cell and are crucial for an efficient ROS detoxification (Red Laboratories, 2008).

These enzymes also require trace metal cofactors for maximal efficiency, including selenium for GPx, copper, zinc or manganese for SOD and iron for catalase (Fouad, 2008a).

(c) Catalase enzyme

Catalase is a member of the peroxidases that contains haem at its active site. It is found in peroxisomes in most tissue and they are believed to cross membranes easily (Gilgun-Sherki *et al.*, 2001). Catalase is present in all body organs being especially concentrated in the liver and erythrocytes. The brain, heart and skeletal muscle contain only low amounts. Catalase and GPx seek out H_2O_2 and convert it to H_2O and O_2 (Fouad, 2008a).

An increase in the production of SOD without a subsequent elevation of catalase or GPx leads to the accumulation of H_2O_2 which is converted into the $OH^{\bullet}$ (Fouad, 2008a).

The respective enzymes, GPx, catalase and SOD, that interact with $O_2^{\bullet-}$ and H_2O_2 are tightly regulated through a feedback system. Excessive $O_2^{\bullet-}$ inhibits GPx and catalase to modulate the equation from H_2O_2 to H_2O. Likewise, increased H_2O_2 slowly inactivates SOD. Meanwhile, catalase and GPx, by reducing H_2O_2, conserve SOD and SOD, by reducing $O_2^{\bullet}$, conserves catalase and GPx. Through this feedback system, steady low levels of SOD, GPx and catalase, as well as $O_2^{\bullet-}$ and H_2O_2 are maintained which keeps the entire system in a fully functioning state. When catalyse activity is insufficient to metabolise the H_2O_2 produced

SOD will increase the tissue oxidant activity. Hence, it was found that the antioxidant enzymes function as a tightly balanced system, any disruption of this system would lead to promotion of oxidation (Fouad., 2008a).

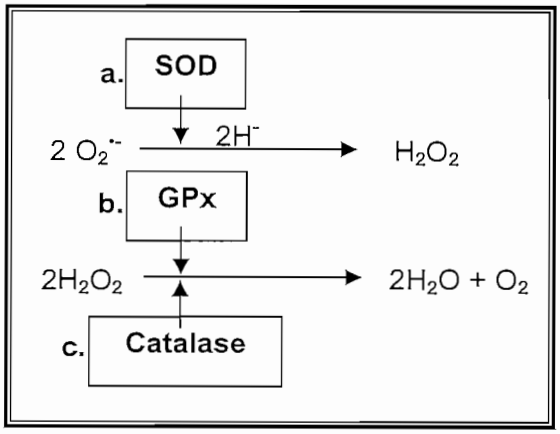


Figure 2.4 The three major enzymatic antioxidant defence system and the role of antioxidant enzymes in the inactivation of superoxide anions (Menvielle-Bourg, 2005).

These three antioxidant enzymes, glutathione peroxidase, catalase and superoxide dismutase require micronutrients as cofactors such as selenium, iron, copper, zinc and manganese for optimum catalytic activity and effective antioxidant defence (Singh *et al.*, 2004).

2.2.2.3 Other antioxidants

(a) Glutathione (GSH)

Glutathione (γ-L-glutamyl-L-cysteinyl-glycine) is a major intracellular antioxidant and its antioxidant activity depends upon the thiol group within the molecules. GSH is the most abundant thiol-reducing agent in cells (Andersen, 2004). GSH acts as a radical scavenger ($OH^{\cdot}$ and $^1O_2$) through two different mechanisms, either by directly reducing free radicals or enzymatically as the donor of electrons for the reduction of peroxides catalysed by GPx. The GSH redox cycle regenerates GSSG (the oxidised form of GSH) to GSH through the catalytic action of GSSG reductase together with the oxidation of NADPH (Contestabile, 2001).

The weakening of the antioxidant defences, related to inadequate levels of GSH, are a cause of some functional changes occurring in the aging brain and neurodegenerative diseases. Therefore, to increase the availability of GSH to neurons is a logical therapeutic target in neural impairment to oxidative stress (Contestabile, 2001).

(b) Coenzyme Q_{10} (CoQ_{10})

Coenzyme Q_{10} (ubiquinone) is a mobile and lipid-soluble compound in the hydrophobic core of the phospholipid bilayer of the inner membrane of the mitochondria. CoQ_{10} is found in almost every living cell. CoQ_{10} serves as an important antioxidant in both mitochondria and lipid membranes (Gilgun-Sherki *et al.*, 2001). Far beyond producing energy, CoQ_{10} can protect the body from destructive free radicals and enhance immune defences (Fouad, 2008a).

Based on findings that energy metabolism and oxidative damage in the mitochondria play a role in the pathogenesis of neurodegenerative diseases, several studies have suggested that CoQ_{10} could exert a beneficial therapeutic effect. CoQ_{10} levels are known to decrease with aging (Gilgun-Sherki *et al.*, 2001).

(c) Uric acid

Uric acid acts as an endogenous radical scavenger and antioxidant. Uric acid is a powerful scavenger of $^1O_2$, peroxyl radical and $OH^{\cdot}$ (Fouad, 2008a).

(d) Albumin

Albumin is an antioxidant that can bind copper tightly and iron weakly to its surface. The bound metals would still be available for participation in Harber-Weiss reaction, but any generated $OH^{\cdot}$ would immediately react with and be scavenged by albumin. The resultant protein damage is biologically insignificant because of the large amount of available albumin and free radicals would be inactivated before reacting with other more vital protein structures (Fouad, 2008a).

Other plasma proteins namely ceruloplasmin and transferrin have also shown antioxidant activity. For example, in human plasma there is an excess of the iron-binding protein transferrin, leading to spare iron ion binding capacity so that no iron exists "free". Similarly, most, if not all, plasma copper is attached to ceruloplasmin or other copper-binding proteins and does not occur in a "'free" state. Within cells, excess iron is stored in ferritin and copper in metallothionein. Some iron ions and presumably also copper ions are always in transit within the cell (Cui *et al.*, 2004).

When cells are mechanically damaged or undergo necrotic death, these metal ions can be released to propagate free radical reactions in the surrounding area, therefore the importance of these plasma proteins (Halliwell, 2001).

(e) Melatonin

Melatonin (N-acetyl-5-methoxytryptamine) is a strong endogenous free radical scavenger. Its potency in neutralising $OH^{\bullet}$ is at least fivefold higher than GSH. However, its low plasma concentration makes it, at first glance, a not very likely candidate for an endogenous role in physiological antioxidant systems (Contestabile, 2001). It prevents lipid peroxidation by interfering with both chain initiation and propagation, a dual effect that has not been shown to occur with other known antioxidants. Other neurophysiologic aspects of melatonin are that it stimulates the activity of the antioxidant enzyme, GPx. Melatonin has also been reported to inhibit the activity of NOS which produces $NO^{\bullet}$ in neurones (Cui *et al.*, 2004).

(f) Synthetic antioxidants (drugs)

Several pharmaceutical agents have been found to exert an antioxidant effect:

- Xanthine oxidase inhibitors: allopurinol, folic acid (Fouad, 2008a).
- NADPH inhibitors: adenosine, Ca^{2+}-channel blockers (Fouad, 2008a).
- Recombinant SOD and catalase (more stable conjugated forms of these enzymes) (Fouad, 2008a).
- Selenium supplements (Cui *et al.*, 2004).
- Albumin (Fouad, 2008a).
- Inhibitors of iron redox cycling: deferoxamine, apotransferrin, caeruloplasmin (Fouad, 2008a).
- Some drugs such as probucol (hypocholesterolemic), salicylates (anti-inflammatory), 21-aminosteroids or "lazaroids" (structurally related to glucocortecoids), dimethyl sulfoxide and dimethylurea have all been found to be able to scavenge free radicals under certain experimental conditions (Cui *et al.*, 2004).

(g) Plants

Several natural products, often part of the human diet, are sources of antioxidants and therefore of potential interest in prevention and possibly treatment of brain aging and

neuropathology related to oxidative stress (Contestabile, 2001). Through the years many plants have been identified as natural resources of compounds conveying potential antioxidant properties. These compounds such as flavonoids, anthocyanins and carotenoids could contribute to the antioxidant defence mechanism as free radical scavengers (Cui *et al.*, 2004). Plants and plant compound(s) as antioxidants are discussed in section 2.5.

Table 2.2 ROS and their corresponding neutralising antioxidants and also additional antioxidants (Singh *et al.*, 2004).

ROS	Antioxidants (endogenous)		Antioxidants (exogenous)
	Direct role	Indirect role	
$OH^{\bullet}$	Glutathione peroxidase (cofactor selenium)		Vitamin C, lipoic acid
Lipid peroxide (LOO•)	Glutathione peroxidase (cofactor selenium)		Vitamin E, β-carotene
$O_2^{\bullet-}$	Superoxide dismutase (cofactor Cu/Zn/Mn)	Ceruloplasmin (Cu) Metallothionin (Cu) Albumin (Cu)	Vitamin C
H_2O_2	Catalase (cofactor iron)	Transferin (iron) Ferritn (iron) Myoglobin (iron)	Vitamin C, β-carotene, lipoic acid
Pro-oxidant/antioxidant equilibrium	Thiols (GSH, lipoic acid, N-acetyl cysteine) NADPH and NADH Ubiquinone	Billirubin Uric acid	Flavonoids

2.3 Oxidative stress

The various antioxidant defence systems of the human body do not completely prevent damage to biomolecules via reactive species, they control the levels of such species but do not eliminate them (Halliwell, 2001). Problems occur when production of ROS exceeds their elimination by the natural antioxidant defence system or when the latter is damaged. This imbalance between cellular production of ROS and the ability of cells to efficiently defend against them is called oxidative stress (Cui *et al.*, 2004). Oxidative stress is the natural consequence of the oxygen metabolism (Menvielle-Bourg, 2005).

Oxidative stress explains the relation between free radicals and diseases. In a normal healthy human body, the generation of pro-oxidants in the form of ROS and RNS are effectively kept in check by the various levels of antioxidant defences. However, when it is exposed to adverse physicochemical, environmental or pathological agents this delicately maintained balance is shifted in favour of pro-oxidants resulting in oxidative stress

(Devasagayam *et al.*, 2004). Oxidative stress result from the generation of reactive species at an abnormally high rate, insufficient antioxidant defence, liberation of transition metal ions so that more $OH^{\bullet}$ are formed from H_2O_2 and any combination of the above (Cui *et al.*, 2004).

It can cause cellular damage and subsequent cell death mainly by apoptosis in neurodegeneration (Gilgun-Sherki *et al.*, 2001). Because of their high chemical reactivity, levels of ROS in excess of the normal needs of the cell, may indiscriminately damage the cell's structural and functional integrity. This they do, as indicated earlier, either by directly modifying cellular DNA, proteins and lipids or by initiating chain reactions that can bring about extensive oxidative damage to these critical molecules (Cui *et al.*, 2004).

Oxidative stress has been implicated in the process of ageing and in the etiology of several human diseases (Devasagayam *et al.*, 2004). This includes diseases such as atherosclerosis, diabetes, ischemia/reperfusion (I/R) injury, inflammatory diseases (rheumatoid arthritis, inflammatory bowel disease and pancreatitis), cancer, neurodegenerative diseases, hypertension, ocular diseases (cataract, senile muscular degeneration and retrolental fibroplasia), pulmonary diseases and haematological diseases (Cui *et al.*, 2004).

Cells subjected to oxidative stress may adapt, raising their antioxidant defences and/or repair capacity, so that they are more resistant to subsequent injury (Cui *et al.*, 2004). Apart from antioxidant defences, the body is also equipped with several repair systems that is very important in preventing the accumulation of oxidative damaged biomolecules e.g. multiple enzymes exist that can repair free radical damage to DNA bases, oxidised lipids are rapidly removed from membranes during normal membrane turnover (Halliwell, 2001).

If the body cannot fight oxidative stress by antioxidant defence or repair, the end is cell death. Ultimately, oxidative stress can lead to the death of neurons either by necrosis or by programmed cell death, called apoptosis (Del Rio *et al.*, 2002). Not only might oxidative stress result in accidental damage to cells but it might also actively trigger intracellular signalling pathways that lead to cellular demise, in other words programmed cell death/apoptosis (Andersen, 2004).

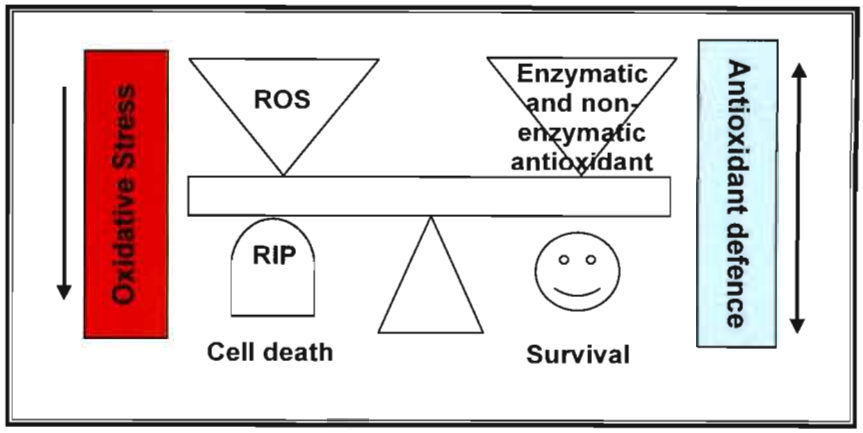


Figure 2.5 The balance of ROS generation and antioxidant systems. An imbalance of both systems due to either excessive production of ROS (left) or reduced antioxidant defence (right) leads to oxidative stress (Schulz *et al.*, 2000).

2.3.1 Consequences of oxidative stress

(a) Apoptosis

Apoptosis, or programmed cell death, is a completely normal process in living organisms and describes the morphological processes leading to controlled cellular self-destruction/death. In the body several hundred thousand cells are produced every second by mitosis and a similar number die by apoptosis for the maintenance of homeostasis upon physiological and pathological conditions and for removal of defect and therefore harmful cells. Deregulation of apoptotic signalling can play a role in various diseases like cancer, persistent infections, neurodegenerative disease's, acquired immunity defect syndrome (AIDS) and ischemia (Gewies, 2003).

A wide range of stimuli can trigger apoptosis; it constitutes a system for the removal of unnecessary, aged or damaged cells that is regulated by the interplay of proapoptotic and antiapoptotic proteins Bcl-2 and Bcl-xl forming heterodimers that block the survival-promoting activity of Bcl-2 and Bcl-xl. The pro-apoptotic proteins act at the surface of the mitochondrial membrane to decrease the mitochondrial trans-membrane potential and promote leakage of cytochrome *c*. In the presence of dATP cytochrome *c* complexes with and activates Apaf-1. Activated Apaf-1 binds to downstream caspase and processes them into proteolytically active forms. This begins a caspase cascade resulting in apoptosis. The removal of dying cells by phagocytes occurs in an orderly manner without eliciting an inflammatory response (figure 2.6**)** (Sigma-Aldrich, 2008b).

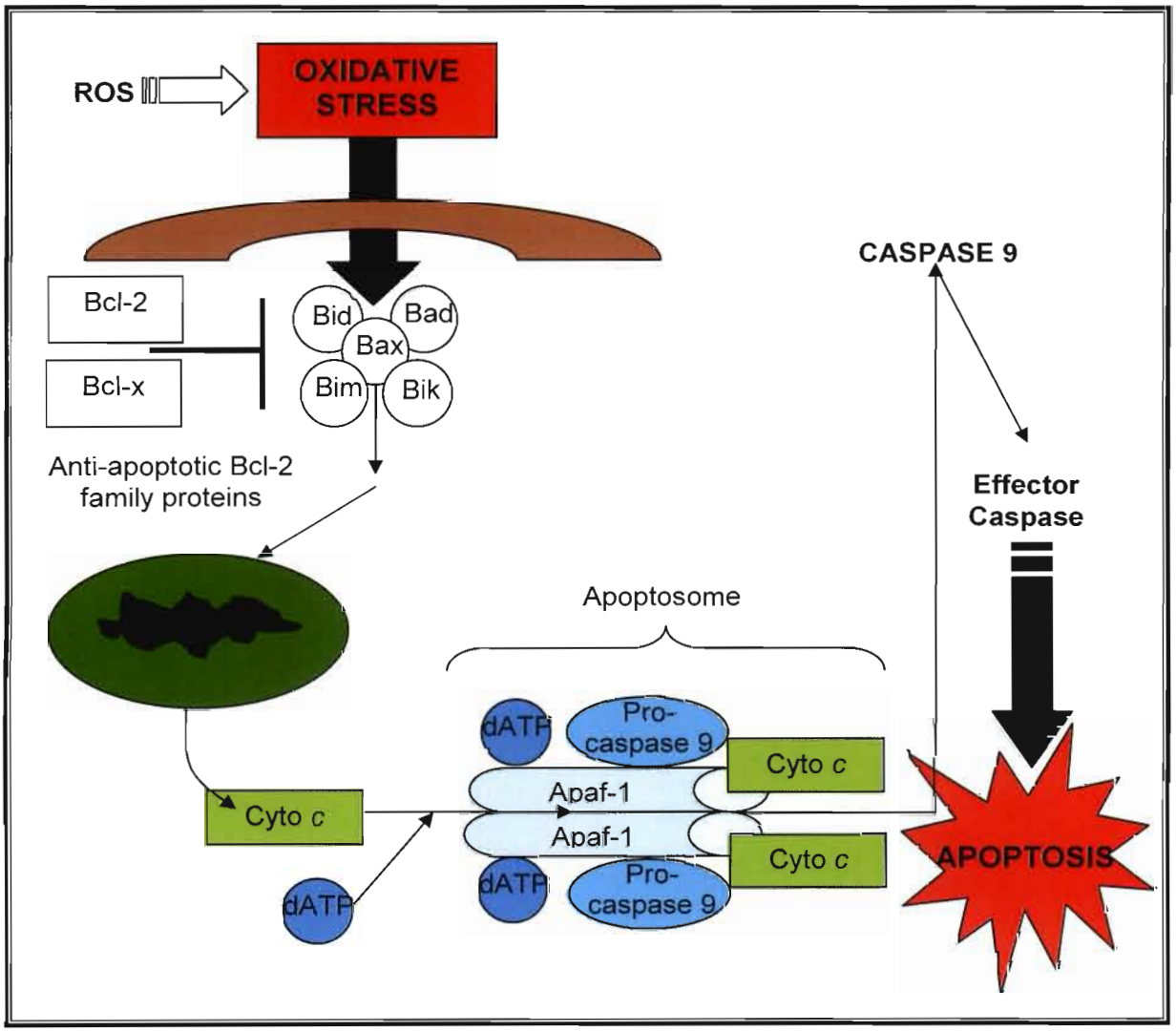


Figure 2.6 Major oxidative stress end-routes to apoptosis (Sigma-Aldrich, 2008b).

(b) Necrosis

In contrast to the controlled, programmed manor of apoptotic cell death, the necrotic mode of cell death is an accidental, major insult to cells, resulting in a loss of membrane integrity, swelling and disruption of the cells. During necrosis, the cellular contents are released uncontrolled into the cell's environment which results in damage of surrounding cells, a so-called "cascade of effects" and a strong inflammatory response in the corresponding tissue. There are many causes of necrosis, including prolonged exposure to injury, infection, cancer, infarction, poisons and inflammation (Gewies, 2003).

2.3.2 Compounds inducing oxidative stress

Thus far, among the various accepted experimental models of Parkinson's disease, neurotoxins have remained the most popular tools to produce selective neuronal death in both *in vitro* and *in vivo* systems (Bové *et al.*, 2005). These neurotoxins provoke the formation of ROS (Dauer *et al.*, 2003). Exposure to dopamine, 6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induces characteristic biochemical, histochemical and morphological changes typical of apoptosis in various neuronal cell cultures (Lev *et al.*, 2003).

(a) 6-Hydroxydopamine (6-OHDA)

6-Hydroxydopamine is a selective catecholaminergic neurotoxin and the cytotoxicity of this neurotoxin is based on the damage of dopaminergic neurons in two ways, it easily forms free radicals and it is a potent inhibitor of the mitochondrial respiratory chain complexes I and IV (Nie *et al.*, 2002). 6-OHDA-induced toxicity is relatively selective for monoaminergic neurons, resulting from preferential uptake by dopamine and noradrenergic transporters (Dauer *et al.*, 2003). The brains of Parkinson's disease patients show evidence of impaired mitochondrial complex I function and of the generation of oxidative stress and these might, therefore, be crucial components of nigral dopamine neurodegeneration (Shimohama *et al.*, 2003). Inside neurons, 6-OHDA accumulates in the cytosol, generating ROS and inactivating biological macromolecules by generating quinines that attack nucleophillic groups (Dauer *et al.*, 2003).

(b) 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)

In 1976, a college student who attempted to manufacture 1-methyl-4-phenyl-4-propionoxypiperidine (MPPP) made an error in his synthesis and produced MPTP. He developed parkinsonian symptoms and an autopsy after his suicidal death revealed degeneration of cells within the substantia nigra. In 1982, MPPP abuse again occurred (Khandhar & Marks, 2007); young drug users developed a rapidly progressive parkinsonian syndrome traced to intravenous use of a street preparation of MPPP, an analogue of the narcotic meperidine (Demerol). MPTP was the responsible neurotoxic contaminant, inadvertently produced during the illicit synthesis of MPPP in a basement laboratory. In humans, MPTP produces an irreversible and severe parkinsonian syndrome characterised by all of the features of Parkinson's disease including, tremor, rigidity and slowness of movement, postural instability and freezing. MPTP damage the dopaminergic pathways in a pattern similar to that seen in Parkinson's disease (Dauer, 2003). MPTP is oxidised to

1-methyl-4-phenyl-2,3-dihydropyridinium ($MPDP^+$) by monoamine oxidase B (MAO-B) and then converted to MPP^+ (1-methyl-4-phenylpyridinium), the active toxic molecule. Absorbed MPP^+ concentrates in mitochondria, where it inhibits complex I of the electron transport chain, thereby reducing ATP generation and causing the production of ROS, inducing apoptotic death of dopamine neurons (Shimohama *et al.*, 2003).

(c) Cyanide

The brain is the primary target organ for cyanide toxicity. Cyanide toxicity may produce cellular anoxia in the brain and also tonic and clonic seizures and convulsions while in some individuals a Parkinson-like condition may develop as a post toxicity sequel. Cyanide also produces dopaminergic toxicity accompanied by impaired motor function. Due to a number of antioxidant enzymes being inhibited by cyanide, it is also believed that oxidative stress plays an important role in cyanide induced neurotoxicity. It is proposed that increased intracellular calcium after cyanide treatment generates ROS leading to peroxidation of lipids and subsequent neuronal damage. The primary function of the mitochondria is to generate ATP that is needed for transport across membranes for all synthetic processes and for the mechanical work involved in motor activities of the cell. Compromised mitochondria may have detrimental effects on the survival of the cell, leading to potential apoptosis. Mitochondrial respiratory chain defects have been implicated in the pathogenesis of Alzheimer's disease and mitochondrial dysfunction has been associated with the neurodegeneration of Parkinson's disease. Cyanide, implicated as a mitochondrial electron transport inhibitor, is also an inhibitor of complex IV and causes severe depletion of cellular ATP (Maharaj *et al.*, 2003). The inhibition of complex IV of the mitochondrial respiratory chain produces $O_2^{\bullet-}$ through the reduction of oxygen (Ottino & Duncan, 1997a). The final transport of electrons across the inner mitochondrial membrane is inhibited with cyanide by inhibition of cytochrome a_1a_3 that reduces the number and rate of electron production by mitochondrial metabolism (Maharaj *et al.*, 2003).

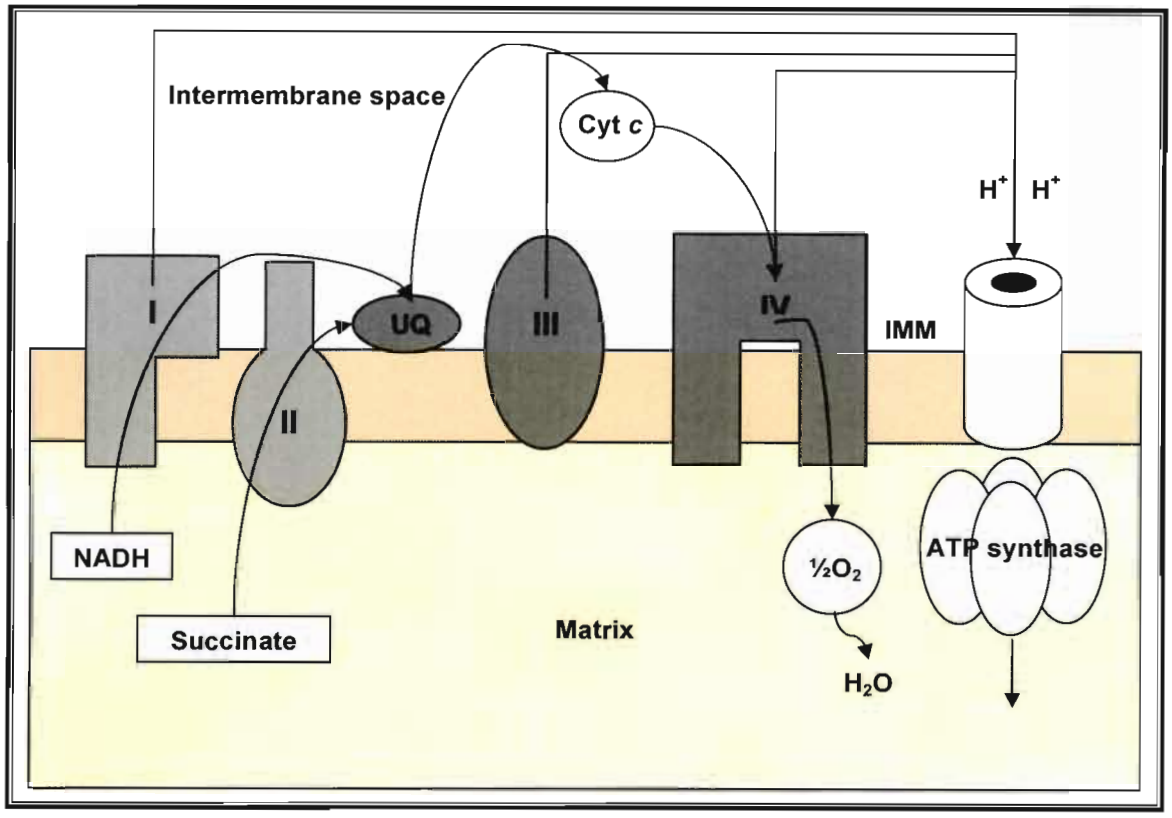


Figure 2.7 The different complexes the neurotoxins can act upon in the mitochondrial electron transport chain (Jackson *et al.*, 2002).

2.3.3 Susceptibility of the brain to oxidative damage

All human tissue suffers oxidative damage, yet the brain is often said to be especially sensitive (Halliwell, 2001). Some of the most important reasons for this are summarised below:

- The brain has a high O_2 consumption. Since oxygen consumed by cells can be reduced to ROS, relatively higher amounts of ROS may be generate in the brain as compared to other tissues that use less oxygen (Cui *et al.*, 2004; Halliwell, 2001).

- A potential problem for the brain is its extensive use of glutamate as a neurotransmitter. The release of excitatory neurotransmitters such as glutamate induces a cascade of reactions in the postsynaptic neuron resulting in the formation of ROS (Cui *et al.*, 2004; Halliwell, 2001).

- Iron is found throughout the brain. Important iron-containing proteins in brain include cytochromes, ferritin, aconitases, mitochondrial non-haem iron proteins, cytochrome *P450* and the tyrosine and tryptophan hydroxylase. Most of the 'total' iron in healthy

brain is apparently stored in ferritin which should limit its capacity to catalyse oxidative damage. However, damage to brain tissue readily release iron (and copper) ions in forms that are capable of catalysing such free radical reactions as $OH^{\bullet}$ formation from H_2O_2 and lipid peroxidation. The brain is also poor in the antioxidants transferrin and ceruloplasmin that normally help bind and segregate these transition metals (Cui *et al.*, 2004; Halliwell, 2001).

- Ascorbic acid, which can act as an antioxidant as well as a pro-oxidant, is present at elevated levels in the brain. It acts as a pro-oxidant when the free iron in brain regions increases due to intracerebral hemorrhage (Cui *et al.*, 2004).

- Neuronal membrane lipids contain highly PUFAs side-chains, especially docosahezaenoic acid residues. These highly PUFAs are extremely susceptible to lipid peroxidation, at least *in vitro* (Cui *et al.*, 2004; Halliwell, 2001)

- Brain metabolism generates H_2O_2. A major source of H_2O_2 is the oxidation of dopamine by monoamine oxidases (MAOs), flavoprotein enzymes located in the outer mitochondrial membrane. RCH_2NH_2 (monoamine) + O_2 + H_2O $\rightarrow$ RCHO (aldehyde) + H_2O_2 +NH_3 (Halliwell, 2001).

- Antioxidant defences in the brain are modest. The brain contains almost no catalase and less GPx and vitamin E, as compared to liver (Cui *et al.*, 2004; Halliwell, 2001)

- Some of the glial cells found in the brain are microglia which is resident macrophage-type cells. Like other macrophages, microglia can produce $O_2^{\bullet-}$ and H_2O_2 (and in some cases $NO^{\bullet}$) upon activation (Halliwell, 2001)

- Cytochromes *P450* (CYPs) are present in certain brain regions. Reactive species can 'leak away' from the catalytic intermediates in the *P450* cycle, generating $O_2^{\bullet-}$ and H_2O_2 (Halliwell, 2001).

- An endogenous antioxidant produced in the pineal gland, called melatonin, is found to be a good scavenger of ROS but its concentration decreases markedly with age (Cui *et al.*, 2004).

- Neurons are non-replicating cells and any damage to brain tissue by ROS tends to be cumulative over time (Cui *et al.*, 2004).

2.4 Neurodegenerative diseases

The brain is exposed throughout life to oxidative stress and certain diseases of the brain and nervous system are thought to involve free radical processes and oxidative damage, either as a primary cause or as a consequence of disease progression (Gilgun-Sherki *et al.*, 2001). There have been many suggestions that reactive species play an important role in and may even be causative of the neurodegenerative diseases, in particular Parkinson's disease, Huntington's disease etc (Halliwell, 2001). In cases of Parkinson's disease, Alzheimer's disease and Amyotrophic lateral sclerosis various indices of ROS damage have been reported within the specific brain region that undergoes selective neurodegeneration. For example, markers for lipid peroxidation, including 4-HNE and MDA, have been identified in the cortex and hippocampus of patients with Alzheimer's disease, the substantia nigra of patients with Parkinson's disease and in spinal fluid from patients with Amyotrophic lateral sclerosis. Protein nitration, a marker of protein oxidation, has been demonstrated to be elevated in the hippocampus and neocortex of individuals with Alzheimer's disease, in Lewy bodies in cases of Parkinson's disease and within motor neurons in Amyotrophic lateral sclerosis. Surprisingly, several of these oxidative events seem to be fairly target specific. For instance, nitration of tyrosine residues within the α-synuclein protein is found to accumulate in the Lewy bodies that are associated with Parkinson's disease and other synucleopathies and within the tau protein in Alzheimer's disease. Oxidative stress is therefore consistently associated with these diseases. One might also indicate that it is due to reduced antioxidant defence potential that contribute to the increase oxidative stress that is associated with these diseases (Andersen, 2004). The diseases discussed below only view the oxidative stress related causes of these neurodegenerative diseases.

2.4.1 Parkinson's disease

Parkinson's disease, a common movement disorder, is characterised by the loss of dopaminergic neurons of the substantia nigra, specifically the substantia nigra pars compacta (SNpc), among other (Barnham *et al.*, 2004). Normal human aging is associated with a progressive loss of nigrostriatal neurons and an age-related exponential increase in Parkinson's disease prevalence. One of the basic mechanisms proposed for nigrostriatal cell loss in Parkinson's disease is an oxidative stress/injury resulting from a production of ROS exceeding the cellular antioxidant capacity (Gilgun-Sherki *et al.*, 2001). In post-mortem studies of Parkinson's disease, lipid peroxidation was elevated in the substantia nigra, while the activity of glutathione peroxidase and glutathione levels was reduced. Furthermore, the content of Fe^{3+}, but not Fe^{2+}, is remarkably increased in the substantia nigra but is unaltered

in other brain regions while ferritin levels are reduced. Thus, there is an ideal environment that could promote and sustain ROS formation in the substantia nigra of Parkinson's disease (Facchinetti *et al.*, 1998). The substantia nigra produces dompamine for neurotransmission. Apart from being an essential neurotransmitter, it is a catechol, a good metal chelator and a potential electron donor (that is, a metal reductant). During dopamine metabolism it can coordinate with metal such as Cu^{+} and Fe^{2+}, reduce the oxidation state of the metal and subsequently engender production of H_2O_2 and setting up conditions for Fenton chemistry. The result is the formation of $OH^{\bullet}$ *in vivo* in the nigrostriatal pathway (Barnham *et al.*, 2004). Another factor implicated as cause of oxidative stress in Parkinson's disease is a defect in mitochondrial respiratory chain function, involving complex I (Cui *et al.*, 2004). A decrease in complex I activity (NADH dehydrogenase) have been found in the substantia nigra (probably in neurons and glia) but not in other brain regions, whereas activities of the other mitochondrial electron-transport complexes seem unaltered. Since the decreases are specific to complex I they are unlikely to be related to cell death or loss of mitochondria. Complex I inhibition could conceivably lead to generation of more reactive species, and reactive species (including $O_2^{\bullet-}$, $OH^{\bullet}$ and $ONOO^{-}$) that can then in turn further damage complex I. Therefore, it is possible that oxidative stress and mitochondrial defects form a 'vicious cycle' in Parkinson's disease (Halliwell, 2001).

2.4.2 Alzheimer's disease

Alzheimer's disease is a progressive neuropsychiatic disorder; it is characterised by neuronal degeneration and cognitive deterioration, especially in the elderly. Oxidative stress has been implicated in the pathogenesis of Alzheimer's disease by the finding of several characteristics such as enhanced lipid peroxidation, increased levels of protein oxidation (carbonyl content) and decreased glutamine synthetase activity in specific areas of the brain in post-mortem studies (Gilgun-Sherki *et al.*, 2001; Cui *et al.*, 2004). Alzheimer's disease is characterised by the deposition of amyloid plaques, the major constituent being the amyloid-β that is cleared from the membrane-bound amyloid precursor protein. Amyloid is a protein peptide deposited in diseased tissue, with high β-sheet structure. It is proposed by Barnham *et al* (2004) that the major source of oxidative stress and free-radical production in the brain in Alzheimer's disease is the transition metals Cu and Fe when bound to amyloid-β. When Cu^{+2} or Fe^{3+} coordinate amyloid-β, extensive redox chemical reactions take place that reduce the oxidation state of both metals and produce H_2O_2 from O_2 in a catalytic manner. The generation of H_2O_2 in the presence of the reduced form of the metal creates conditions in which Fenton chemistry occurs with the generation of highly toxic $OH^{\bullet}$ (Barnham *et al.*, 2004).

2.4.3 Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis, also known as motor neuron disease or Lou Gehrig's disease, is characterised by a selective and progressive degeneration of the lower motor neurons in the spinal cord and the upper motor neurons in the cerebral cortex, usually beginning in midlife (Cui *et al.*, 2004; Gilgun-Sherki *et al.*, 200). This neuron loss causes muscles to weaken and waste away leading to paralysis (Andersen, 2004). A large number of different mutations in the SOD gene have so far been identified but it is not clear whether the disease is related to the inactivation of the SOD enzyme or the appearance of new functions as a result of the mutations (Cui *et al.*, 2004). The nature of this gain in function is widely debated and there are two main theories: one suggest that the toxicity is due to misfolded aggregated forms of SOD, whereas the other proposes that SOD becomes a pro-oxidant protein generating ROS (Barnham *et al.*, 2004). This has been explained on the basis that mutations of the SOD gene may have two adverse effects on the cell: (a) reduced ability to scavenge the $O_2^{\bullet-}$ and (b) enhanced affinity to peroxynitrite which could have the consequence of inactivating, through nitration, several critical components of motor neurons such as the neurofilaments and tyrosine kinase receptors (Cui *et al.*, 2004). Thus, the mutant polypeptide may fail in binding or shielding transition metals effectively. They are then free to support the Fenton reaction leading to the production of $OH^{\bullet}$ (Facchinetti *et al.*, 1998).

2.5 Plants in Medicine

2.5.1 Ethnomedicine

A broad definition of ethnomedicine is the use of plants for medicinal purposes (Fabricant & Farnsworth, 2001). Another term, pharmacognosy is defined as the study of crude drugs from a natural origin for example plants. This entails the use of medicinal plants as crude herbs, extracts or pure natural compounds; it also studies the physical, chemical, biochemical and biological properties thereof and aids in the search for new drugs from natural sources (Phillipson, 2007).

Plants have been utilised as medicines for thousands of years, fossil records date human use of plants as medicines at least to the Middle Palaeolithic age some 60 000 years ago. In the grave of a Neanderthal man pollen analysis indicated that the numerous plants buried with the corpse were all of medicinal value (Fabricant & Farnsworth, 2001; Kong *et al.*, 2003). Plant medicines initially took the form of crude drugs such as tinctures, teas, poultices, powders and other herbal formulations. The specific plants to be used and the

methods of application for particular ailments were passed down through oral history. Eventually information regarding medicinal plants was recorded in herbals (Kong *et al.*, 2003). Further on in the history, the use of plants as medicines have involved the isolation of active compounds, beginning with the isolation of morphine from opium by Friedrich Serturner in the early 19th century. The first purely synthetic drugs based on natural products were formulated in the middle of the 19th century. In 1839, salicylic acid was identified as the active ingredient in a number of plants known for their pain-relieving qualities and was first synthesised in 1853. This led to the development of aspirin which is a widely used synthetic drug today (Kong *et al.*, 2003). A pure extract of a herb's "active component" is more reliable and safer than administration of the herb itself (Karthikumar *et al.*, 2007). The isolation and characterisation of pharmacologically active compounds from medicinal plants continue to date.

Fabricant and Farnsworth (2001) stipulates the goals of using plants as sources of therapeutic agents as;

- to isolate bioactive compounds for direct use as drugs e.g. digoxin, digitoxin, morphine, reserpine, taxol, vinblastin and vincristine;

- to produce bioactive compounds of novel or known structures as lead compounds for semi synthesis to produce patentable entities of higher activity and/or lower toxicity e.g. metformin, nabilone, oxycodom (and other narcotic analgesics), taxotere, teniposide, verapamil and amiodarone which are based, respectively, on galegine, Δ^9-tetrahydrocannabinol, morphine, taxol, podophyllotoxin and khellin (Fabricant & Farnsworth, 2001);

  For many years, natural molecules have acted as templates for the synthesis of new drugs and there are numerous examples of synthetic drugs that are based on the structure of a natural product molecule e.g. the analgesic pethidine was based on morphine and the antimalaria mefloquine on quinine (Phillipson, 2007).

- to use agents as pharmacologic tools e.g. lesergic acid diethylamide, mescaline, yohimbine and

- to use the whole plant or part of it as a herbal remedy e.g. cranberry, echinacea, feverfew, garlic, ginkgo biloba, St. John's Wort and Saw Palmetto (Fabricant & Farnsworth, 2001).

According to Balunas and Kinghorn (2005) research on natural products accounts for approximately 48 % of the new chemical entities reported from 1981 – 2002. Many structural features common to natural products (e.g. chiral centres, aromatic rings and complex ring systems, degree of molecule saturation and number and ratio of heteroatoms) have been shown to be highly relevant to drug discovery efforts (Balunas & Kinghorn, 2005).

Research into and development of therapeutic materials from plant origin is a difficult and expensive task (Rates, 2001). Natural products discovered from medicinal plants (and derivatives thereof) have provided numerous clinically used medicines. Even with all the challenges facing drug discovery from medicinal plants, natural products isolated form medicinal plants can be predicted to remain an essential component in the search for new medicines (Balunas & Kinghorn, 2005).

2.5.1.1 Ethnomedicine in South Africa

South Africa boasts remarkable biodiversity and rich cultural traditions of plant use. Medicinal plants are commonly used in South African traditional healthcare to treat a range of ailments. Of the 24 300 higher plant taxa recorded in the Flora of Southern Africa region, approximately 15 % are used for medicinal purposes (Clarkson *et al.*, 2004). The five most important plant products from South Africa that have become popular worldwide, according to George *et al* (2001) are:

1. Devils's claw (*Harpagophytum procumbens*) which is widely used for its thick, fleshy secondary roots that is valued for their efficacy in osteoarthritis, fibrositis, rheumatism and small-joint pains. The iridoids harpagoside, harpagide and procumbide possess anti-inflammatory and analgesic activity and these, along with β-sitosterol are believed to be responsible for its activity.

2. "African potato" (*Hypoxis hemerocallidea*), the extracts of the tuberous rhizome of this locally and internationally popular plant are widely used as a cure for all ailments from benign prostate hypertrophy to rheumatoid arthritis.

3. Buchu (*Agathosma betulina*), is used for treating wounds and stomach complaints by Khoi's in the Cape Province. The leaves have diuretic, antiseptic and antispasmodic properties. Essential oils from buchu are used for treating colds, flu and rheumatism. Isomenthone A and diosphenol are the major compounds.

4. Aloe products (*Aloe ferox*), are widely used for cosmetic and as general health tonics. The concentrated yellow leaf juice has laxative properties owing to anthraquinones.

The gel contains complex polysaccharides and has been reported to possess immunomodulatory and anti-inflammatory activity.

5. Rooibos tea (*Aspalathus linearis*) is a popular health beverage. Devoid of caffeine, low in tannin and containing several minerals, this tea has been claimed to possess antioxidant and antispasmodic activity owing to the presence of several unique flavanoid C-glycosides such as aspalathin and nothofagin. Apart from being used as a refreshing beverage, rooibos tea is also used in cosmetics, slimming products, cocktails and as a milk substitute for infants prone to colic (George *et al.*, 2001).

2.5.1.2 The safety of ethnomedicine

It is important to note briefly on the safety of medicinal plants. The use of traditional medicine in South Africa is not regulated properly, with the result that there is always the danger of misadministration, especially of toxic plants. Plants commonly used in traditional medicine are assumed to be safe. This safety is based on their long usage in the treatment of diseases according to knowledge accumulated over centuries. However, research has shown that many plants used in traditional medicine are potentially toxic. This raises the concern for toxicological testing before a plant-based remedy is prohibited for use (Fennell *et al.*, 2004).

For instance some medicinal herbs, when ingested, affect either cytochrome *P450* isoenzymes by which drugs are metabolised or phosphoglucoprotein transporter systems that affect drug distribution and excretion. Concurrent use of some herbal medicines with other medicines may either lower blood plasma concentrations of medicinal drugs, possibly resulting in suboptimal therapeutic amounts or lead to toxic concentrations in the blood. St. John's Wort (*Hypericum perforatum*) commonly use for the treatment of mild to moderate depression interacts with digoxin, HIV inhibitors, theophylline and warfarin among others and is therefore not advisable to use without proper consultation (Phillipson, 2007).

Rates (2001) mentions a few toxic plant-derived compounds, toxic plant species, as well as plants that interfere with conventional pharmacological therapy:

- Plants containing coumarinic derivatives such as dicoumarol and the sodium coumarins, which have the potential to lead to haemorrhagical accidents due to chronic use or synergistic effects with oral anti-coagulants. Among the coumarin-rich plants used in folk medicine are *Mykania* spp, *Melilotus officinalis* and *Dypterix odorata.*

- Plants with a high tyramine content, tyramine is phenylethylamine found in yeast and wine and can be responsible for hypertensive accidents in patients treated with monoamine oxidase inhibitors. Mushrooms and higher plants such as *Portulacca* spp, *Phoradendron* spp and *Psittacanthus* spp are also potentially dangerous.

- Plants containing oestrogenic compounds such as gingseng can have important oestrogenic effects and its use in combination with steroid drugs is not recommended.

- Plants can cause irritations, allergic problems and allergic reactions. Pollen and plant secretions are typical causes of these reactions. The folk literature reports many plants that cause irritations, these include all species from the families *Urticaceae*, *Euphorbiaceae* and *Leguminoseae*, the sesquiterpene lactones found in *Asteraceae* and other plants generally considered as harmless such as camomile (*Matricharia recutita*) and *Arnica montana* which can cause dermatitis.

- Plants can contain photosensitive compounds like furocoumarins in *Psoralea corylifolia*, *Conilla glauca*, *Ficus carica* and several species of *Citrus*. Another plant containing photosensitive compounds is *Hypericum perforatum* and it is possible that other plants of the same genus have similar photosensitive properties because of the presence of hypericin and analogues.

- Other toxic plants include the "arruda" (*Ruta graveolens*), popularly known to cause abortion and plants form the genus *Atropa*, *Brugmansia* and *Datura* which are cultivated to produce tropanic alkaloids. These plants are used in folk medicine against asthma but have toxic effects due to their content of yioscyamine, scopolamine and atropine and are responsible for accidental or voluntary intoxication and causing atropinic hallucination (Rates, 2001).

However, it is more important to note that of the nearly 4 000 ethnomedicinal plant taxa used in South African traditional healthcare (Arnold *et al.*, 2002) relatively few are considered likely to give rise to serious toxicity (Fennell *et al.*, 2004).

2.5.2 The value of plants in neurodegeneration as antioxidants

Recent health concerns draw much attention to the use of natural antioxidants (Karthikumar *et al.*, 2007). The plant-based antioxidants are believed to have an important role in the maintenance of human health because our endogenous antioxidants provide insufficient protection against the constant and unavoidable challenges of ROS (Benzie, 2003). Diets rich in bioactive phytochemicals reduce the risk of degenerative disorders such as cancer,

diabetes, cardiovascular and oxidative dysfunction. Foods containing these phytochemicals not only provide our diet with certain antioxidant vitamins like vitamin C and vitamin E but also a complex mixture of other natural substances with antioxidant capacity (Natarajan *et al.*, 2005). Thus plant derived antioxidants are viewed as promising therapeutic drugs for free radical pathologies (Kang *et al.*, 2003).

Over the past few years, a number of medicinal plants have been investigated for their quenching activity of specific ROS; especially since the use of some synthetic antioxidant compounds such as butylated hydroxyl toluene (BHT) and butylated hydroxyanisole (BHA) have been suggested to have side effects (Pietta *et al.*, 1998).

Several Indian medicinal plants have been extensively used in the Indian traditional (Ayurveda) system of medicine as rejuvenators slowing the process of aging and related disorders including neurodegenerative diseases. These plants have already been highlighted; *Emblica officinalis*, *Curcuma longa*, *Mangifera indica*, *Santhum album*, *Withania somnifera* for their antioxidant activity. Active principles isolated from the plants include, mangiferin from *M. indica*, emblicanin A&B, two tannins from *Phyllanthus emblica* and curcumin a well-known compound isolated from *C. longa* (Auddy *et al.*, 2003). Numerous studies were carried out on rosemary, sage, oregano which resulted in the development of natural antioxidant formulations for foods, cosmetics and other applications (Miliauskas *et al.*, 2004). Plants native to Europa and North America (*Crataegus oxyacantha*, *Hamamelis virgiana*, *Hydrastis canadensis*) have long been used in medicine and in bio-assay guided studies showed antioxidant potential. *Ginkgo biloba* a reputed medicinal plant has shown to possess very interesting pharmacological properties these include, antioxidant/free radical scavenging effects and neuroprotective action (Shah *et al.*, 2003).

The general conception for the specific plant constituents responsible for these antioxidant properties is that it is mainly due to the phenolic components such as flavonoids, phenolic acids and phenolic diterpenes (Pietta *et al.*, 1998). Polyphenols, all phenolic compounds, are very important constituents because of their scavenging ability with ROS and chelating ability with divalent cations due to their hydroxyl groups and redox properties which can play an important role in absorbing and neutralising free radicals, quenching singlet and triplet oxygen or decomposing peroxides (Al-Dabbas *et al.*, 2006; Kang *et al.*, 2003). More than 4 000 naturally occurring flavonoids have already been described (Kang *et al.*, 2003). Not only flavonoids received considerable attention but also terpenoids and steroids due to their diverse pharmacological properties including antioxidant activity (Karthikumar *et al.*, 2007).

Phenolic antioxidants have been successfully isolated from rosemary and oregano (Al-Dabbas *et al.*, 2006). Tea (black and green tea) is one of the most commonly consumed beverages in the world and is rich in polyphenolic compounds collectively known as the tea flavonoids (Katalinic *et al.*, 2006). Another example of a plant derived compound that has been researched for antioxidant activity in the literature is; resveratrol, a polyphenolic antioxidant present in red wine. Resveratrol can scavenge free radicals and suppress mitochondrial-induced ROS production in the rat brain, inhibit lipid peroxidation and protect against oxidative DNA damage in stroke-prone hypertensive rats (Hwang *et al.*, 2004).

2.5.3 Plant constituents with proposed antioxidant properties

As was discussed briefly in section 2.5.2 the most well known group of compounds with antioxidant properties are the phenolic-based constituents. Phenolic compounds or polyphenols constitute one of the most numerous and widely distributed substances in the plant kingdom with more than 8 000 phenolic structures currently known. The expression "phenolic compounds" embraces a range of substances that possess an aromatic ring bearing one or more hydroxyl substituents. Most of the classes of plant polyphenols are listed in table 2.3 according to the number of carbon atoms of the basic structure. Natural polyphenols varies from simple molecules such as phenol acids to highly polar compounds such as condensed tannins (Urquiaga & Leighton, 2000).

Table 2.3 The major classes of phenolic compounds in plants (Urquiaga & Leighton, 2000).

Number of C-atoms	Basic skeleton	Class	Examples
6	C6	Simple phenols Benzoquinones	Catechol, hydroquinone, 2,6-Dimethoxybenzoquinone
7	C6-C1	Phenolic acids	Gallic, salicylic acid
8	C6-C2	Acetophenones Tyrosine derivatives Phenylacetic acids	Tyrosol
9	C6-C3	Hydroxycinnamic acids Phenylpropenes Coumarins Isocoumarins Chromones	Caffeic, ferulic Myristicin Umbelliferone Bergenon Eugenin
10	C6-C4	Naphthoquinones	Juglone, plumbagin
13	C6-C1-C6	Xanthones	Mangiferin
14	C6-C2-C6	Stilbene Anthraquinones	Resveratrol Emodin
15	C6-C3-C6	Flavonoids Isoflavonoids	Quercetin Genistein
18	(C6-C3)2	Lignans Neolignans	Pinoresinol Eusiderin
30	(C6-C3-C6)2	Biflavonoids	Amentoflavone
n	(C6-C3)n (C6)n (C6-C3-C6)n	Lignins Catechol melanins Flavolans (Condensed tannins)	

2.5.3.1 Flavonoids

The flavonoids are responsible for the colour or flowers, fruits and sometimes leaves (Gurib-Fakim, 2006). They are the largest group of polyphenols, consisting of an aromatic ring that is condensed to a heterocyclic ring and attached to a second aromatic ring (Weinreb *et al.*, 2004). Small differences in basic substitution patterns give rise to several subgroups (Gurib-Fakim, 2006). The group is divided into chalcones, flavonones, flavones, biflavonoids, dihydroflavonols, anthrocyanidins, flavonols and other phenolic compounds such as tannins, coumarins, xanthones, procyanidins (Kang *et al.*, 2003). This large class of compounds are ubiquitous in plants and usually occur as glycosides (Rice-Evans *et al.*, 1997). The *in vitro* antioxidant activities of flavonoids have been recognised for decades (Harborne & Williams, 2000) and studies on the free radical-scavenging properties of flavonoids have permitted characterisation of the major phenolic components of naturally occurring phytochemicals as antioxidants (Rice-Evans *et al.*, 1997). Flavonoids have been shown to act as scavengers of various oxidising species e.g. $O_2^{\bullet-}$, $OH^{\bullet}$ or peroxyl radicals (Harborne & Williams, 2000).

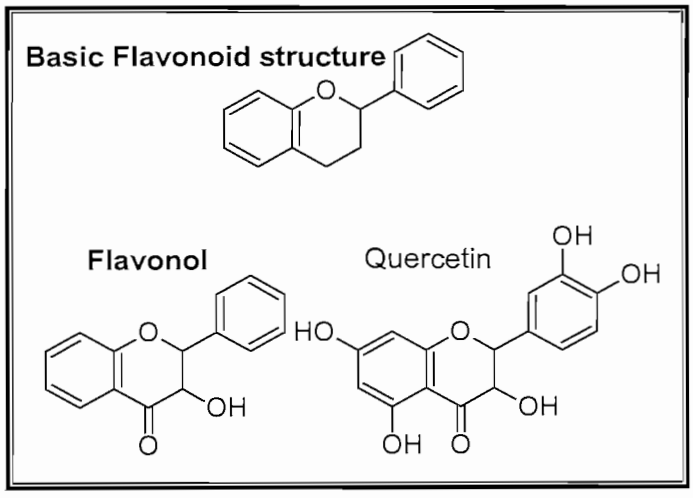


Figure 2.8 The basic structure of the flavonoids and the flavonol, quercetin exhibiting antioxidant activity (Van Acker *et al.*, 1996; Galotta *et al.*, 2008).

2.5.3.2 Anthocyanidins

The anthocyanidins are also part of the flavonoid class and contribute greatly to the antioxidant properties of certain colourful foods such as grapes and cranberries. Cyanidin is the most common anthocyanidin and the most active antioxidants are the 3-glycosides anthocyanins (Einbond *et al.* 2004). Along with other flavonoids, the anthocyanins can directly scavenge molecular species of active oxygen, including H_2O_2, singlet oxygen and

$O_2^{\bullet-}$, OH^- and peroxyl radicals. They are also effective scavengers of peroxynitrite, a highly reactive oxidant formed when $O_2^{\bullet-}$ reacts with nitric oxide. Anthocyanins have an inherent potential to protect cell membranes from the effects of oxidative damage (Gould *et al.*, 2002).

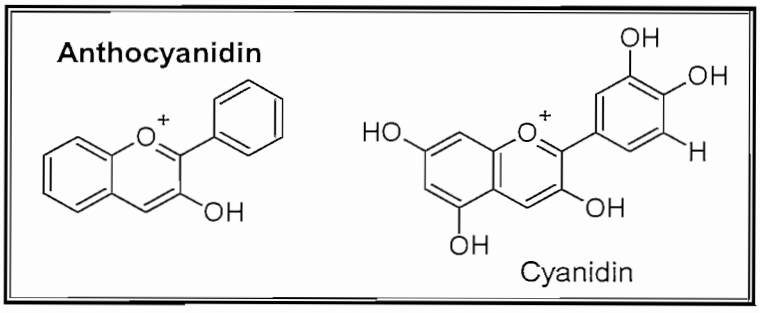


Figure 2.9 The basic structure of anthocyanidins, a subclass of the flavonoid, and cyanidin exhibiting antioxidant activity (Van Acker *et al.*, 1996; Kähkönen & Heinonen, 2003).

2.5.3.3 Tannins

The chemistry of these compounds is very complex (Gurib-Fakim, 2006). Two basic groups of tannins are found in plants, namely hydrolysable tannins and condensed tannins. Hydrolysable tannins are compounds where one or more sugar is bonded to phenolic acid molecules (gallic acid, ellagic acid) for example geraniin from *Geranium* spp. Condensed tannins, also called proanthocyanidins are quite different. They are made up of two or more flavonoid units which break down into anthocyanidins when treated with acids at high temperature. An example is procyanidin C from *Sclerocarya* species. The pharmaceutical value of tannins is linked to their ability to form complexes with other molecules. The antioxidant abilities are similar to those of flavonoids (Van Wyk *et al*, 2002). Recent studies have reported that tannins have anti-cancer and anti-HIV activities (Gurib-Fakim, 2006).

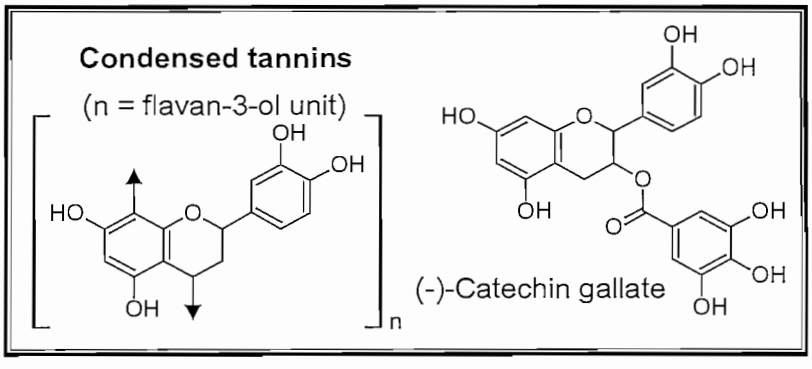


Figure 2.10 The basic structure of condensed tannins and an example exhibiting antioxidant activity (Cai *et al.*, 2006; Rahim *et al.*, 2008).

2.5.3.4 Coumarins

The coumarins are compounds with oxygen containing ring structures. Coumarins are sometimes present in essential oils because they are sufficiently volatile to be extracted through the process of steam distillation (Van Wyk *et al*, 2002). They are found in the following plant families; Apiaceae, Rutaceae, Asteraceae and Leguminosae (Gurib-Fakim, 2006). Numerous biological activities, similar to the flavonoids, have been attributed to them, such as antimicrobial, anticancer, anti-inflammotory and antioxidant (Borges *et al.*, 2005). Simple coumarins include umckalin isolated from *Pelargonium reniforme* and furanocoumarins (additional furan ring attached to the first ring of the basic coumarin structure) include xanthotoxin isolated from *Peucedanum galbanum* (Van Wyk *et al*, 2002).

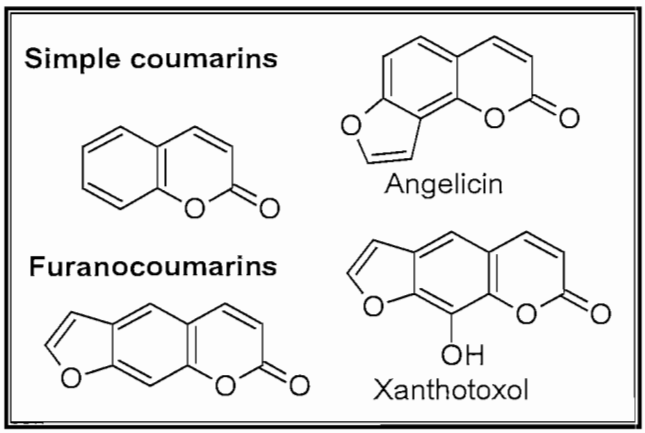


Figure 2.11 The basic structure of the coumarins and examples exhibiting antioxidant activity (Cai *et al.*, 2006; Ng *et al.*, 2000).

2.5.3.5 Quinones

The quinones are similar to phenolic compounds but they are oxidised and have carbonyl rather than hydroxyl groups. Various types are distinguished of which naphthoquinones (plumbagin isolated from *Plumbago* spp.) and anthraquinones (chrysophanol isolated from *Bulbine natalensis* and aloin, an anthrone C-glycoside isolated from *Aleo ferox*) are the best known. Naphthoquinones are yellow or orange pigments from plants and found in these families; Bignoniaceae, Ebenaceae, Droseraceae, Juglandaceae, Plumbaginaceae, Boraginaceae, Lythraceae, Proteaceae and Verbenaceae (Gurib-Fakim, 2006). Quinones

have proven antibacterial, antifungal and antioxidant activity and various other properties (Van Wyk *et al*, 2002).

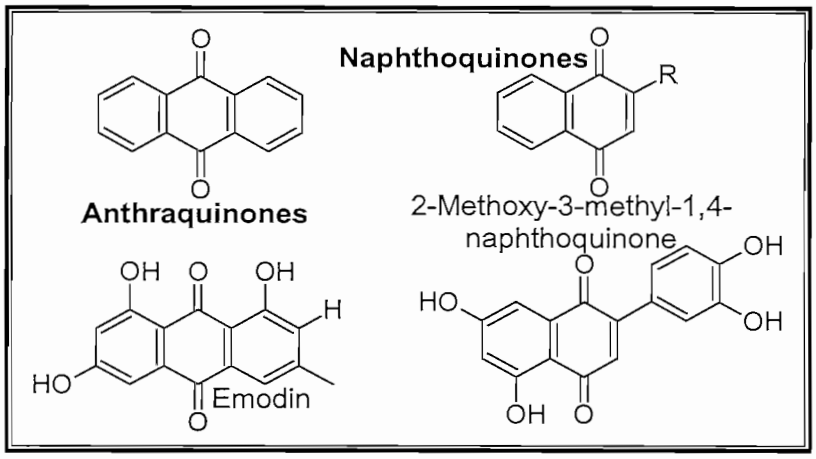


Figure 2.12 The basic structure of the quinones and examples with antioxidant activity (Cai *et al.*, 2006; Yen *et al.*, 2000; Mikhaeil *et al.*, 2004).

2.5.3.6 Terpenoids

Terpenoids are formed by the linking together of a number of five carbon units, the so-called isoprene units. The various classes of terpenoids have the following numbers of isoprene units: monoterpenoids (2 isoprene units, 10 carbon atoms) such as menthol, sesquiterpenoids (3 isoprene units, 15 carbon atoms) such as zingiberene, triterpenoids (6 isoprene units, 30 carbon atoms) such as madecassic acid. Monoterpenoids and sesquiterpenoids are common constituents of volatile oils (Van Wyk *et al.*, 2002). Normally the antioxidant activity of plants can be accredited to these compounds (Ruberto & Baratta, 1999). Diterpenes constitute a vast group of 20 carbon compounds. These compounds have some therapeutic applications. For example, taxol and its derivatives are anti-cancer drugs (Gurib-Fakim, 2006). Another pharmaceutical important group of the terpenoids are the sesquiterpenoid lactones, particularly common in the *Asteraceae* species (Van Wyk *et al*, 2002). These compounds possess a broad range of activities due to the α-methylene lactone moiety and epoxides (Gurib-Fakim, 2006).

Some secondary modifications occur in steroids (Van Wyk *et al.*, 2002). For the most part, tetracyclic terpenes and steroids have similar structures. Steroids contain a ring system of three 6-membered and one 5-membered ring. Many natural steroids are employed in medicine due to the profound biological activities encountered in these constituents (Gurib-

Fakim, 2006). Both triterpenoids (30 carbon compounds) and steroids are often present in plants in the form of saponins with one or more sugar molecule attached to them (Van Wyk *et al.*, 2002). Saponins constitute a vast group of glycosides, which occur in many plants and exhibit various pharmacological activities (Gurib-Fakim, 2006).

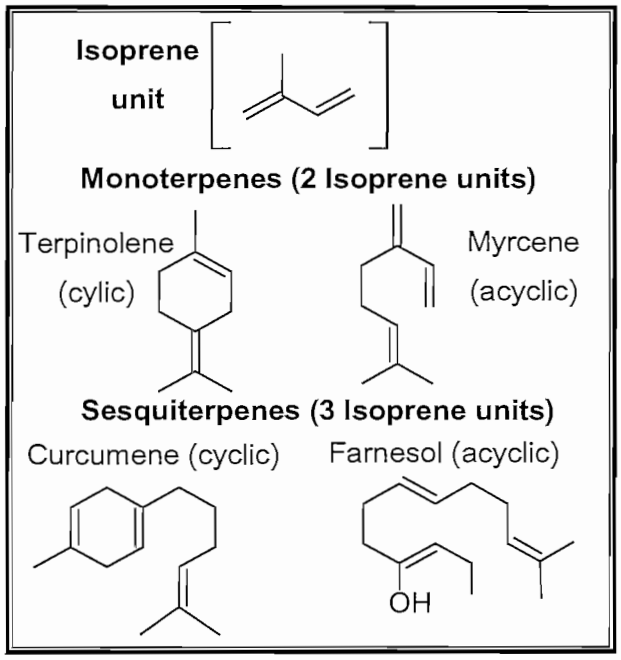


Figure 2.13 The terpenoids with 2 and 3 isoprene units (Cyberlipid Center, 2008; Ruberto & Baratta, 1999).

2.5.3.7 Carotenoids

The carotenoids contain eight isoprene (40 carbons) units and are responsible for the orange and yellow colours of some vegetables and fruits. Among these compounds the hydrocarbons are collectively referred to as carotenes and the hydroxylated derivatives as xanthophylls. Carotenoids are either acyclic (e.g. lycopene) or comprise of one or two pentacyclic or hexacyclic rings at one end or the other (e.g. β,ψ-carotene) or at both ends (e.g. β,β -carotene) (Gurib-Fakim, 2006). A wide range of carotenoids (β-carotene, lutein, zeaxanthin) is found in human diets; many of them are precursors of vitamin A (Gilgun-Sherki *et al.*, 2001). In the intestine β-carotenes are converted to retinol (Vitamin A). They can be used for the treatment of photosensitisation, retinal diseases and glaucoma. Carotenoids became interesting agents after the discovery of a negative correlation between the plasma concentration or β-carotene and the prevalence of certain forms or cancer. Some doctors prescribe β- carotene for cancer patients (Gurib-Fakim, 2006). They are known to possess antioxidant activity somewhat analogous to that of vitamin E (Gilgun-Sherki *et al.*,

2001). Due to its ability to quench $^1O_2$ and scavenge free radicals it protects the cell membrane lipids from the harmful effects of oxidative degradation. The ability of β-carotene and other carotenoids to quench excited oxygen however is limited because the carotenoid itself can be oxidised during the process (autoxidation) (Fouad, 2008a).

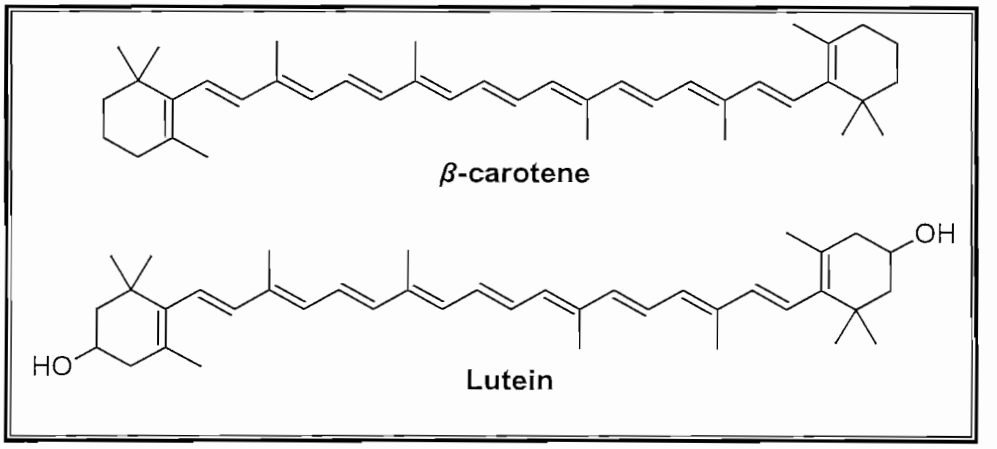


Figure 2.14 The basic structure of carotenoids as shown by selected compounds, β-carotene and lutein (Rock, 1997).

CHAPTER 3. PLANT SELECTION AND EXTRACTION

3.1 Introduction

According to Rates (2001) the approach to drug development from plant resources depend on the aim of the proposed study, in this particular case, the antioxidant properties of plant species and compound(s) (Rates, 2001). After the aim is set Silva *et al.* (1998) identify the next steps in a phytochemical investigation as the selection, collection and identification of the plant material to be studied, as it will be discussed in this chapter.

There are several ways in which a selection can be made including traditional use, chemical content, toxicity, randomised selection or a combination of several criteria. The most common strategy is careful observation of the use of natural resources in folk medicine in different cultures; this is known as ethnobotany or ethnopharmacology. Information on how the plant is used by an ethnic group is extremely important. The preparation procedure may give an indication of the best extraction method. Another method of selecting a plant is that the investigator decides on a well-defined pharmacological activity and performs a randomised search, resulting in active species to be considered for further study. The latter being the route of action for this study. Once the plant is chosen, the next step is its collection and botanical identification. Here the need for a botanist is important to be able to correctly identify the species (Rates, 2001). Care should also be taken to avoid the gathering of mixtures of plants, since many similar species grow side by side (Harborne, 1984). The basis for all phytochemical research is raw material – various plant parts that are either underground (roots, bulbs, corms and rhizomes) or aerial parts (stem, bark, leaves, inflorescence, fruit and seed), in other words the plant material to be studied. For sustainability purposes, only the leaves of all plant species were screened for possible antioxidant properties. This decision of the specific part to be researched could be derived from ethnobotanical literature containing varied bioactivity of plant parts, as well as their reported usage in traditional pharmacopoeia (George *et al.*, 2001). After the plant material is collected and the parts to be used separated, it should be subjected to a suitable extraction process. Most likely the chemical composition would be unknown, then the extraction procedure can be based on how the plant is used in folk medicine or several extractions with solvents of increasing polarity can be performed (Rates, 2001). The chosen extraction method was with solvents of increasing polarity using a Soxhlet apparatus.

3.2 Selections of Plants

Twenty one plant species were identified and subjected to screening in our laboratory after an extensive literature study.

The selected species were as follows;

1. *Acacia karroo*
2. *Berula erecta*
3. *Clematis brachiata*
4. *Elephantorrhiza elephantine*
5. *Erythrina zeyheri*
6. *Gymnosporia buxifolia*
7. *Heteromorpha arborescens*
8. *Leonotis leonurus*
9. *Lippia javanica*
10. *Physalis peruviana*
11. *Plectranthus ecklonii*
12. *Plectranthus rehmanii*
13. *Plectranthus ventricillatus*
14. *Plumbago auriculata*
15. *Salvia auritia*
16. *Salvia rincinata*
17. *Solenostemon latifolia*
18. *Solenostemon rotundifolius*
19. *Tarchonanthus camphoratus*
20. *Vagueria infausta*
21. *Vernonia Oligocephala*

Two plant species were selected from the list based on the ferric reducing ability (FRAP) and oxygen radical absorption capacity (ORAC) results obtained from fellow researchers.

3.2.1 Ferric reducing ability of plasma (FRAP)

The FRAP assay is a method for assessing "antioxidant power" by measuring the ferric reducing ability of a sample. The method relies on the reduction of ferric-tripyridyltriazine (Fe^{3+}-TPTZ) to ferrous-tripyridyltriazine (Fe^{2+}-TPTZ) and the development of an intense blue colour with an absorption maximum at 539 nm. This occurs at a low pH (Singh & Rajini, 2004).

The reaction is non-specific and any half-reaction which has a less-positive redox potential, under reaction conditions, than the Fe^{3+}/Fe^{2+}-TPTZ half-reaction will drive the Fe^{3+}-TPTZ reduction. Test conditions favour reduction of the complex and thereby colour development, if a reductant (antioxidant) is present (Singh & Rajini, 2004).

The highest value is interpreted as the sample, in this case plant extract, with the best antioxidant ability. A modified method of Singh and Rajini was used (Singh & Rajini, 2004).

The results in figure 3.1 and table 3.1, as generated by colleagues, were used for determining which plant species to select for experimentation.

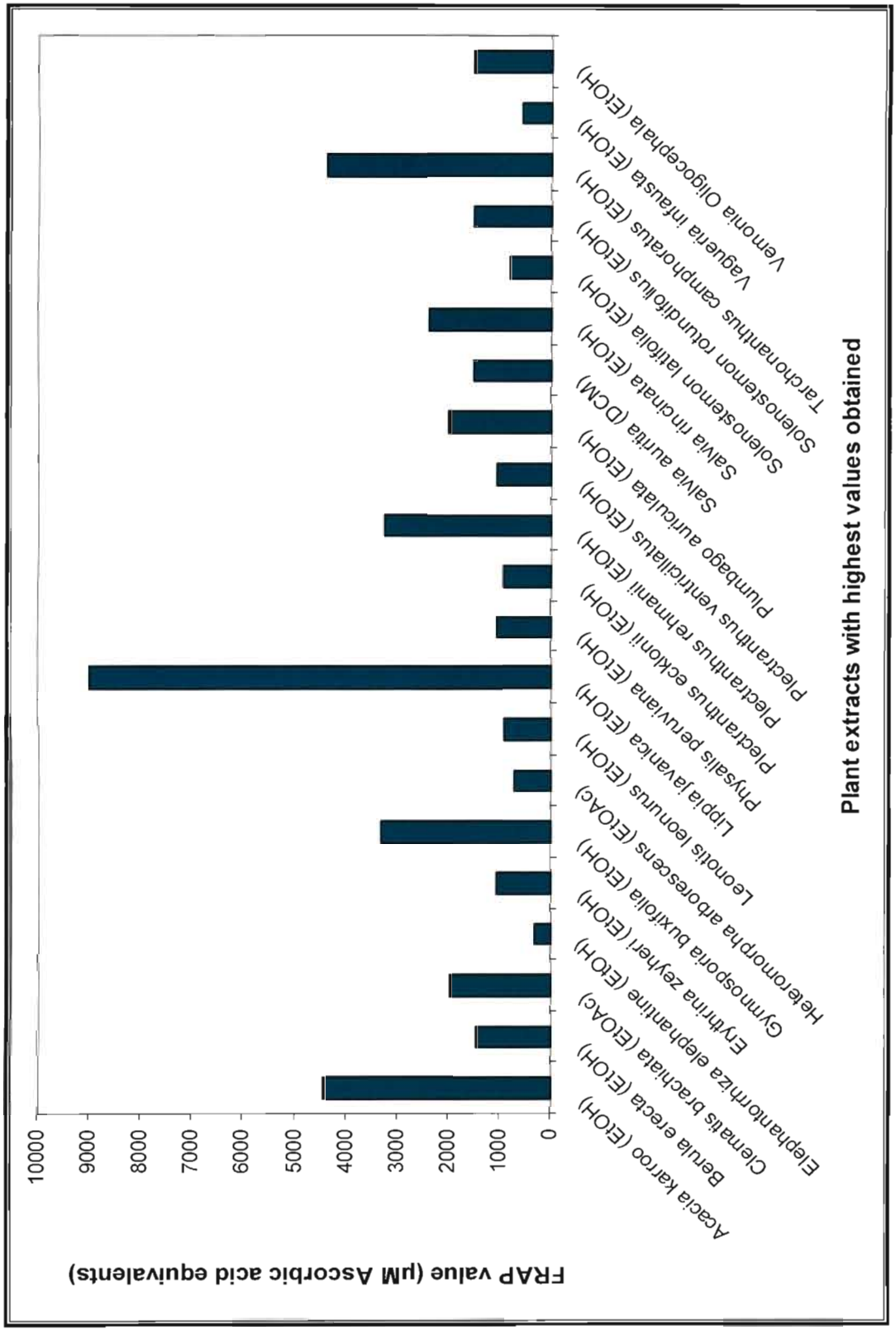


Figure 3.1 FRAP results of the 21 plant species selected for antioxidant activity screening.

Table 3.1 The FRAP results for the seven best plant species according to the highest value obtained for an extract.

Plant species	**Extract**	**Value** (µM Ascorbic acid equivalents)
L. javanica	EtOH	9 009.32
A. karroo	EtOH	4 421.69
T. camphoratus	EtOH	4 384.31
G. buxifolia	EtOH	3 310.74
P. rehmanii	EtOH	3 235.99
S. rincinata	EtOH	2 387.20
P. auriculata	EtOH	1 982.16

3.2.2 Oxygen radical absorption capacity (ORAC)

The ORAC assay is a method for the quantitative measuring of the total antioxidant capacity of a sample (Cao & Prior, 1998).

The effectiveness of various antioxidants present in a sample, in this case plant extracts, is measured in preventing the loss of the fluorescence intensity of a fluorescent marker, during oxygen radical induced damage; fluorescien was used as a fluorescence marker. The time-dependant decrease in the fluorescence intensity of the fluorescien marker is measured and interpreted to the antioxidant ability of the sample (Genox Corporation, 2003).

A high value represents a better antioxidant capacity. The activity of the sample against oxygen radicals is compared to a synthetic standard, in this case the reputed antioxidant Trolox (Genox Corporation, 2003). A modified method from Cao and Prior (1998) was used (Cao & Prior, 1998).

The results in figure 3.2 and table 3.2, as generated by colleagues, were used for determining which plant species to select for experimentation.

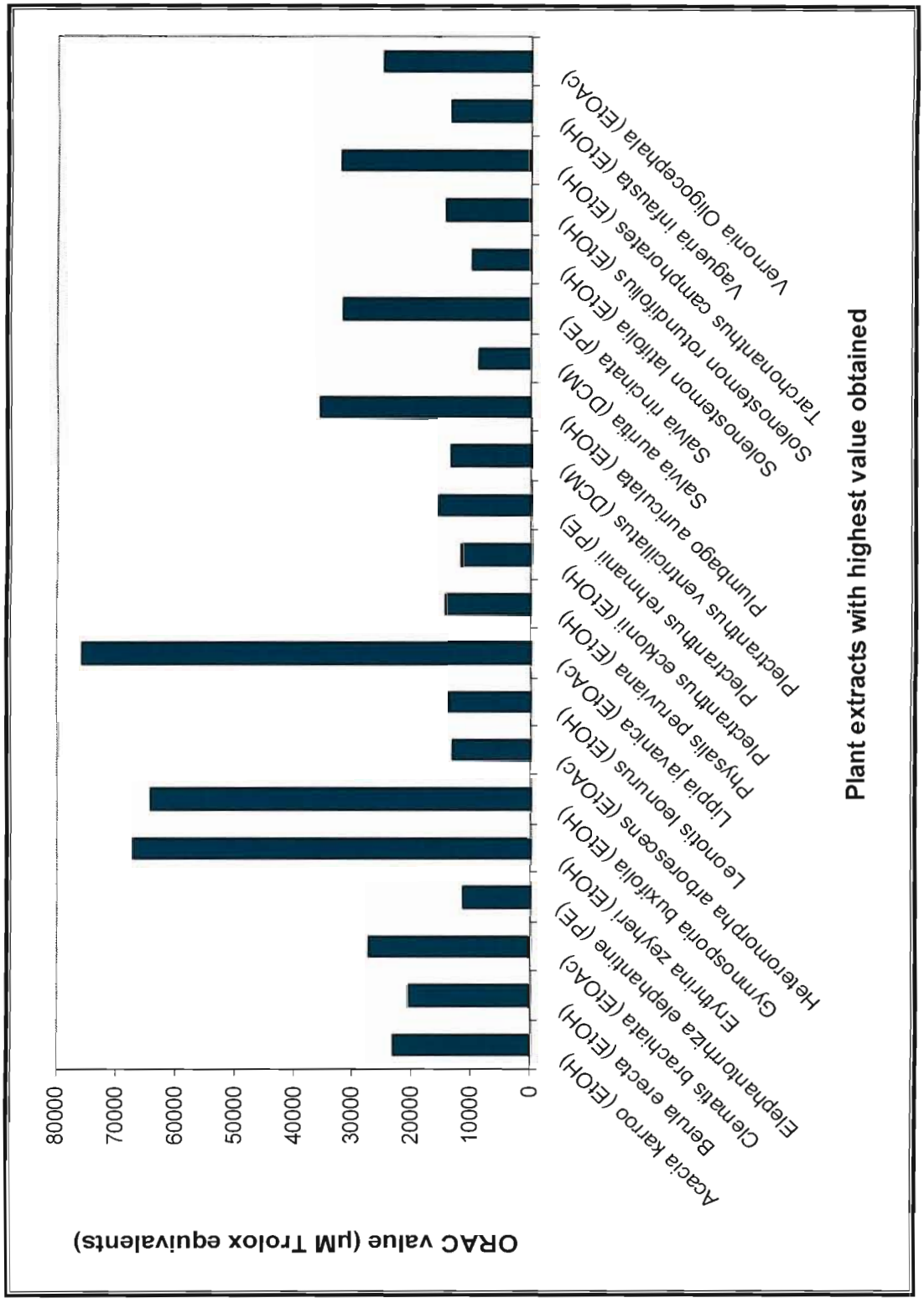


Figure 3.2 ORAC results of the 21 plant species selected for antioxidant activity screening

Table 3.2 The ORAC results for the seven best plant species according to the highest value obtained for an extract.

Plant species	**Extract**	**Value** (μM Trolox equivalents/ℓ)
L. javanica	EtOAc	75 908.10
E. zeyheri	EtOH	67 362.90
G. buxifolia	EtOH	64 318.80
P. auriculata	EtOH	35 586.70
T. camphoratus	EtOH	32 077
S. rincinata	EtOH	28 229.60
C. brachiata	EtOAc	27 462.30

The elucidation of both the FRAP and ORAC results determined the selection of *P. auriculata* and *T. camphoratus* for investigation into possible antioxidant properties with specified biological assays.

Other researchers, in the same research group, investigated *L. javanica* and *G. buxifolia*. *E. zeyheri* and *S. rincinata* were not selected due to the unavailability of plant material in large quantities. *A. karroo* and *P. rehmanii* obtained much lower results in ORAC than in FRAP. *P. auriculata* and *T. camphoratus* were the only other plant species in the best 7 category for both FRAP and ORAC.

Table 3.3 The results of the different extracts of *P. auriculata* and *T. camphoratus* with the EtOH-extracts obtaining the highest values.

***P. auriculata* extracts**				
	PE	**DCM**	**EtOAc**	**EtOH**
ORAC (μM ORAC/mg extract)	3 185.30	6 801.90	5 406.80	35 586.70
FRAP (μM FRAP/mg extract)	28.66	86.17	43.76	1 982.16
***T. camphoratus* extracts**				
	PE	**DCM**	**EtOAc**	**EtOH**
ORAC (μM ORAC/mg extract)	6 285.40	25 822.60	18 347.80	32 077
FRAP (μM FRAP/mg extract)	111.47	1 283.63	861.93	4 384.31

3.3 Selected Plants

3.3.1 Plumbaginaceae

Table 3.4 The scientific classification of the Plumbainaceae family (USDA, 2006a).

	Scientific classification
Kingdom:	Plantae
Division:	Magnoliophyta (flowering plants)
Class:	Magnoliophyta (dicotyledons)
Order:	Plumbaginales
Family:	Plumbaginaceae (leadwort family)
Genus:	*Plumbago*
Species:	*Plumbago auriculata* Lam. (cape leadwort) previously known as *Plumbago capensis*

Figure 3.3 *P. auriculata* bush and flower (Wittman, 2005; UF, 2008).

Other:

Plumbago indica L. (whorled plantain)

Plumbago scandens L. (doctorbush)

Plumbago zeylanica L. (wild leadwort)

The family Plumbaginaceae is a small group of flowering plants, sometimes referred to as the leadwort family or the plumbago family. They are mostly herbs and small shrubby plants found in many different climatic regions form arctic to tropical conditions. The most likely place to encounter leadwort is in the Mediterranean and western Asia (UCMP, 2008).

Some of the other genera in this family include *Acantholimon*, *Bamiana*, *Chaetolimon*, *Dictyolimon*, *Eremolimon*, *Ghasnianthus*, *Ikonnikovia*, *Limoniastrum*, *Meullerolimon*, *Neogontscharovia*, *Plumbagella*, *Vassilczenkoa* (Watson & Dallwitz, 2008a).

Species in this family researched for antioxidant properties include; *Limonium brasiliense*. Murray et al. (2004) detected the presence of antioxidant metabolites in the methanolic extract from the roots of *L. brasiliense* in a preliminary screening for radical scavenging activity. This led to the isolation of active compounds; one flavonoid identified as myricetin 3-*O*-α-rhamnopyranoside, 3 flavan-3-ols, (-)-epigallocatechin 3-*O*-gallate, (-)-epigallocatechin, (+)-epigallocatechin and gallic acid. These polyphenolic compounds are some of the major constituents of the green tea extract and are well recognised to be responsible for the antioxidant properties of tea. They are effective scavengers of radicals generated in aqueous phases and against the propagation of lipid peroxyl radicals (Murray *et al.*, 2004).

The genus *Plumbago* is known for its variety of biological uses, most of them attributed to the presence of naphthoquinones (Charles Dorni *et al.*, 2006). The roots and leaves are powdered and snuffed to relieve headaches. Various other uses have been recorded, such as the removal of warts and the treatment of fractures (Van Wyk *et al.*, 2002; De Paiva *et al.*, 2004). Traditionally, in India roots are used to treat diarrhoea, dyspepsia, piles, skin disease including leprotic lesions and for birth control. In China, the whole plant and its root are used for rheumatic pain, menostatis, carbuncles and bruises (Hazra *et al.*, 2004).

The chemical profile of the genus is marked by the presence of naphthoquinones, flavonoids and terpernoids. Plumbagin (2-methyl-5-hydroxy-1,4-naphthoquinone), one of the major naphthoquinones, is a naturally occurring yellow pigment produced by members of Plumbaginaceae and accumulated mostly in the roots. Lin *et al.* (2003) reported that plumbagin significantly suppressed growth of HeLa tumour cell lines (Lin *et al.*, 2003). It is generally accepted that oxidative stress is involved in the tumour promotion stage because organic peroxides or radical-generating agents, such as benzoylperoxide are tumour promoters in mouse skin; while conversely, some radical scavengers can counteract them (Palasuwan *et al.*, 2005).

Plumbagin is claimed to possess anticarcinogenic, anti-tumour, antispasmodic, hypolipidaemic, antibacterial and antifungal activities (De Paiva *et al.*, 2004; Van Wyk *et al.*, 2002; Tilak *et al.*, 2004). Plumbagin is toxic at high doses (Van Wyk *et al.*, 2002). Among the species of *Plumbago*, *P. zeylanica* has been the most widely assigned to various medicinal properties (Charles Dorni *et al.*, 2006).

P. zeylanica is an important medicinal plant, distributed in the tropical regions of India. It is also found throughout Tropical Africa and some parts of Oriental Asia, Australia, Yemen and the Pacific islands (Ali *et al.*, 2008). The root of *P. zeylanica* is used for menstrual disorder, hemorrhoids, as an appetiser, an antimicrobial agent, an anti-tumour agent and for cholesterol reduction (Palasuwan *et al.*, 2005). The herb *P. zeylanica* in Amrita Bindu is known for its inhibitory effects against superoxide and nitric oxide production. As mentioned by Ali *et al.* (2008) this antioxidant probability was also reported by Natarajan *et al.* (2005) (Ali *et al.*, 2007; Natarajan *et al.*, 2005). Tilak *et al.* (2004) also conclude that *P. zeylanica* have antioxidant effects, due to the flavonoid and phenolic content but more so due to plumbagin (Tilak *et al.*, 2004). Previously isolated classes of constituents from this species include naphthoquinones, flavonoids, steroids, sugars, naphthalenones, alkanes, triterpenes and amino acids (Nguyen *et al.*, 2004; Lin *et al.*, 2003).

Some of the other species in the *Plumbago* genus include; *P. scandens*, suggested as a plant with possible therapeutic value for Parkinson's disease by Morais *et al.* (2004). *P. scandens* has shown activity on tremorine-induced tremor. Both the total acetate fraction and crude ethnolic extract showed an anti-tremor activity with some potency differences (Morais *et al.*, 2004).

3.3.1.1 *Plumbago auriculata* L.

P. auriculata is native to South Africa and occur in the northern and eastern parts of South Africa, as far south as George and Knysna (Van Wyk *et al.*, 2002).

P. auriculata is an attractive shrub or scrambler with a height of up to two meters or more. The leaves are oblong, thin in texture and often minutely gland-dotted. A characteristic feature is the leaf stalk, which is winged at the base, partly clasping the stem. The sticky, glandular flowers are pale blue or rarely white and occur in short clusters at the branch tips (Morais *et al.*, 2004).

P. auriculata (previously *P. capensis*) is not as well known as *P. zeylanica* in ethnopharmacognosy. Previous studies reported the presence of antifungal protein acting

against *Trichosporium vesiculosum* and *Macrophomina phaseolina* in the crude extract of *P. auriculata*. In the study by Charles Dorni *et al.* (2006), *P. auriculata* showed significant anti-inflammatory activity comparable to the standard non-steroidal anti-inflammatory drug indomethacin. The hydroalcoholic extract showed inhibitory action on carrageenan induced paw edema and inhibited the leukocyte migration (Charles Dorni *et al.*, 2006).

The crude chloroform extract from roots of *P. auriculata* was analysed by chromatography and the isolation of the naphthoquinones plumbagin and *epi*-isoshinanolone, the steroids sitosterol and 3-*O*-glucosylsitosterol, plumbagic and palmitic acids was easily achieved. Naphthoquinones are typical components of *Plumbago* species, including *P. auriculata* and these compounds show interesting biological activities (De Paiva *et al.*, 2005).

3.3.2 Asteraceae

Table 3.5 The scientific classification of the Asteraceae family (USDA, 2006b).

	Scientific classification
Kingdom:	Plantae
Division:	Magnoliophyta (flowering plants)
Class:	Magnoliophyta (dicotyledons)
Order:	Asterales
Family:	Asteraceae/Compositae (aster, daisy, or sunflower family)
Genus:	*Tarchonanthus*
Species:	*Tarchonanthus camphoratus* Lin. (camphorbush)

Figure 3.4 *T. camphoratus* bush and close-up of the leaves (Wakfer, 2008).

Other:

Tarchonanthus trilobus L. (broad-leaved camphorbush)

The Asteraceae is the largest family in the fynbos biome of the Cape Floral Kingdom. Descendants of the Khoi and San people have long used many fynbos plants as herbal remedies. The fynbos, with its richness and diversity, represents an important potential source of new drugs (Salie *et al.*, 1996). Asteraceae is the second largest family of flowering plants in terms of number of species, with more than 25 000 species world wide, growing from sea level to the highest mountain peaks. In Southern Africa it is also one of the biggest families of flowering plants with about 246 genera and 2 300 species. The Orchidaceae is the largest family of flowering plants (Herman, 2004).

Asteraceae species include shrubs or small trees of rarely more than six meters in height, with a greyish appearance, hence the Afrikaans vernacular name ("vaal" = grey, "bos" = bush) (Van Wyk *et al.*, 2002).

Many species have traditionally been used medicinally as they are so easily obtained. Well-known medicinal plants are "wilde-als" or African wormwood (*Artemisia afra*), "kapokbos" or wild rosemary (*Eriocephalus africanus*) and wild camphor bush (*T. camphorates*) (Herman, 2004). Most traditional medicines used by the indigenous communities are in fact derived from plants belonging to this family (Salie *et al.*, 1996).

Some species of Asteraceae are poisonous to stock e.g. "vermeerbos" (*Geigeria* species), "kaalsiektebos" (*Chrysocoma ciliate*) and "bloubietou" (*Dimorphotheca spectabilis*) (Herman, 2004).

Asteraceae contains iso/chlorogenic acid, arthroquinones, sesquiterpene lactones, pentacyclic triterpene alcohols, various alkaloids, acetylenes (cyclic, aromatic with vinyl end groups), tannins, flavonols, kaempferol and quercitin. They have terpenoid essential oils that never contain iridoids (Watson & Dallwitz, 2008b). In some taxa, some segments of the family accumulate sesquiterpene lactones which are important natural products responsible for the pharmacological activity of many botanical drugs e.g. Fever few (*Chrysanthemum parthenium*) and Arnica (*Arnica montana*) (Gurib-Fakim, 2006).

Some of the largest genera in this family include *Senecio*, *Vernonia*, *Cousinia* and *Centaurea* (Watson & Dallwitz, 2008b).

Some species of the Asteracea family with antioxidant activity found in the literature include; *Helichrysum arenarium* containing a number of different components; flavonoids, coumarins, phtalides, α-pyron derivatives, terpenoids, essential oils, volatile and fatty acids, among which the most important are the flavonoids. The therapeutic activity of the crude drug is

probably first due to these substances. Research by Czinner *et al.* (2000) concluded that the bioactive compounds of *H. arenarium* can act as primary and secondary antioxidants, scavenge free radicals and therefore inhibit lipid peroxidation and may have beneficial effects on prevention of liver and gall blabber diseases where ROS are involved. Antioxidant properties of its phenolic and flavonoid compounds can be at the origin of these effects (Czinner *et al.*, 2000).

Achillea millefolium is a well-known species of the Asteraceae family. Candan *et al.* (2003) screened the crude water-soluble extract of *A. millefolium* and the main polyphenolic components of the oil, eucalyptol, camphor, β-pinene, borneol, terpinen-4-ol, α-pinene for antioxidant activity. The crude extract showed antioxidant activity, although the individual compounds did not. These results imply that the main components in the oil might synergise each other or that other components are involved in these activities (Candan *et al.*, 2003).

Calendula officinalis (rich in carotenoids), *Matricaria recutita* (contains a great number of polyphenolic compounds), *Echinacea purpurea* and *Rhaponticum carthamoides* were all previously screened for antioxidant activity by Miliauskas *et al.* (2004). The ethyl acetate, acetone and methanol extracts of these species were tested and found to inhibit radical formation to a small extent, with the exception of *R. carthamoides* with good inhibition results. The ethyl acetate and acetone extracts were considerably less effective radical scavengers compared to methanolic extracts (Miliauskas *et al.*, 2004).

Five flavonoids, kaempferol, apigenin, luteolin, quercetin and tiliroside and two sesquiterpene lactones, were isolated from *Lychnophora passerine*. Chicaro *et al.* (2004) showed that quercertin and kaempferol, present in the ethnolic extract, were efficient antioxidants (Chicaro *et al.*, 2004).

The genus *Tarchonanthus* consists of the species *T. camphoratus* and *T. trilobus* (Letsela *et al.*, 2002).

3.3.2.1 *Tarchonanthus camphoratus* L.

T. camphoratus occurs widespread in Southern Africa. It grows in thickets of bushveld, grassland, forest and semi-desert. *T. camphoratus* grows from 2-9 m high. It is a semi-deciduous small tree that grows mostly in large uniform groups, but it grows larger and more densely when it grows alone among other trees in the bush (Letsela *et al.*, 2002). *T. camphoratus* is found in almost any part of South Africa. The leaves are oblong in shape, with the upper surface dark green and strongly net-veined and the lower surface pale grey

and densely velvety. The small, whitish flower heads are followed by small woolly fruits (Van Wyk *et al.*, 2002).

The camphor bush is used for many medicinal purposes. Problems such as blocked sinuses and headaches can be healed by inhaling the smoke from the burning green leaves. Leaves can be used for massaging body stiffness and as a perfume (Letsela *et al.*, 2002). The leaves of the plant are smoked, the fumes from the fresh or dried plant inhaled or either used as decoctions, tinctures or infusions for stomach trouble, bronchitis, asthma, headache, toothache, inflammations, rheumatism, chilblains or abdominal pains. There are also historic records of the Khoi and San people smoking the dried leaves like tobacco, apparently with a slight narcotic effect (Matasyoh *et al.*, 2007; Van Wyk *et al.*, 2002). Splinters are reputed to be poisonous, causing septic sores that heal with difficulty. Women have used the leaves to perfume their hair (Coates Palgrave, 2002).

It is aromatic with a strong smell of camphor (Coates Palgrave, 2002). Despite this camphor-like smell, the plant contains only very small amounts of camphor (Van Wyk *et al.*, 2002). Aromatic and medicinal plants provide a wide variety of volatile terpene hydrocarbons and their corresponding oxygenated derivatives known as essential oils. The antimicrobial activities of essential oils have been well recognised for many years. The oil is dominated by monoterpenes. According to Matasyoh *et al.* (2007) T. *camphoratus* leave oil has a high percentage of oxygenated monoterpenes of which the main constituents are fenchol, 1,8-cineole and α-terpineol. Other components are α-pinene, trans-pinene hydrate, terpinen-4-ol and camphene. β-eudesmol is the major oxygenated sesquiterpene present in the oil. Other sesquiterpene hydrocarbons, such as δ-curcumene, α-carbinol and *ar*-curcumene are also present (Matasyoh *et al.*, 2007). The volatile oil is highly complex and variable, showing large differences between localities. Material form North Africa yield α-fenchyl alcohol, 1,8-cineole and α-terpineol as major compounds, together with a large number of minor constituents. The plant also contains a flavanone – pinocembrin. It is possible that flavonoids and ingredients of the volatile oil are responsible for the reported analgesic, diaphoretic, decongestant and antispasmodic effects (Van Wyk *et al.*, 2002).

In a study done by Matasyoh *et al.* (2007), *T. camphoratus* showed antimicrobial activity against gram positive and negative bacteria and marked antifungal activity against *Candida albicans* (Matasyoh *et al.*, 2007). Many antimicrobial activities have been described for *T. camphoratus*, but no reports on antioxidant activities for this plant could be found.

3.4 Collection and Storage of Plant Materials

The leaves of *P. auriculata* and *T. camphoratus* showed significant antioxidant activity as was reported from a routine screening in our laboratory. The leaves were the only morphological parts examined for antioxidant activity. The selected species were collected from the North-West University Botanical Gardens at the Potchefstroom Campus. Voucher specimens are kept at the A.P. Goossens Herbarium (PUC), North-West University, Potchefstroom. Accession numbers PUC 9764 and PUC 8761. Care was taken not to run out of plant material due to *P. auriculata* seasonal availability and before winter, enough was collected. *T. camphoratus* was available through-out the year. Both species were positively identified by Mr. P. Mortimer, the Curator of the Botanical Garden, North-West University (Potchefstroom Campus).

Different drying methods of plant material exist and avoidance of harsh conditions retains valuable constituents (George *et al.*, 2001). Good ventilation and homogeneous distribution of plant material are important to avoid fungal infestation (Harborne, 1984). Fresh or dried plant material can be a source of secondary plant constituents. Dried material is preferred in cases where there is a delay between collection and processing and dry material has fewer problems with large-scale extraction (George *et al.*, 2001). In the present study, dried leaves of *P. auriculata* and *T. camphoratus* were used. The leaves were spread out on a desktop on tissue paper and left to dry at room temperature (25 °C) for ± 5 days. Plant materials were stored in sealed brown paper bags in a cool, dry place away from direct sunlight, until use.

3.5 Preparation of Extracts and Solvent Extraction

The dried leaves were grounded to increases the surface area accessible to the solvents and for an extraction that was more efficient.

Soxhlet extraction was employed, as it is a convenient way to prepare plant extracts. The most salient advantages of conventional Soxhlet are as follows: (a) plant material is separated from the extract and is repeatedly brought into contact with fresh solvent for continuous, exhaustive extraction; (b) the solvent is recycled and therefore not a lot of solvent is needed; (c) the temperature of the system is close to the boiling point of the solvent, providing energy in the form of heat that helps to increase the extraction kinetics of the system. Technical drawbacks of this technique are; (a) the sample is diluted in large volumes of solvent; (b) the long time required for extraction; (c) loss of compounds occur due

to thermal degradation and volatilisation because of the heat supplied (Ganzler & Salgó, 1987; Silva *et al.,* 1998).

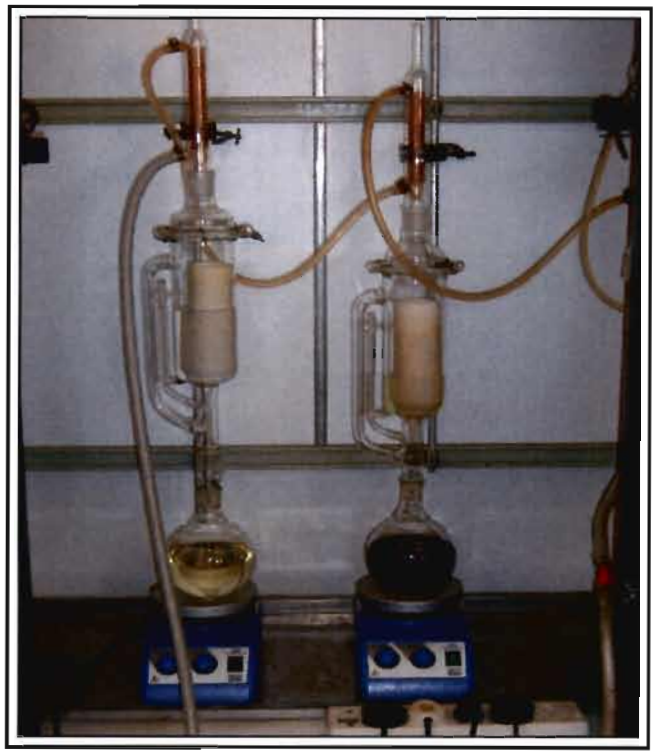


Figure 3.5 The Soxhlet apparatus set-up in laboratory.

According to Farnsworth (1985), the biggest problem in drug development from plants is to choose the appropriate solvents for extraction (Farnsworth *et al.*, 1985). If the type of compounds being isolated is known selective solvent extraction will make the process safe (Williamson *et al.,* 1996). If all the constituents are to be examined a series of solvents with increasing dielectric constant should be used, e.g. hexane-chloroform-ether-ethyl acetate in turn. The solvents used in the extraction of plant material must be inert, easy to remove and of high quality. Plant material was extracted using a range of pure solvents in an increasing order of polarity. The following solvents were used successively:

- Petroleum ether (PE)
- Dichloromethane (DCM)
- Ethyl acetate (EtOAc)
- Ethanol (EtOH)

↓ Increasing polarity

Petroleum ether removed the fixed oils and waxes, dichloromethane separated lipids, terpenoids, and ethyl acetate and ethanol was used to extract the polar compounds. By using a wide range of solvents, it was ensured that all possible plant constituents with different polarities were present in the screened extracts. The plant material was extracted for ± 24-48 hours with each solvent (starting with PE, DCM, EtOAc and finally EtOH), after which the extracts were concentrated using a rotary vacuum evaporator (BUCHI Rotavapor RII) and allowed to dry completely in a fume hood. At the end four extracts were obtained for each plant species; PE-extract, DCM-extract, EtOAc-extract and EtOH-extract. The % yield of extracts were not determined, although there was an obvious pattern of how much of the extracts were produced each time after a Soxhlet extraction with a specific solvent, the order being DCM > PE > EtOH > EtOAc.

CHAPTER 4: BIOLOGICAL TESTING; *IN VITRO* ASSAYS - METHODS, RESULTS & CONCLUSIONS

4.1 Introduction

Natural products have served as an important source of drugs since ancient times and a significant part of today's drugs are somehow derived from natural sources. In recent years, a renewed interest in obtaining biologically active compounds from natural sources has been observed. Therefore, a broad field of bioactive assays, isolation methods and spectroscopic techniques have been developed (Pérez-Bonilla *et al.*, 2006). Various assays can be used to test for biological activity, firstly *in vitro* and later, for promising natural products, *in vivo* (Choi *et al.*, 2002; Kang *et al.*, 2003). As mentioned by Masoko & Eloff (2007), the most widely used methods, for screening antioxidant activity, are those that involve generation of free radical species which are then neutralised by antioxidant compounds (Masoko & Eloff, 2007).

From the previous chapter (chapter 3), *P. auriculata* and *T. camphoratus* were selected for further *in vitro* biological testing. All extracts were subjected to two different *in vitro* assays to determine their antioxidant properties. The biological assays employed were chosen because of their simplicity, reproducibility and relatively low cost while being rapid and simple at the same time (Kaur & Geetha, 2006). The assays were qualitative and were used to identify active extracts. The following spectrophotometrical methods were used for the determination of antioxidant activity of plant extracts: the thiobarbituric acid (TBA) assay (section 4.1) and the nitro-blue tetrazolium (NBT) assay (section 4.2). Both screening methods were used for comparative purposes and for identifying different mechanism of actions.

The selected assays were chosen due to their application on biological samples, based on this the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay was not used. Although, the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay provides an easy and rapid way to evaluate antioxidants (Mensor *et al.*, 2001), some disadvantages limit its applications. DPPH, a long-lived nitrogen radical, which bears no similarity to the highly reactive and transient peroxyl radicals involved in lipid peroxidation. Many antioxidants that react quickly with peroxyl radicals may react slowly or may even be inert to DPPH. Consequently, the antioxidant capacity is not properly rated. The assay is strictly based on a chemical reaction and bears no similarity to biological systems. The validity of the data is limited to a strict chemical sense with context interpretation. Any claims about bioactivity of a sample based solely on this assay would be exaggerated, unscientific and out of context (Huang *et al.*, 2005).

Screening of plant extracts identified the bioactive extracts; the most active extract was selected for isolation of the possible active principle (discussed in chapter 6).

4.2 TBA Assay

The TBA (thiobarbituric acid) assay is one of the most widely used methods for determination of lipid peroxidation in biological samples and involves the reaction between TBA and malondaldehyde (MDA) equivalents (Ottino & Duncan, 1997a&b). MDA, a major degradation product of lipid peroxidation, serves as a marker for assessing the extent of lipid peroxidation. MDA reacts with two molecules of TBA via an acid-catalysed nucleophilic-addition reaction yielding a pinkish-red chromagen, which can be extracted with butanol and measured by spectrophotometric quantisation due to an absorbance maximum at 532 nm (Milton Roy Spectronic 1201) (Hodges *et al.*, 1999; Botsoglou *et al.*, 1994; Ottino & Duncan, 1997a&b). Although the method has been criticised for its lack of specificity and its tendency to overestimate the MDA content, it has been shown to be sensitive to small TBA changes in animal and plant tissue and is recognised as a reliable estimator of lipid peroxidation (Hodges *et al.*, 1999). In this case, a modification of the TBA assay described by Ottino and Duncan (1997a&b) was used for antioxidant activity screening of the plant extracts, thus assessing the amount of lipid peroxidation, via hydroxyl radical ($OH^{\bullet}$) scavenging, using the concentrations of rat brain MDA as an index of induced membrane oxidative damage (Ottino & Duncan, 1997a&b; Sewerynek *et al.*, 1995).

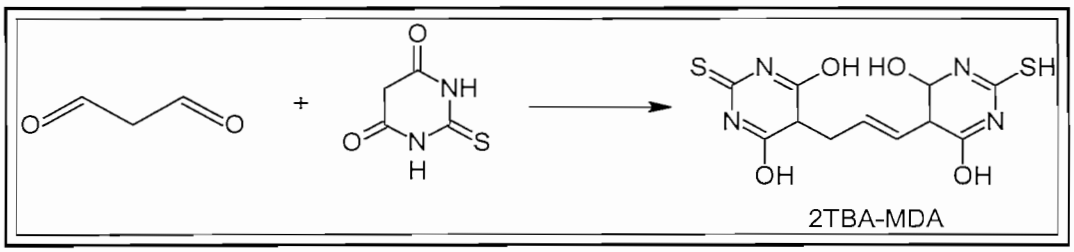


Figure 4.1 Pink chromogen – allegedly a $[TBA]_2$-malondialdehyde adduct (Halliwell & Chirico, 1993).

4.2.1 Extract preparation

The extracts were bleached before beginning with the assay, using an ultra-violet irradiation apparatus to eliminate the interference of the dark green colour resulting from chlorophyll.

Dried extracts were reconstituted in concentrations of 0.625, 1.25 and 2.5 mg/ml.

Dimethyl sulphoxide (DMSO) was used as solvent. The literature reported DMSO to have antioxidant activity (Cui *et al.*, 2004; Reid & Moody, 1994). Therefore, DMSO at a concentration of 10 % after dilution was tested for antioxidant activity (figure 4.2).

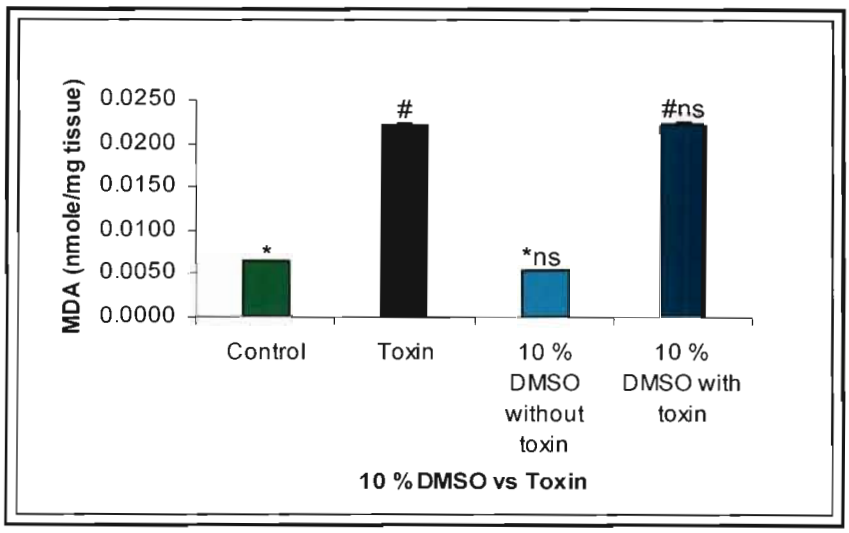


Figure 4.2 The effect of 10 % DMSO on toxin-induced lipid peroxidation, indicating that 10 % DMSO would have no additional effect on the antioxidant ability of the extracts. Each bar represents the mean ± S.E.M.; n = 5; *ns $p > 0.05$ vs. control; #ns $p > 0.05$ vs. toxin.

The extracts were reconstituted in 100 % DMSO and diluted to desired concentrations, thus after 10 x dilution the final concentration of DMSO was 10 %.

4.2.2 Animals

In vitro experiments were performed on whole rat brain homogenates from adult male Sprague-Dawley rats weighing between 200 – 250 g. The animals were housed in a windowless, well-ventilated constant environment room under a diurnal lightning cycle: 12 hours light; 12 hours darkness. Ambient temperature of the animal room was maintained at 21 °C ± 1 °C, with a humidity of 55 % ± 5 %. The animals received standard laboratory chow and water ad libitum. The North-West University Ethics Committee approved the experimental protocol.

4.2.3 Chemicals and reagents

Ascorbic acid (Vit. C), dimethyl sulfoxide (DMSO) and iron(III)chloride (Fe_3Cl) were purchased from Merck-Chemicals (Saarchem, 259 Davidson Rd, Wadeville, Gauteng, South Africa). 1,1,3,3-Tetramethoxypropane (98 %) (TEP), 2-thiobarbituric acid (98 %) (TBA),

butylated hydroxytoluene (BHT), trichloroacetic acid (TCA) and trolox (Vit. E) were purchased from Sigma-Aldrich (Reidstr. 2, D-89555 Steinheim, Germany). Hydrogen peroxide was purchased at a local pharmacy. All other chemicals and reagents used were of the highest chemical purity.

Phosphate buffer (PBS) consisted of 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 2 mM KH_2PO_4 in 1 L Milli-Q water. The pH of the solution was ascertained to be 7.4 and the solution was stored in the refrigerator.

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

Hydrogen peroxide (5 mM H_2O_2) was used, as the toxin, to generate $OH^{\cdot}$ and initiate lipid peroxidation in the rat brain homogenates (Sewerynek *et al.*, 1995 & Garcia *et al.* 2000). Ascorbic acid (1.4 mM) and $FeCl_3$ (4.88 mM) were added to increase the generation of $OH^{\cdot}$ according the following reactions (Cui *et al.*, 2004; Kang *et al.*, 2003; Bhat *et al.*, 2001; Zhu *et al.*, 2006);

Fe^{3+} + ascorbic acid $\rightarrow$ Fe^{2+} + oxidised ascorbic acid

$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH + OH^{\cdot}$ (Fenton reaction)

Trolox was used throughout experimentations as the positive control.

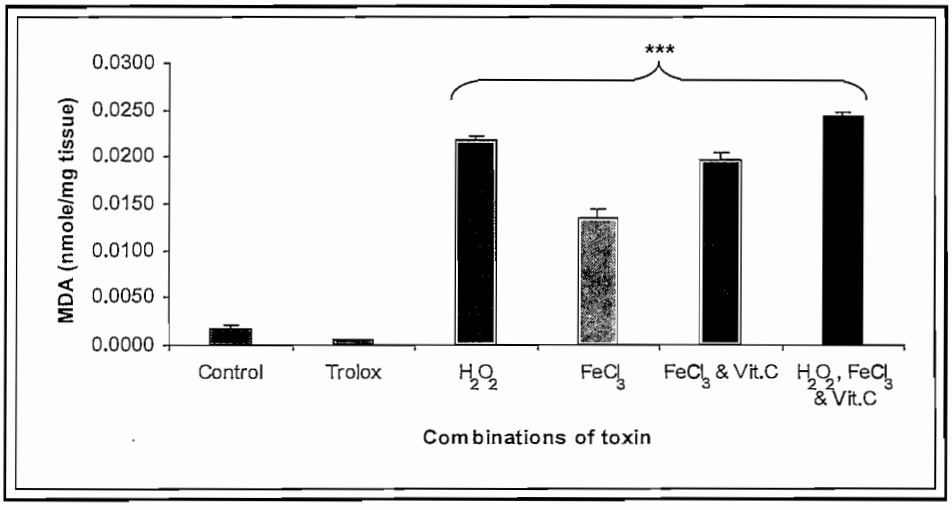


Figure 4.3 The increase of MDA induced by combinations of proposed toxins, with H_2O_2, $FeCl_3$ and Vit.C resulting in the most damage on brain homogenate and the comparison to trolox. Each bar represents the mean ± S.E.M.; n = 5; ***p < 0.001 vs. control.

4.2.4 Preparation of standard

1,1,3,3-Tetramethoxypropane (TEP)/malondealdehyd (MDA) 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 1 ml. A calibration curve was generated by measuring the absorbance at 10 nmole/ml intervals, in the range of 0 – 50 nmole/ml at a detection wavelength of 532 nm using an ultraviolet-visible spectrophotometer. The absorbance of the TBA/MDA-complex was plotted against the known concentration of MDA (figure 4.4).

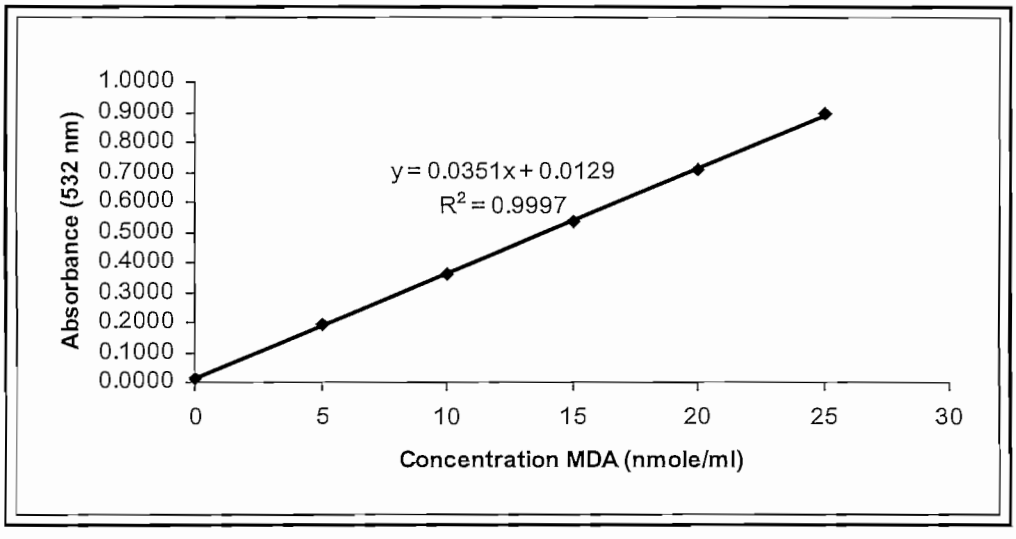


Figure 4.4 MDA standard curve generated from TEP.

4.2.5 Tissue preparation

Rat brain homogenate is a useful model for determining the efficacy of agents to attenuate or potentiate lipid peroxidation and is widely used as a rich source of membrane lipids to assess general lipid peroxidation. Rats were sacrificed by decapitation and the whole brain of each rat was rapidly excised. The whole brain was homogenised in 0.1 M PBS, pH 7.4 to give a final concentration of 10 % (w/v). PBS buffer was used as it has been shown not to scavenge free radicals (Auddy *et al.*, 2003; Kang *et al.*, 2003).

4.2.6 Method

To determine the possible antioxidant activity of extracts, 1 ml rat brain homogenate containing toxin combination and varying concentrations (0.625, 1.25 and 2.5 mg/ml) of extracts were incubated in an oscillating water bath for 60 min at 37 °C, in order to induce lipid peroxidation. After incubation the content was centrifuged at 2000 x g for 20 min, this was done to remove all insoluble protein. The supernatant was removed from each tube and the termination of the incubation period was followed by the addition of 0.5 ml methanolic BHT (0.5 mg/ml), 1 ml TCA (10 %) and 0.5 ml TBA to this fraction. Amplification of lipid peroxidation during the assay was prevented by adding the chain-breaking antioxidant BHT to the sample, TCA to start the acid-heating hydrolysis reaction (acid-catalysed nucleophillic addition reaction) and to precipitate proteins and TBA to bind to the formed MDA and form the pink chromogen (Halliwell & Chirico, 1993). The tubes were sealed (marbles) and the mixtures heated to 60 °C in a water bath for 60 min, to release the protein-bounded MDA through hydrolysis. Following the incubation, the samples were cooled on crushed ice until it reached room temperature and the TBA-MDA complexes were extracted with 2 ml buthanol and centrifuged at 2000 x g for 10 minutes. The absorbance was read at 532 nm.

4.2.7 Data collection

The absorbance values obtained were converted to MDA levels (nmole MDA) from the calibration curve generated with TEP (figure 4.4). Results and the extent to which lipid peroxidation occurred were expressed as nmole MDA/mg tissue in tabel 4.1.

4.2.8 Results

The levels of lipid peroxidation indices in brain homogenates are shown in table 4.1, figure 4.5 and 4.6.

Table 4.1 The effect of extracts on toxin induced lipid peroxidation in rat brain homogenate.

Test Compounds	Concentration	Lipid peroxidation (nmole MDA/mg tissue	± S.E.M.
Control		0.006	0.0003
Toxin	5 mM H_2O_2 4.44 mM Vit. C 1.68 mM Fe_3Cl	0.027	0.0004
Trolox		0.0002	0.00003
P. auriculata	mg/ml		
PE	0.625	0.014	0.0003
	1.25	0.009	0.0003
	2.5	0.008	0.0001
DCM	0.625	0.01	0.0003
	1.25	0.0094	0.0004
	2.5	0.0091	0.001
EtOAc	0.625	0.014	0.0004
	1.25	0.01	0.001
	2.5	0.007	0.0003
EtOH	0.625	0.012	0.001
	1.25	0.008	0.001
	2.5	0.006	0.0002
T. camphoratus	mg/ml		
PE	0.625	0.022	0.001
	1.25	0.021	0.001
	2.5	0.017	0.001
DCM	0.625	0.013	0.001
	1.25	0.01	0.001
	2.5	0.007	0.001
EtOAc	0.625	0.01	0.001
	1.25	0.007	0.001
	2.5	0.0072	0.001
EtOH	0.625	0.009	0.001
	1.25	0.005	0.001
	2.5	0.004	0.0001

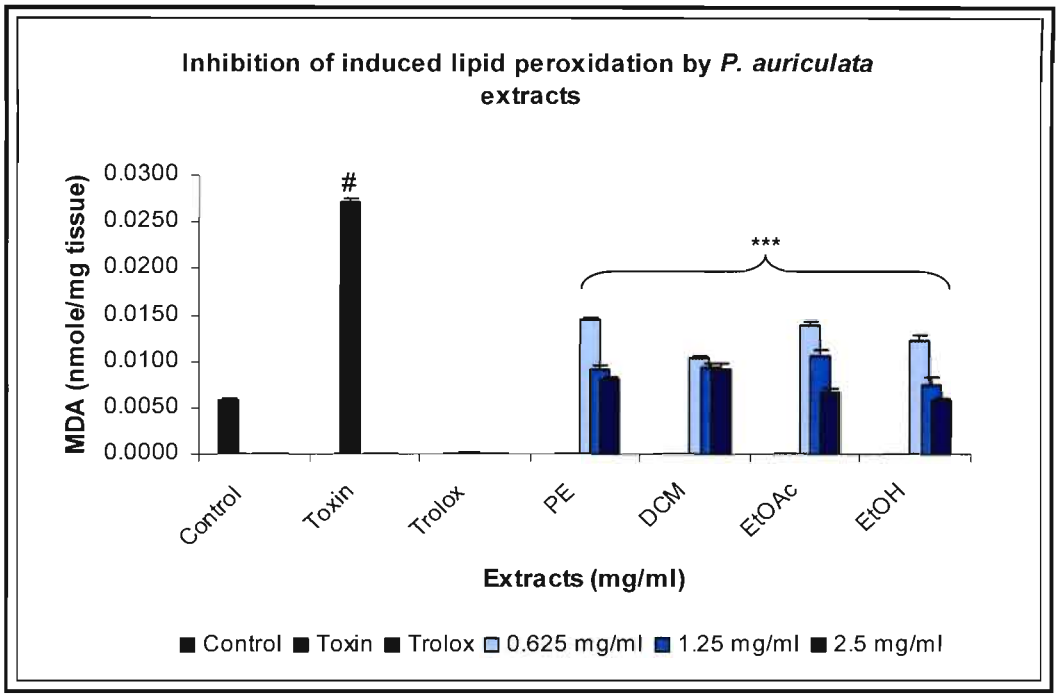


Figure 4.5 The attenuation of lipid peroxidation by different concentrations of *P. auriculata* extracts in whole rat brain homogenates *in vitro*. Each bar represents the mean ± S.E.M.; n=5. ***p < 0.001 vs. toxin (#).

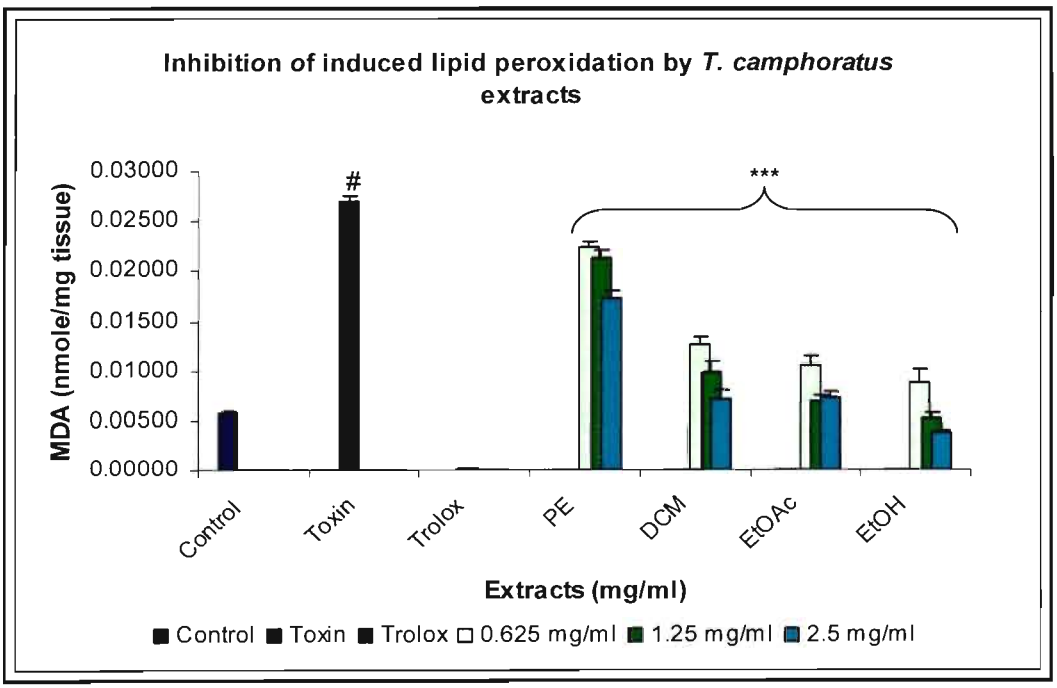


Figure 4.6 The attenuation of lipid peroxidation through $OH^{\bullet}$ scavenging by different concentrations of *T. camphoratus* extracts in whole rat brain homogenates *in vitro*. Each bar represents the mean ± S.E.M.; n=5. ***p < 0.001 vs. toxin (#).

4.2.9 Statistical analysis

Graphpad instat was used for the statistical analysis of data. Results are given as the mean ± S.E.M. of 5 repeats. Data were analysed by one-way analysis of variance (ANOVA) followed by the Student-Newman Keuls test for multiple comparisons. Difference between groups were considered to be significant when $p < 0.05$.

4.2.10 Discussion

All the plant extracts of *P. auriculata* and *T. camphoratus* showed *in vitro* antioxidant activity with the TBA-assay.

In comparison to the toxin (0.027 ± 0.0004 nmole MDA/mg tissue) the extracts showed significant decrease in MDA formation in rat brain tissue *in vitro*. This attenuation in 2TBA-MDA complex formation indicates less lipid peroxidation and the $OH^{\bullet}$ radical scavenging abilities of the extracts. The extracts fell in the range of 0.022 – 0.004 nmole MDA/mg tissue. However, none of the extracts could be compared to the results obtained for Trolox ($0.0002 \pm 3x10^{-5}$ nmole MDA/mg tissue).

The EtOH-extracts of *P. auriculata* and *T. camphoratus* (concentration range 0.625 – 2.5 mg/ml) showed the best results overall. The highest concentration (2.5 mg/ml) showed the most promise for antioxidant activity, 0.006 ± 0.0002 nmole MDA/mg tissue achieved for *P. auriculata* and 0.004 ± 0.0001 nmole MDA/mg tissue for *T. camphoratus*. This can also be compared to the highest values obtained for, *P. auriculata* (EtOH extract) and *T. camphoratus* (EtOH extract), from the ORAC and FRAP assays.

As evident from the results, the *T. camphoratus* extracts had the lowest value and attenuated lipid peroxidation the best. The results were correlated to the antioxidant activity of extract.

4.3 NBT Assay

The assay is based on the ability of free radicals to reduce the yellow dye NBT to insoluble nitroblue diformazan (NBD) (blue dye), which can be extracted with glacial acetic acid (GAA) and the relative absorbance values can then be measured at 560 nm using a spectrophotometer (Ottino & Duncan, 1997a&b).

A modification of the NBT assay from Ottino and Duncan (1997a&b) was used for assaying the ability of extracts to scavenge superoxide radicals ($O_2^{\bullet-}$) (Ottino & Duncan, 1997a&b). $O_2^{\bullet-}$ was generated *in vitro* by the toxin potassium cyanide (KCN) (Zhu *et al.*, 2006).

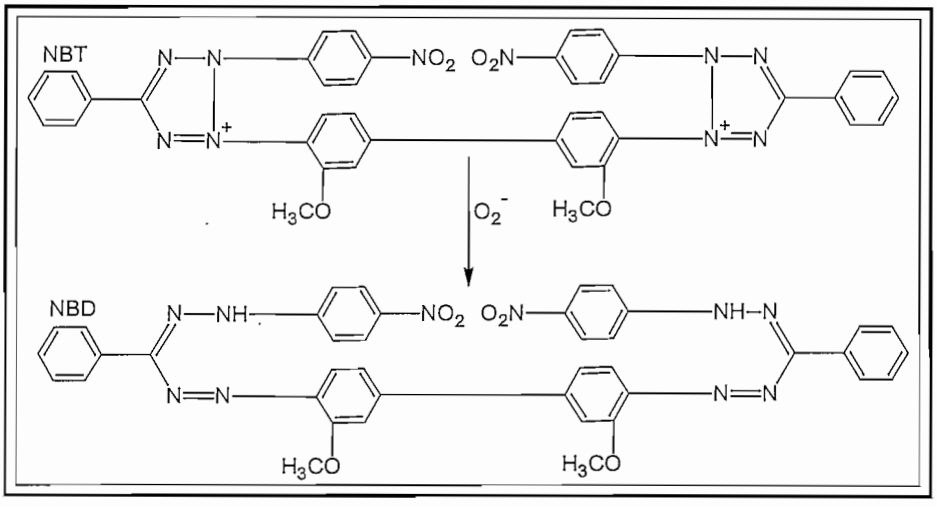


Figure 4.7 The reaction of NBT reduction to NBD via $O_2^{\bullet-}$ (Kaur & Geetha, 2006).

4.3.1 Extract preparation

As described in 4.2.1.

4.2.2 Animals

As described in 4.2.2.

4.3.3 Chemicals and reagents

Potassium cyanide was purchased from Merck-Chemicals (SAARCHEM, 259 Davidson Rd, Wadeville, Gauteng, South Africa). Bovine serum albumin (BSA), nitro-blue diformazan (NBD) and nitro-blue tetrazolium (NBT) were purchased from Sigma-Aldrich (Reidstr. 2, D-89555 Steinheim, Germany). All other chemicals used were of the highest chemical purity.

Stock solutions of potassium cyanide (KCN) were prepared by dissolving KCN in Milli-Q water. Concentrations of 0.25, 0.5 and 1 mM were tested to determine the most effective concentration for $O_2^{\bullet-}$ generation *in vitro* in rat brain homogenate.

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

0.1 % NBT solution was prepared by dissolving NBT in ethanol before diluting to the required volume with Milli-Q water. The final ethanol concentration in the incubation flasks was less than 0.5 %. Fresh solutions were prepared daily and the solution was protected from light by covering the container with aluminium foil.

4.3.4 Preparation of standards

4.3.4.1 BSA standard

In order to express the scavenging of $O_2^{\bullet-}$ in terms of µmoles diformazan/mg protein, an estimation of the protein content of each brain was determined prior to the NBT assay.

Increasing concentrations of bovine serum albumin (BSA) was used as a standard for determining the protein content of each brain. Protein standards containing 0 – 300 µg/ml of BSA at intervals of 60 µg/ml were used to generate a standard curve (figure 4.8). These standards were assayed in the same manner as described by Lowry *et al.* (1951).

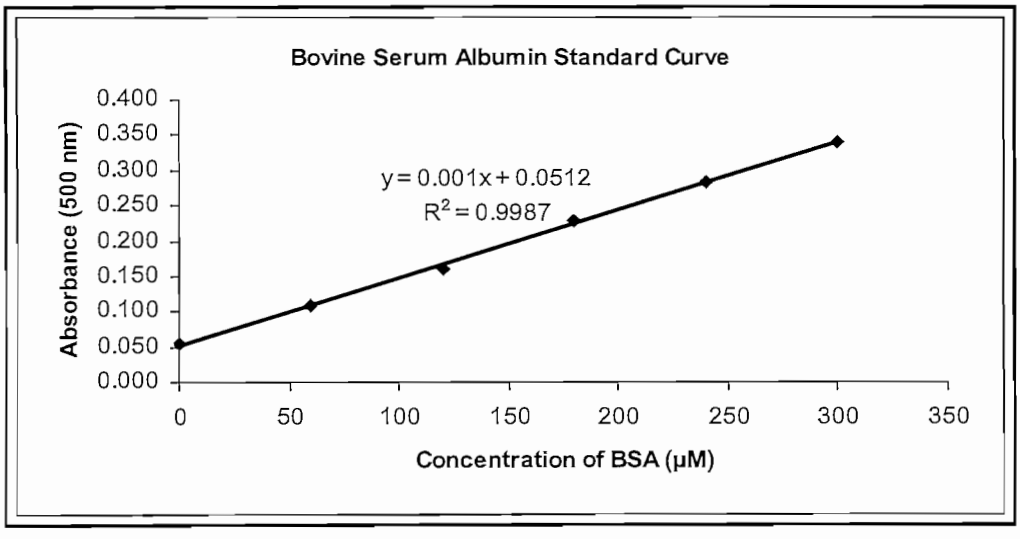


Figure 4.8 Protein standard curve generated from bovine serum albumin.

4.3.4.2 NBD standard

Nitroblue diformazan (NBD) was used as a standard measuring the level of induced $O_2^{\bullet-}$ with the NBT assay. A series of reaction tubes, each containing appropriate aliquots of NBD dissolved in acetic acid was prepared to a volume of 1 ml. A standard curve was generated by measuring the absorbance at 560 nm in 100 µmole/ml increments to the range of 0 – 400 µM (figure 4.9). The absorbencies were read using a spectrophotometer.

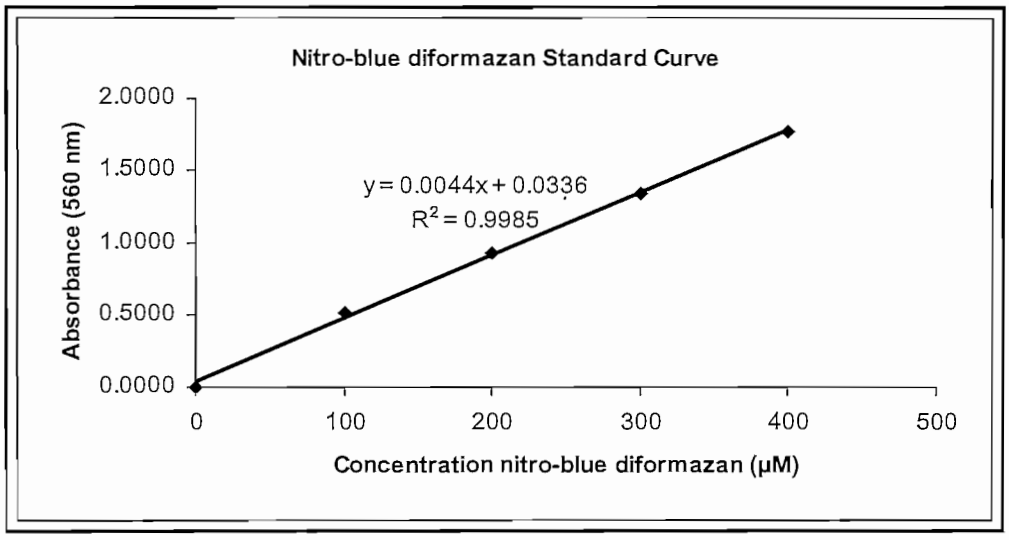


Figure 4.9 Nitro-blue diformazan standard curve.

4.3.5 Tissue preparation

As described in 4.1.1.5.

4.3.6 Method

To determine $O_2^{\bullet-}$ scavenging ability of extracts, homogenate (1 ml) containing 1 mM KCN, inducing $O_2^{\bullet-}$ anions, in the presence of varying concentrations of extracts (0.625, 1.25 and 2.5 mg/ml) were incubated with 0.4 ml 0.1 % NBT solution for 1 hour at 37 °C in an 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 min. The supernatant was decanted and the pellet was resuspended with 2 ml GAA, into which the reduced NBT dye was extracted. The absorbencies of the GAA fractions were measured at 560 nm and converted to µmoles diformazan using a standard curve generated from NBD (figure 4.9). Final results were expressed as µmole/mg protein.

Protein estimation for each brain was performed prior to the NBT assay using the method described by Lowry *et al.* (1951).

Briefly, an aliquot of 0.1 ml homogenate was added to 4.9 ml PBS. Of this mixture, 1 ml was added to 6 ml alkaline copper reagent solution, vortexed and left to stand at room temperature for 10 min. To this 0.3 ml Folin-Ciocalteau's phenol reagent was added and the tubes were left to stand in the dark at room temperature for 30 min. After the incubation, the absorbance was measured at 500 nm and converted to mg protein. The protein content of each brain used was measured in duplicate.

4.3.7 Data collection

Absorbance values of the protein assay were converted to mg protein, using the calibration curve generated from increasing concentrations of BSA (figure 4.8). These values were used in expressing the superoxide anion scavenging results.

Absorbance values of each NBT assay was converted to µmoles diformazan produced using the standard curve generated from increasing concentrations of NBD (figure 4.9). Results were expressed as µmoles diformazan/mg protein.

4.3.8 Results

The results obtained from the NBT assay are presented in table 4.2, figure 4.10 and 4.11.

Table 4.2 The *in vitro* effect of selected compounds on KCN-induced superoxide anion formation in rat brain homogenate.

Test Compounds	Concentration	Diformazan (μM/mg protein)	± S.E.M.
Control		22.55	0.77
Toxin	1 mM KCN	30.5	0.78
Trolox		19.05	0.58
P. auriculata	mg/ml		
PE	0.625	29.02	0.52
	1.25	17.84	0.64
	2.5	15.32	0.71
DCM	0.625	20.65	0.73
	1.25	18.51	0.55
	2.5	17.74	0.38
EtOAc	0.625	18.87	0.54
	1.25	13.24	0.88
	2.5	11.44	0.97
EtOH	0.625	19.17	1.04
	1.25	18.42	1.52
	2.5	14.89	0.73
T. camphoratus	mg/ml		
PE	0.625	24.39	2.79
	1.25	14.88	0.42
	2.5	14.24	0.66
DCM	0.625	20.24	1.23
	1.25	15.37	0.87
	2.5	10.94	0.36
EtOAc	0.625	12.66	1.03
	1.25	10.17	1.01
	2.5	3.59	0.5
EtOH	0.625	20.9	0.87
	1.25	17.56	1.44
	2.5	10.89	1.24

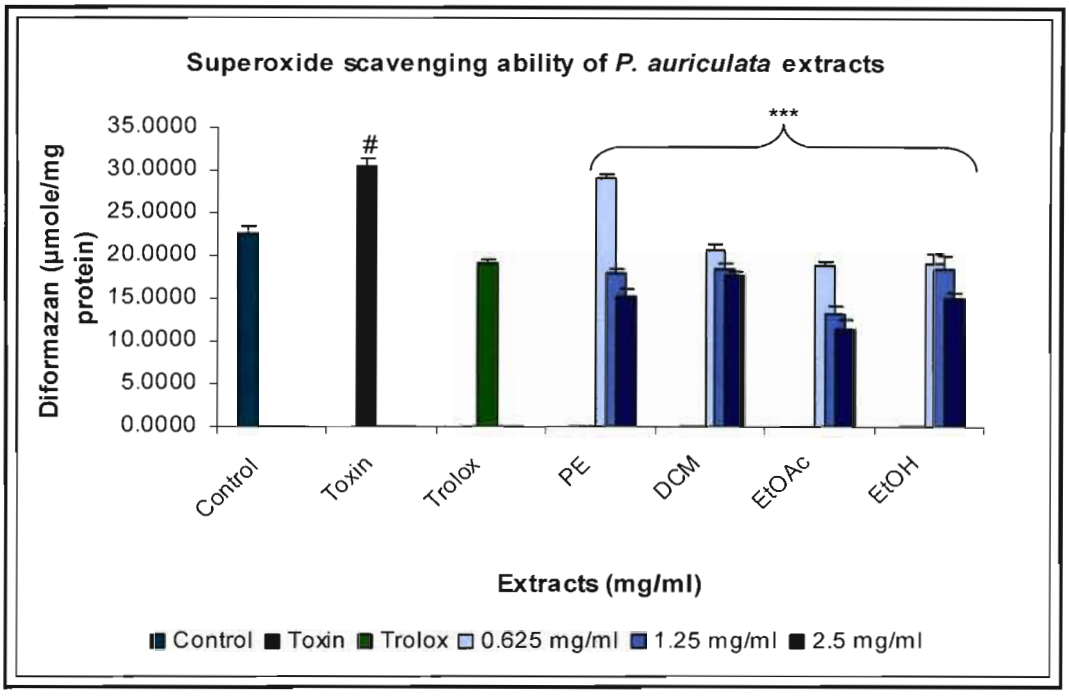


Figure 4.10 The superoxide scavenging properties of extracts in the presence of KCN in rat brain homogenate. Each bar represents the mean ± S.E.M.; n = 5. ***p < 0.001 vs. toxin (#).

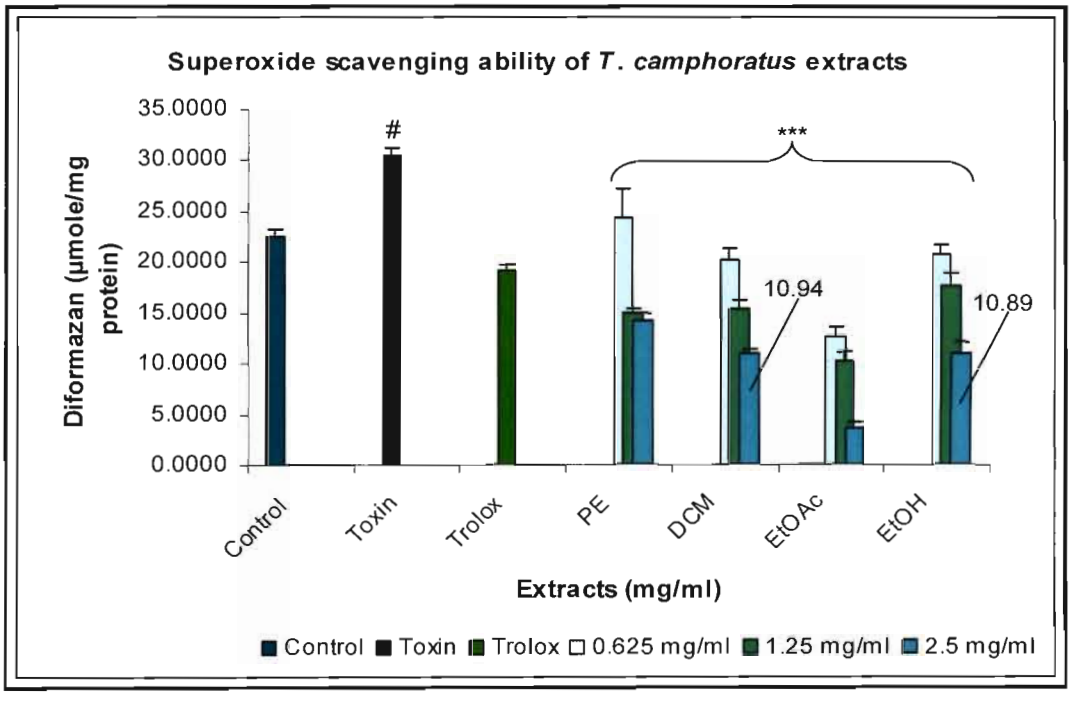


Figure 4.11 The superoxide scavenging properties of extracts in the presence of KCN in rat brain homogenate. Each bar represents the mean ± S.E.M.; n = 5. ***p < 0.001 vs. toxin (#).

4.3.9 Statistical analysis

Graphpad instat was used for the statistical analysis of data. Results are given as the mean ± S.E.M. of 5 repeats. Data were analysed by one-way analysis of variance (ANOVA) followed by the Student-Newman Keuls test for multiple comparisons. Difference between groups were considered to be significant when $p < 0.05$.

4.3.10 Discussion

All the plant extracts of both, *P. auriculata* and *T. camphorates*, showed *in vitro* antioxidant activity with the NBT assay.

The plant extracts were compared to the toxin, 30.5 ± 0.78 μmole/mg proteins and showed significant reduction of NBD formation in rat brain protein content and thus $O_2^{\bullet-}$ scavenging ability, as $O_2^{\bullet-}$ is responsible for the reduction of NBT to NBD. Some of the extracts even showed better results than that of Trolox (19.05 ± 0.58 µmole/mg protein). The values for the extracts ranged from 29.02 – 3.59 μmole/mg protein.

The EtOAc-extracts of *P. auriculata* and *T. camphoratus* (concentration range 0.625 – 2.5 mg/ml) showed the best results overall. The highest concentration (2.5 mg/ml) showed the most promise for antioxidant activity, 11.44 ± 0.97 µmole/mg protein achieved for *P. auriculata* and 3.59 ± 0.5 µmole/mg protein for *T. camphoratus*.

As evident from the results, the *T. camphoratus* extracts had the lowest values and thus the most $O_2^{\bullet-}$ scavenging ability which correlated to the best antioxidant activity.

4.3 Toxicity Testing

Valuable pharmacological and toxicological knowledge has been gained from *in vivo* and *in vitro* studies and hence the importance of assessing the safety of medicinal plants and compounds isolated (Van Wyk *et al.*, 2002). The determination of cell growth rates is widely used in the testing of drug action, cytotoxic agents and screening of other biological active compounds (Freimoser *et al.*. 1999; Mosmann, 1983).

The cytotoxic effects of *P. auriculata* and *T. camphoratus* extracts were evaluated using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, first described by Mosmann (1983), a quantitative colorimetric assay to determine the toxicity against mammalian cells. The MTT assay is among one of the most versatile and popular assays fast, simple, cheap and accurate (Freimoser *et al.*, 1999; Mosmann, 1983).

The MTT assay is based on the cleavage of yellow MTT, a tetrazolium salt, which is reduced by the respiratory chain and other electron transport systems to form non-water-soluble purple formazan crystals within metabolic active cells, just like in the NBT assay. The formazan crystals are then solubilised (usually in an organic solvent) to obtain the insoluble purple formazan product in a homogeneous, coloured solution suitable for measurement. The absorbance of this coloured solution can be quantified by measuring at a certain wavelength (usually between 500 and 600 nm) by a spectrophotometer. The absorption maximum is dependant on the solvent employed. The colorimetric signal is proportional to the cell number and therefore conversion can be directly related to the number of viable cells. Results are measured on a multi-well scanning spectrophotometer (in this case a 96-well okate reader) that insures a high degree of precision (Freimoser *et al.* 1999; Mosmann, 1983; Wilson, 2000). This method is thus an indication of cytotoxicity, proliferation or activation (Mosmann, 1983). The MTT assay was used to evaluate the growth or survival of human epithelial cells (HeLa) to evaluate the toxicity of extracts, the same as in Trivedi *et al.* (1990).

HeLa cells – the first continuous cancer cell line – have been a mainstay of cancer research ever since their isolation from the aggressive glandular cervical cancer of a young woman, Henrietta Lacks, more than 50 years ago. Knowledge of almost every process that occurs in human cells has been obtained using HeLa cells and the many other cell lines that have since been isolated (Masters, 2002). The mechanism of MTT conversion has been questioned by Berridge *et al.* (1996), whose study indicated that most of the cellular conversion of MTT to formazan occurs outside the mitochondria and that the pyridine cofactors NADH and NADPH are better reducing agents for MTT than mitochondrial enzymes (Berridge *et al.*, 1996). However, the assay system is not invalidated from a cytotoxicity viewpoint and still has the advantages of being simple, inexpensive and widely applicable (Cookson *et al.* 1995).

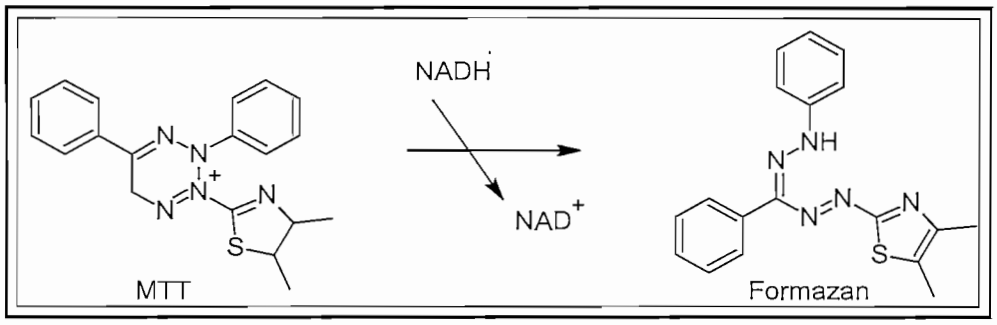


Figure 4.12 Yellow MTT reduction to purple formazan in the mitochondria of living cells (Mossman, 1983).

4.3.1 Chemicals and reagents

Dulbeccos's Modified Eagle's Medium (DMEM), foetal bovine serum (FBS) and trypsin were purchased from Scientific Group (Midrand, South Africa). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and isopropanol were all purchased form Sigma-Aldrich (Reidstr., D-89555 Steinheim, Germany). HeLa cells were purchased from Adcock Ingram (Gibbco, Scotland). Other chemicals used were of highest grade available commercially.

4.3.2 Cell culture preparation

The toxicity evaluations of the prepared extracts were performed on HeLa cells in DMEM medium containing 10 % FBS, 100 IU/ml penicillin, 0.1 mg/ml streptomycin and 0.25 µG/ml fungizone. Two confluent flasks were used for preparing the cell culture. The cells were examined daily; the growth medium was changed twice a week to maintain the highest levels of sterility and to avoid infecting the cells and the cells were trypsinised weekly and then allowed to reach confluence before being used in the toxicity assays. After trypsinisation, a single cell suspension was obtained. The cells were counted using a haemocytometer, visualising the cells under the microscope and ensuring that at least 95 % of the cells were viable. The stock cell solution was then adjusted to 0.75 million cells per milliliter for 1.5 million cells, by diluting with the culture media using equation 4.1;

$$VCR = \frac{CD \times FVSS}{NCSC}$$

CD = Cell density (1.5 x log cell/ml), FVSS = Final volume of single cell + DMEM required to seed, NCSC = Number of counted single cells available, VCR = Volume of cells required and Single cell = Cell suspension + trypsin.

4.3.3 Extract preparation

The prepared extracts were dissolved in 1 % DMSO in distilled water and concentrations of 10 mg/ml, 2 mg/ml, 0.4 mg/ml and 0.8 mg/ml were made for each extract. There were some dissolution problems therefore the concentrations only present an estimate range of high to lower concentrations. The extracts were filtered and sterilised before use.

4.3.4 Preparation of microplates

24-Well plates were used in the toxicity assay. The plate was set-up with 18 columns and 3 rows in each column. The following were added: 1 000 µl of standardised cell cultures (1.5 million cell/ml), 400 µl of DMEM media and 100 µl of each extract concentration to wells of column 3 through to 18. Column 1 contained only 1 000 µl of cells and 500 µl DMEM, acting as the 0 % growth control and to give an indication of contamination. Column 2 contained 1 000 µl cells, 500 µl DMEM and 100 µl 1 % DMSO (solvent), acting as the 100 % growth control to ensure that normal growth occurred. To each column a blank was added, with no cells. The plates were incubated under humidified conditions at 37 °C in 5 % CO_2 for 24 hours. The assay was conducted in triplicate.

4.3.5 Preparation and addition of MTT

The stock solution of MTT (5 mg/ml PBS) was filter sterilised and stored at 4 °C until required. After a 24 hour incubation period, 200 µl of the prepared MTT solution (0.25 mg/ml PBS) was added to all wells and the plates were incubated for a further 2 hours to terminate cell growth and MTT cleavage. This was performed in the laminar flow chamber with the light off. After a 2 hour incubation period at 37 °C and 5 % CO_2, the MTT supernatant was aspirated from each well. The reaction was stopped and the formazan crystals solubilised with the addition of 250 µl of isopropranol to each well. The plates were shaken to allow the isopropranol to dissolve the formazan crystals completely. From each well 100 µl of the solution was transferred to a 96 well plate. The absorbance of each well was measured at a test wavelength of 560 nm and a reference wavelength of 650 nm using a microplate reader. Results were expressed as a percentage of cellular viability of the controls, using equation 4.2.

Equation 4.2

$$\% \text{ cellular viability} = \frac{\Delta \text{ Absorbance} - \Delta \text{ Blank} \times 100}{\Delta \text{ Control} - \Delta \text{ Blank}}$$

Where; Δ Control (mean cell control) = cell $control_{550}$ – cell $control_{560}$

Δ Blank = mean $blank_{550}$ – mean $blank_{560}$

Δ Absorbance = $absorbance_{550}$ – $absorbance_{560}$

4.3.5 Results

The results obtained from the MTT assay are presented in table 4.3, figure 4.13 and 4.14.

Table 4.3 The % cell viability results of *P. auriculata* extracts against the cellular growth of HeLa cells.

Test Compounds	Concentration	Cellular Growth % Cell Viability	± S.E.M.
0 % Growth		0	0
100 % Growth		101.99	3.5490
P. auriculata	mg/ml		
PE	0.08 mg/ml	99.97	0.78
	0.4 mg/ml	97.29	1.5
	2 mg/ml	93.77	2.44
	10 mg/ml	72.69	1.36
DCM	0.08 mg/ml	96.47	2.04
	0.4 mg/ml	92.68	2.03
	2 mg/ml	89.77	2.51
	10 mg/ml	88.08	2.84
EtOAc	0.08 mg/ml	97.76	0.54
	0.4 mg/ml	95.43	1.43
	2 mg/ml	94.35	2.25
	10 mg/ml	89.95	0.68
EtOH	0.08 mg/ml	97.54	0.42
	0.4 mg/ml	90.46	0.77
	2 mg/ml	88.13	0.57
	10 mg/ml	88.15	1.39

Table 4.4 The % cell viability results of *T. camphoratus* extracts against the cellular growth of HeLa cells.

T. camphoratus	mg/ml		
PE	0.08 mg/ml	103.71	1.94
	0.4 mg/ml	93.76	1.35
	2 mg/ml	85.14	2.26
	10 mg/ml	65.6	4.06
DCM	0.08 mg/ml	92.28	2.59
	0.4 mg/ml	87.36	1.95
	2 mg/ml	82.42	1.76
	10 mg/ml	69.33	3.0
EtOAc	0.08 mg/ml	91.41	1.36
	0.4 mg/ml	91.32	1.31
	2 mg/ml	89.98	1.8
	10 mg/ml	82.11	3.6
EtOH	0.08 mg/ml	93.75	1.85
	0.4 mg/ml	85.88	1.81
	2 mg/ml	85.16	3.63
	10 mg/ml	79.6	4.55

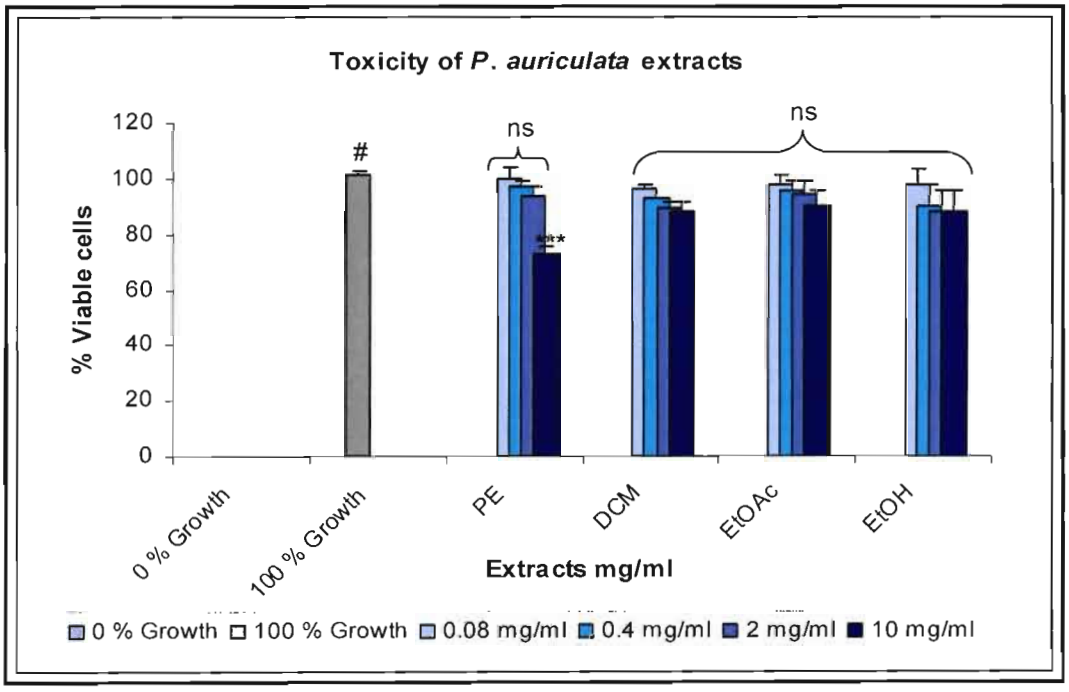


Figure 4.13 Results obtained after exposure of HeLa cells to *P. auriculata* extracts. Each bar represents the mean ± S.E.M.; ***$p < 0.001$, ns $p > 0.05$ vs. control (100 % Growth).

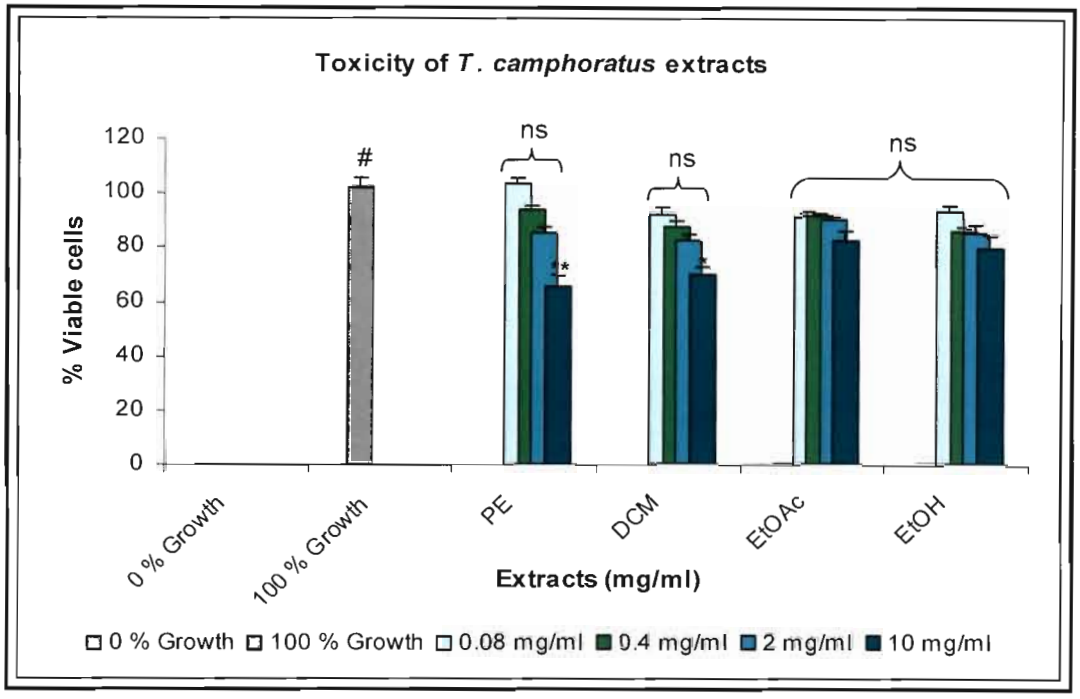


Figure 4.14 Results obtained after exposure of HeLa cells to *T. camphoratus* extracts. Each bar represents the mean ± S.E.M.; **p < 0.01, *p < 0.05, ns p > 0.05 vs. control (100 % Growth).

4.3.6 Statistical analysis

Statistical analysis was done using one-way ANOVA method followed by the Student-Newman Keuls test for multiple comparisons. All absorbance values of each assay (done in triplicate) were statistically analysed (n = 9). The mean was taken of the average of all three to present in the graph and be compared to the control.

4.3.7 Discussion

The control, signified as 100 % cellular growth with a value of 101.99 ± 3.55 % viable cells, was compared to all the extracts of *P. auriculata* and *T. camphoratus*.

All of the *P. auriculata* extracts, except PE (10 mg/ml) with 72.69 ± 1.36 % viable cells, seemed to affect the process of cellular growth non-significantly in comparison to the control (100 % cellular growth). The non-toxic extracts fell in the range of 99.97 – 88.08 % viable cells.

Only the *T. camphoratus* extracts of PE (10 mg/ml), 65.6 ± 4.06 % viable cells, and DCM (10 mg/ml), 69.33 ± 3.0 % viable cells, seemed to reduce the process of cellular growth

significantly in comparison to the control and are thus most toxic to the HeLa cells. The other non toxic extracts fell in the range of 103.71 – 79.6 % viable cells.

Most of the extracts of *P. auriculata* and *T. camphoratus* screened negative for significant cellular growth inhibition and did not remarkably decrease the growth of HeLa cells. The affect on the cellular growth of HeLa cells are correlated to possible toxicity and therefore the results resembled the safe use of the extracts.

4.4 Conclusion

From these results, it was evident that all the plant extracts of both plants have free radical scavenging activity in both of the assays. The NBT assay indicated $O_2^{\bullet-}$ scavenging, as $O_2^{\bullet-}$ was responsible for the reduction of NBT to NBD. The attenuation in the 2TBA-MDA complex formation indicated less lipid peroxidation and $OH^{\bullet}$ radical scavenging in the TBA assay. The extracts ability to scavenge $O_2^{\bullet-}$ and $OH^{\bullet}$ free radicals correlated to antioxidant activity.

The most active extracts came from *T. camphoratus*. Differentiation was observed on their mechanism of action; EtOAc-extract scavenged $O_2^{\bullet-}$ and EtOH-extract scavenged $OH^{\bullet}$ at the beginning and end of the free radical chain reaction *in vitro*. Thus, the EtOAc and EtOH-extracts respectively showed the most promising antioxidant activity.

Both would be suitable for further investigation and bioassay-guided fractionation to obtain the compound(s) responsible for these properties.

The decision to choose the EtOAc or EtOH-extracts for further isolation of compound(s) was supported by the toxicity results obtained by the MTT assay. Neither of these extracts seemed to be extremely toxic to HeLa cells, indicating the non-cytotoxicity of these extracts. Thus it was assumed to be save to continue in isolating active compound(s) for antioxidant properties from these extracts.

However, for isolation, the EtOH-extract of *T. camphoratus* was selected, based on the correlation of the FRAP, ORAC and TBA assays and the second lowest value obtained for the highest concentration (2.5 mg/ml) of the EtOH-extract in the NBT assay. The disregard of the EtOAc-extract for further isolation was also based on the low yield obtained after solvent extraction. The PE and DCM-extracts were disregard based on comparing the results of both *in vitro* assays. The PE and DCM-extracts did not scavenge the $OH^{\bullet}$ and $O_2^{\bullet-}$ (TBA and NBT assays) as effectively and showed some toxicity in the MTT assay.

CHAPTER 5: ISOLATION AND CHARACTERISATION

5.1 Introduction

The isolation of all the constituents from a plant extract, in the process of identifying active compound(s), is nearly an impossible task. Mainly due to hundreds or thousands different constituents present and that only one or a few of these are responsible for the therapeutic action or toxicity of the plant (Hostettmann *et al.*, 2000; Williamson *et al.*, 1996). Upon the selection of the EtOH-extract from *T. camphoratus*, as discussed in chapter 4, a bioassay guided fractionation process was used to isolate a compound.

5.2 Separation Techniques

The selection of adequate mobile phases were based on the principle of the "PRISMA" mobile phase optimisation model and prepared on a volume to volume basis (Nyiredy *et al.*, 1985).

5.2.1 Selective precipitation

Selective precipitation was done to render the EtOH-extract of *T. camphoratus* less complex. The EtOH-extract was evaporated until a little ethanol was left, diethyl-ether (DEE) was added to the solution and caused a selective precipitation (Dhont & Vanden Berghe, 2003). The precipitate was then separated from the liquid phase by filtration. Both fractions were left to dry. The original EtOH-extract was thus divided in two phases; 1.) Precipitate phase (TC1) and 2.) Liquid phase (TC2).

5.2.2 Thin layer chromatography (TLC)

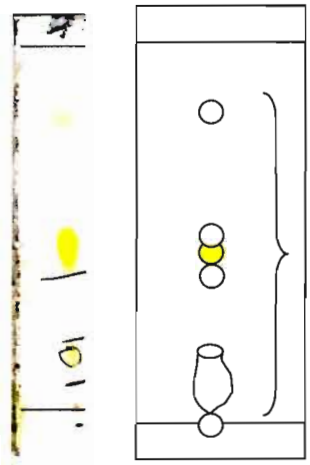
Thin layer chromatography was employed in the selection of suitable mobile phases for the isolation of the compounds. Analytical TLC was performed on 0.25 mm thick aluminium silica gel sheets (Merck® TLC aluminium sheet gel 60 F_{254}). Visualisation was achieved using UV light (254 nm/366 nm), iodine vapours and/or 5 % sulphuric acid (H_2SO_4) in ethanol spray after which the TLC plate was heated for 10 min in 110 °C oven. Figure 5.1 and figure 5.2 are representative of the TLC of the chosen fractions showing the complexity of the plant extract.

The "spots" on top was a green streak visible with the eye, white and pink under UV and brown with heated H_2SO_4-spray.

The yellow streak was visible with the eye, white under UV, showing not one "spot" and brown with heated H_2SO_4-spray (TCY3).

The brown streak was visible with the eye, showing streaking under UV with many "spots" and brownish-black with heated H_2SO_4-spray.

Figure 5.1 The thin layer chromatogram of TC2 and a schematic representation thereof. A mobile phase consisting of petroleum ether : ethyl acetate : tetrahydrofurane (5 : 3 : 7) was used.



The bright yellow "spot" was the only visible spot with the eye, yellow under UV and brownish with heated H_2SO_4-spray (MVHS). The other "spots" were visible as yellow "spots" in iodine and brownish streaking with heated H_2SO_4-spray.

Figure 5.2 The thin layer chromatogram of TCY3 and a schematic representation thereof. A mobile phase consisting of petroleum ether : chloroform : dichloromethane (4 : 1 : 1) was used.

5.2.3 Column chromatography

Fractions were purified using standard glass columns of different sizes. The same stationary phase was used throughout, silica gel (Merck®; 0.063 – 0.2 mm).

TC2 was further separated to fractions using the mobile phase petroleum ether : ethyl acetate : tetrahydrofurane (5 : 3 : 7) (figure 5.1). The fractionation of TC2 yielded three main fractions; TCG4, TCY3 and TCB5.

TCY3 was selected for further fractionation with a mobile phase of petroleum ether : chloroform : dichloromethane (4 : 1 : 1) (figure 5.2). The fractions collected yielded; MVHC7, MVHS6 and MVHR8.

Not all the fractions isolated were of sufficient quantity to obtain the data required for their identification.

The route followed to isolate compounds is described in the flowchart (figure 5.3).

5.2.4 Solid phase extraction

Solid phase extraction has become the most powerful technique available for rapid and selective sample preparation prior to analytical chromatography and improves qualitative and quantitative analysis. This method was used to purify a fraction (Sigma-Aldrich, 2008c). On TLC MVHS6 showed; a yellow "spot" visible with the eye and two other "spots" above and underneath the yellow "spot", they were visible under UV as a white "spot" above and a purple "spot" below. MVHS6 was then purified further with a solid-phase extraction tube. The Discovery® solid phase extraction (SPE) tubes with a silica gel-based bonded phase sorbent (3 ml/500 mg) was used for purification. The tube was conditioned with PE. MVHS6 was dissolved in a small amount of PE (< 1 ml). The tube was washed with PE to collect the spot above. Afterwards the mobile phase petroleum ether : chloroform (7 : 1) was send through to collect the yellow spot and leaving the rest behind. Everything else was collected with ethanol. This left only the yellow spot – compound MVHS9.

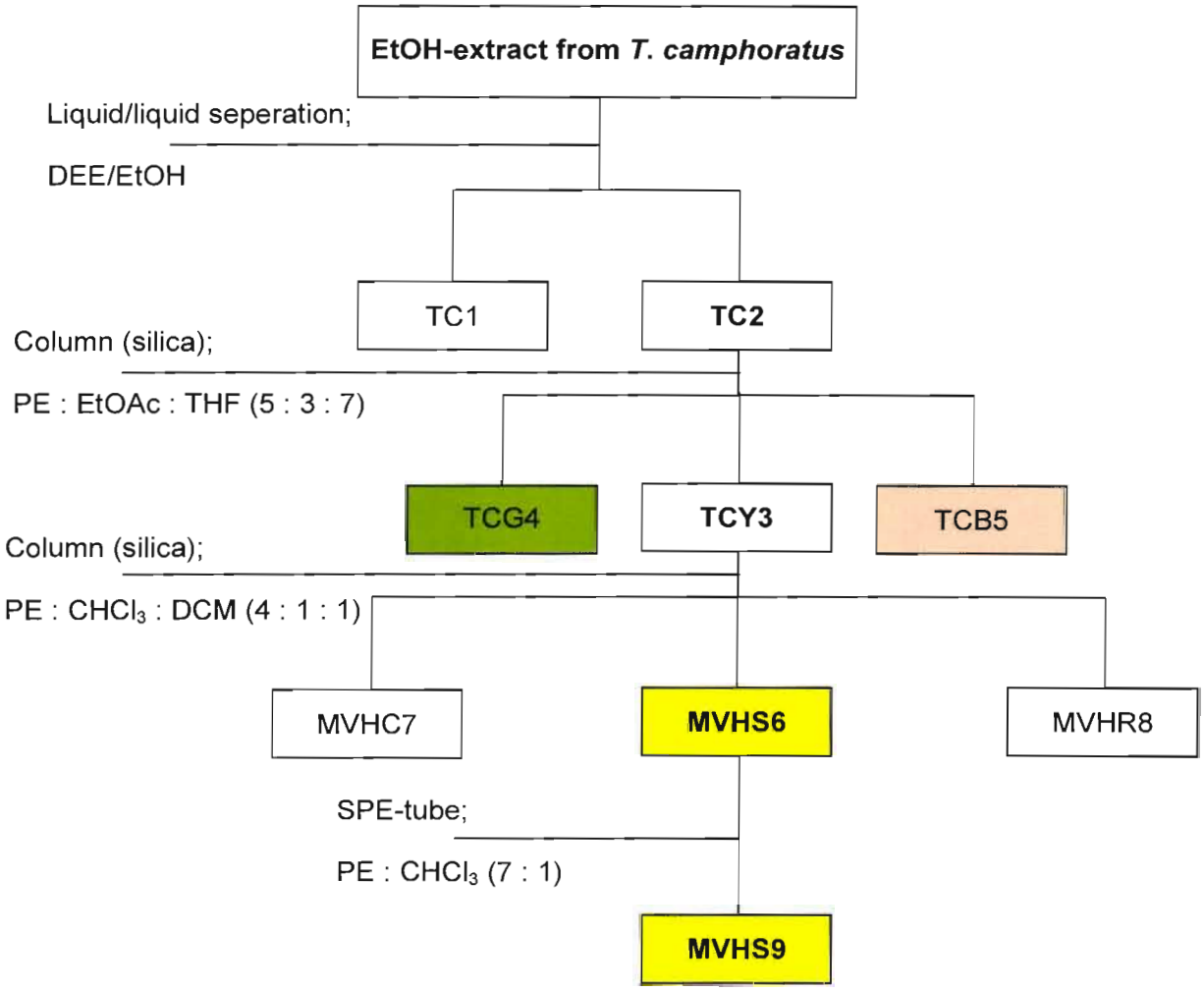


Figure 5.3 Flowchart for the isolation of compound (MVHS9) from EtOH-extract of *T. camphoratus*.

5.3 The Bioassay-guided Fractionation of EtOH-extract from *T. camphoratus*

5.3.1 TBA assay

The TBA assay was used as the best results on the EtOH-extract were found with this test. It was also a quick and easy test for determining the antioxidant properties of the fractions and eventually the isolated compound. The assay was done exactly as described in chapter 4, section 4.2.

5.3.2 Results

The levels of lipid peroxidation indices in brain homogenates are shown in table 5.1 and figure 5.4.

Table 5.1 The lipid peroxidation attenuation of fractions and compound MVHS9.

Test Compounds	Concentration	Lipid peroxidation (nmoles MDA/mg tissue	±S.E.M.
Control		0.003	0.001
Toxin	5 mM H_2O_2 4.44 mM Vit. C 1.68 mM $FeCl_3$	0.034	0.001
Trolox		0.001	0.0003
T. camphoratus	mg/ml		
TC2	0.625	0.006	0.0004
TCY3	0.625	0.0044	0.0005
MVHS9	0.625	0.0041	0.0004

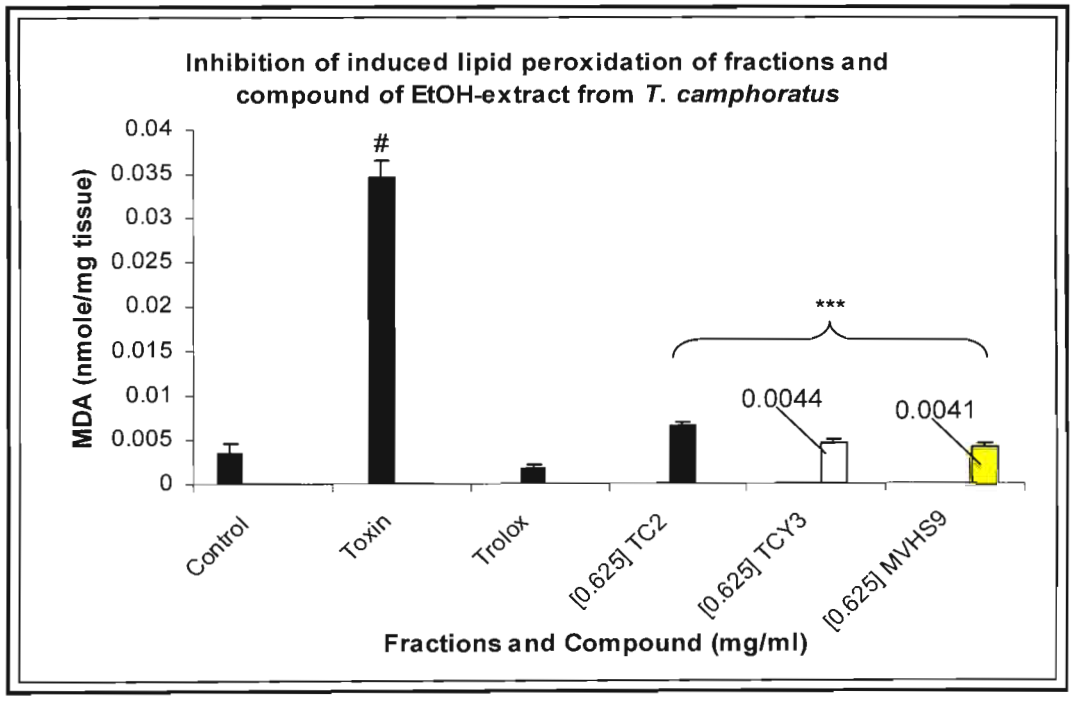


Figure 5.4 The attenuation of lipid peroxidation by the fractions and compound isolated from *T. camphoratus* in whole rat brain homogenates *in vitro*. Each bar represents the mean ± S.E.M.; n = 5. ***p < 0.001 vs. toxin (#).

5.4 Characterisation of Compound Isolated from *T. camphoratus*

5.4.1 Instrumentation

5.4.1.1 Nuclear magnetic resonance spectroscopy (NMR)

^{1}H, ^{13}C and COSY NMR spectra were obtained using a Bruker advance 600 in a 14.09 Tesla magnetic field utilising an ultra shield plus magnet spectrometer. The ^{1}H and ^{13}C frequencies, respectively, were at 600.1724007 MHz and 150.9128712 MHz. Tetramethylsilane (TMS) was used as internal standard. A bandwidth of 1 000 MHz at 24 kG was applied for ^{1}H and ^{13}C-decoupling. All chemical shifts are reported in parts per million (ppm) relative to the signal from TMS ($\delta = 0$) added to an appropriate deuterated solvent. NMR samples were dissolved in deuterated chloroform ($CDCl_3$).

5.4.1.2 Mass spectroscopy (MS)

A Micromass Autospec was used to record the mass spectrum of sample. It is a high resolution magnetic sector based instrument with Electron Ionisation (EI), Chemical Ionisation and Fast Atom Bombardment Ionisation (FAB) capabilities. Only the EI-MS (m/z) spectrum was however obtained for sample.

5.4.1.3 Infrared spectroscopy (IR)

IR spectra were recorded on a Nicolet Nexus 470-FT-IR spectrometer over the range of 400 – 4 000 cm^{-1}. The Multi-Bounce HATR method was used. The sample was applied directly to a ZnSe (Zinc Selenium) crystal plate as a film dissolved in EtOH. Before the spectrum was recorded, the EtOH had to be completely evaporated.

5.4.2 Characterisation of compound (MVHS9)

Comparing data generated for MVHS9 to that of data found in the literature, MVHS9 could possibly be a phatalic acid ester. MVHS9 was compared to didecyl phthalate (SDBS, 2004a), diisononyl phthalate (SDBS, 2004b) and dioctyl phthalate (SDBS, 2004c; Pouchert & Behnke, 1993) on ^{13}C, ^{1}H NMR and IR spectral data relations (table 5.2, table 5.3 and table 5.4). The chemical shifts reported for all compounds were in ppm (δ) in $CDCl_3$ (SDBS, 2004a-c; Pouchert & Behnke, 1993).

Table 5.2 The ^{13}C NMR spectral data for didecyl phthalate, dioctyl phthalate and MVHS9.

^{13}C NMR spectral data			
	Didecyl phthalate	**Dioctyl phthalate**	**MVHS9**
	δ_C	δ_C	δ_C
C=O	167.60	167.66	167.76
=	132.58	132.41	132.42
	130.83	130.82	130.87
	128.89	128.74	128.78
C-O	65.83	68.10	68.14
CH	-	38.10	38.70
CH_2	31.95	-	31.91
	29.59	30.37	30.33
	29.35	-	29.68
	28.68	-	29.34
	-	28.93	28.90
	26.06	-	24.98
	-	23.76	23.72
	-	23.00	-
	-	22.99	22.97
	22.70	-	22.67
CH_3	14.06	14.05	14.04
	-	10.97	10.94

Table 5.3 The ^{1}H NMR spectral data for didecyl phthalate, dioctyl phthalate and MVHS9.

^{1}H NMR spectral data			
	Didecyl phthalate	**Dioctyl phthalate**	**MVHS9**
	δ_H	$\pm \delta_H$	δ_H
Ar-H	7.712	7.7	-
	7.692		7.687
	7.514	7.5	-
	7.503		7.506
$O-CH_2$	4.296	4.2	4.261
	4.28		4.193
CH_2	1.749	1.7	1.756
	1.728		-
	1.417	1.4	1.415
	1.317	1.3	1.317
	1.268		1.233
CH_3	0.896	0.9	0.898
	0.877		0.858

Table 5.4 The IR spectral data for didecyl phthalate, diisononyl phthalate, dioctyl phthalate and MVHS9 (SDBS, 2004a-c).

IR ν_{max} (cm^{-1})			
Didecyl phthalate	**Diisononyl phthalate**	**Dioctyl phthalate**	**MVHS9**
3 443	-	3 444	-
3 069	3 071	3 070	-
2 955	2 959	2 956	2 957
2 926	2 929	2 927	2 924
2 856	2 873	2 871	2 854
1 730	1 728	1 729	1 728
1 601	1 601	1 601	1 602
1 580	1 580	1 580	1 580
1 488	-	1 488	-
1 457	1 464	1 467	1 463
1 449	-	1 449	-
1 379	1 380	1 384	1 378
1 285	1 287	1 286	1 273
1 124	1 124	1 124	1 122
1 073	1 074	1 075	1 073
1 040	1 040	1 040	1 039
968	966	952	965
868	-	881	-
-	-	875	-
-	-	870	799
743	742	744	742
723	-	724	-
705	705	705	704
662	652	652	-
572	-	570	-
-	-	474	-

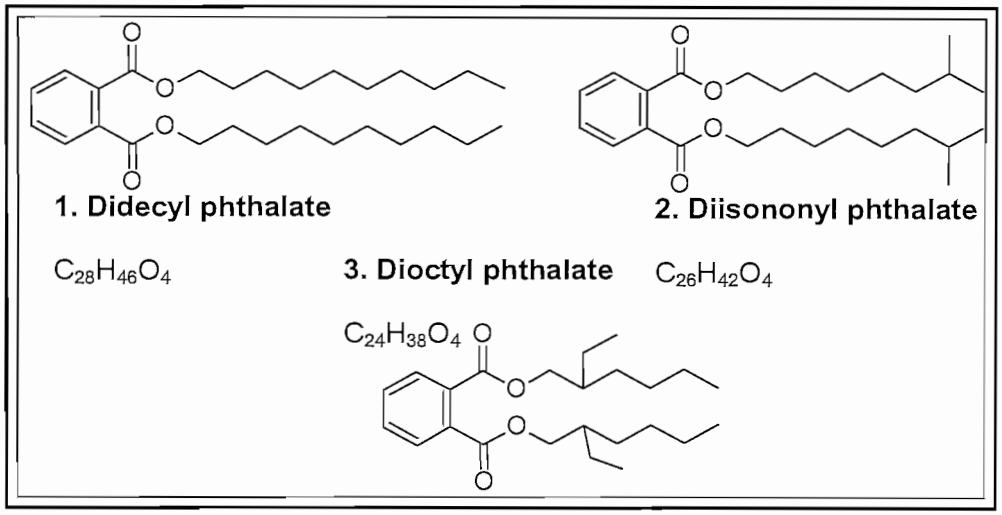


Figure 5.5 The structures of the phthalic acid esters to which MVHS9 were compared to (SDBS, 2004a-c).

Spectra of compound MVHS9 can be referred to in the appendix.

5.5 Discussion

Compound MVHS9 contained possible impurities that made the identification of the compound difficult. The impurities present in the sample were taken into consideration for signals slightly out of bounds.

Data from didecyl phthalate, diisononyl phthalate and dioctyl phthalate correlated well with the spectral data gathered for MVHS9 and therefore it could be assumed that the compound isolated from *T. camphoratus* was a phthalic acid ester. Phthalic acid esters or phthalate esters are 1,2-benzenedicarboxylic acid structures. The basic structure of these phthalic acid esters are a benzene ring ortho-substituted to an ester with carbon side chains of different lengths and with or without branching of the chain.

The spectra were analysed with the help off Silverstein *et al.* (2005) and literature on the phthalic acid esters (Silverstein *et al.*, 2005). The ^{1}H, ^{13}C and IR spectral data for MVHS9 confirmed the ortho-substitution of the bezene ring. The ^{1}H NMR showed multiplets at δ_H 7.687 and δ_H 7.506 indicating a possible ortho-substitution in the aromatic region of the spectrum (table 5.3). The double bonds on the bezene ring was also confirmed by the ^{13}C NMR signals at δ_c 132.42, δ_c 130.87 and δ_c 128.27 that corresponded to the signals on didecyl phthalate and dioctyl phthalate (table 5.2). The IR spectrum showed only a slight signal over 3 000 cm^{-1} indicating the aromatic hydrogens. More positive confirmation for an

aromatic structure on the IR spectrum was the aromatic double bonds signals at 1 602 cm^{-1} and 1 463 cm^{-1} (table 5.4).

The ester was also confirmed via the positive identification on all the spectra available. The IR spectrum indicated a C=O ester (α, β-unsaturated C=O) signal at 1 728 cm^{-1} that corresponded to the signals of didecyl phthalate (1 730 cm^{-1}), diisononyl phthalate (1 728 cm^{-1}) and dioctyl phthalate (1 729 cm^{-1}) (table 5.4). The same correlation could be drawn from the ^{13}C NMR with a carbonyl signal (C=O) at δ_C 167.78 similar to that of didecyl phthalate (δ_C 167.60) and dioctyl phthalate (δ_C 167.66) (table 5.2). Based on a correlation table for ^1H NMR, the hydrogen's bonded to oxygen's and more specific to an ester formation is at δ_H 4.1. The signals for MVHS9 were at δ_H 4.193 and δ_H 4.261 and correlated to the region on the correlation table and to that of didecyl phthalate (δ_H 4.28). The exact values of dioctyl phthalate could not have been obtained and therefore only an indication of the possible region was given, $\delta_H \pm 4.2$ (Sigma Chemical Company, 1986). The presence of the ^1H signals at δ_H 4.261 and δ_H 4.193 also correspond to methylene protons adjacent to an ester oxygen (table 5.3) (Cakir *et al.*, 2003).

It was difficult allocating an exact side chain because of the wide variations possible and the unavailability of an accurate mass for MVHS9. For this reason there was only distinguished between the most feasible of all the possibilities. The possibilities were didecyl phthalate with a long side chain (1), diisononyl phthalate with a slightly shorter branched side chain (2) and dioctyl phthalate with a shorter branched side chain (3) (figure 5.5). Diisononyl phthalate and dioctyl phthalate with fewer carbons fitted better than didecyl phthalate with more carbons. Diisononyl was the best fit based on the IR spectrum, but no information on the ^{13}C and ^1H NMR could be gathered and therefore the correlation similarities were further supported only on dioctyl phthalate. The possibility of a branched side chain was supported by the ^{13}C NMR CH_3 signal at δ_C 10.97 of dioctyl phthalate and at δ_C 10.94 of MVHS9 and also the CH signal at δ_C 38.10 of dioctyl phthalate and δ_C 38.70 of MVHS9 (table 5.2).

The EI-mass spectrum (spectrum 6) for MVHS9 revealed significant fragment ions at m/z 167 and 149, according to the literature this fragmentation ions, 166 [phthalic acid]$^+$ and 149 $[C_8H_5O_4]^+$, indicate that a compound might be a phthalate (Cakir *et al.*, 2003). An accurate mass for MVHS9 was however not obtained and therefore the exact structure was difficult to allocate.

Included in the spectra available for structure elucidation of MVHS9 was the COSY NMR (spectra 4 & 5) and this also supported the idea of a possible Ar-O(C=O)$(CH_2)_xCH_3$ structure.

With regard to the physical properties of MVHS9, it corresponded to dibutyl phthalate as a yellow oily liquid soluble in petroleum ether and ethanol (Saeed *et al.*, 2007).

Certain phthalate esters are known to be natural constituents of some plants (Herring & Bering, 1988). Phthalate esters isolated from plants include, bis(2-ethylhexyl) phthalate isolated from *Eupatorium odoratum* (Amatya & Tuladhar, 2005), bis(2-methylheptyl) phthalate isolated from *Hypericum hyssopifolium* (Cakir *et al.*, 2003), isobutyl-*o*-phthalate isolated from *Anthemis tinctoria* (Saroglou *et al.*, 2006) and dibutyl phthalate isolated from *Helichrysum italicum* (Mastelić *et al.*, 2008). The chemical composition of the essential oil of *Launaea arboresens* indicated the presence of esters as the dominant group with dioctyl phthalate as one of the main constituents (Cheriti *et al.*, 2006). *T. camphoratus* and the plant species mentioned above all belong to the Asteraceae family.

Phthalic acid esters are mainly used as plasticizer in medical tubing (infusion tubings, infusion bag, blood storage bag, intestinal tubing) (Saeed *et al.*, 2007). There is a concern of the safety of these compounds but published literature on the subject has indicated that these chemical agents are relatively non-toxic and should not present any health hazards to man (Autian, 1973; Hill *et al*, 2001). Dibutyl phthalate isolated from *Torreya grandis* and other reports on phthalate ester mention their pharmacological activity as eliminating tumor cells and as a purging agent in autologous bone marrow transplantation (Saeed *et al.*, 2007). Another use of phthalate esters are as insect repellents (Herring & Bering, 1988).

Whenever phthalates are found in a tissue extract, there is generally a question raised concerning their actual presence in the tissue itself, since phthalates have almost invariably been shown to be formed during extraction and are, therefore, artefacts of the procedure (Paré *et al.*, 1981). In this study, this phenomenon cannot be totally eliminated. For the most part of experimentation glass apparatus were used to avoid any plasticizer sources. Since phthalates have been found to occur in plants a biosynthetic origin cannot be excluded (Khan & Schnitzer, 1971).

The TBA assay employed to screen for possible antioxidant properties showed all of the fractions and the final compound MVHS9 to have excellent antioxidant capacity, TC2 < TCY3 < MVHS9. The route followed to isolate an active compound was a success based on the results obtained by the TBA assay.

CHAPTER 6. CONCLUSION

Oxygen is an essential molecule for survival of the majority of living organisms (Singh *et al.*, 2004) but overwhelming evidence suggest that oxidative stress occurs in cells as a consequence of the utilisation of oxygen in normal physiological processes and also environmental interactions (Cui *et al.*, 2004; Young & Woodside, 2001). When oxygen is reduced to water in the electron transport chain several reactive oxygen species (ROS) are formed (Cui *et al.*, 2004). The body's complex web of antioxidant defence systems play a key role in protecting against oxidative damage caused by free radicals (Young & Woodside, 2001). It is becoming increasingly apparent that the inadvertent overproduction of ROS may overwhelm the protective endogenous antioxidant defences resulting in oxidative damage (Haraguchi, 1997). Promising therapeutic interventions targeted against oxidative processes should be explored in clinical trials to eventually relieve the burden of neurodegeneration (Ischiropoulos & Beckman, 2003). The elimination of the free radical involvement offers a therapeutic target in such diseases. Therefore, the therapeutic reward of exogenous antioxidants are likely to be substantial in combating oxidative stress by working to neutralise the excess free radicals and stopping them from starting the chain reactions that contribute to these diseases and premature aging. The strategies aimed at limiting free radical production, oxidative stress and damage may slow progression of neurodegenerative diseases. There are several natural and synthetic compounds already selected and extensively studied (Singh *et al.*, 2004). This evidence and support of cited literature aided the setting of the aim of the present study. The following aims were achieved;

Two plant species were selected from an initial screening of 21 plant species done by co-workers. The process was based on the elimination of plant species on the results obtained with the ORAC and FRAPS assays. The availability of specific plant species was also brought into consideration. Therefore, the final selected plant species were *P. auriculata* and *T. camphoratus* (chapter 3).

In chapter 4 the *in vitro* assays (TBA and NBT assays) were useful in determining whether a particular extract was an effective antioxidant through evaluating the free radical scavenging ability. The ethyl acetate and ethanol extracts of *T. camphoratus* were the best radical scavengers in both assays, among the selected plant species screened. The EtOH-extract was eventually selected for further research into the compound(s) responsible for these antioxidant properties. The decision was based on the correlation between the TBA, FRAP and ORAC assays, all of which indicated the EtOH-extract as the best antioxidant. The extract significantly reduced H_2O_2-induced lipid peroxidation, whilst also scavenging $O_2^{\bullet-}$

formed by KCN. Free radical scavengers have become increasingly popular as a means of reducing or preventing the hazardous effects of free radicals and their inducers (Maharaj *et al.*, 2003). Thus, the findings imply that the EtOH-extract of *T. camphoratus* can be potentially neuroprotective via combating the deleterious effects of free radicals ($OH^{\bullet}$, $O_2^{\bullet-}$). The pathological effects of $O_2^{\bullet-}$ are indirect in the sense that it is the subsequently formed $OH^{\bullet}$ and $ONOO^{\bullet}$ which are involved in different pathological conditions. Thus, if $O_2^{\bullet-}$ production is controlled or scavenged, the formation of $OH^{\bullet}$ and $ONOO^{\bullet}$ can be limited and diseases caused by them can be sufficiently reduced. The $OH^{\bullet}$ derived from H_2O_2 and $O_2^{\bullet-}$ is a preliminary requirement for the prevention of $OH^{\bullet}$ mediated pathologies. $OH^{\bullet}$ cleaves covalent bonds in proteins and carbohydrates causing lipid peroxidation and destroying the cell membranes. Lipid peroxidation and its attenuation is a universal indicator for evaluating the antioxidant activity of compounds (extracts) (Kaur & Geetha, 2006). Further toxicological studies (MTT assay) were required to prove the safety of the selected plant species and more importantly the selected plant extract. The results obtained aided in the decision to continue the investigation of the EtOH-extract as it showed to be not significantly toxic to the growth of HeLa cells (chapter 4).

By implementing analytical techniques, the EtOH-extract was then subjected to bioassay-guided fractionation to separate and isolate compound(s) possibly responsible for the antioxidant activity (chapter 5). Thin-layer chromatography formed the bases for this isolation by selecting appropriate mobile phases for use in columns (silica as stationary phase). Further purification of the fraction, MVHS6, was obtained by solid phase extraction. Finally, compound MVHS9 was isolated and characterised through MS, NMR and IR elucidation and a phthalate ester was eventually proposed. The TBA assay showed that the sample (MVHS9) did indeed have antioxidant properties but on further research, the possibility of this being due to the phthalate ester proposed was not likely. Based on the literature found, no antioxidant activity has been reported for phthalate esters. Therefore, it can not be confirmed whether the antioxidant properties of MVHS9 shown by the TBA assay was due to the phthalate ester or if the impurities present in the sample were responsible for these positive results.

In conclusion, all aims were achieved for the study. A compound was identified from an active sample from a South African plant – *T. camphoratus*. The objective for future research should be the further purification of samples to identify the active compound and eventually the *in vivo* application of the active compound. These results further support the view that some medicinal plants could be promising sources of natural antioxidants.

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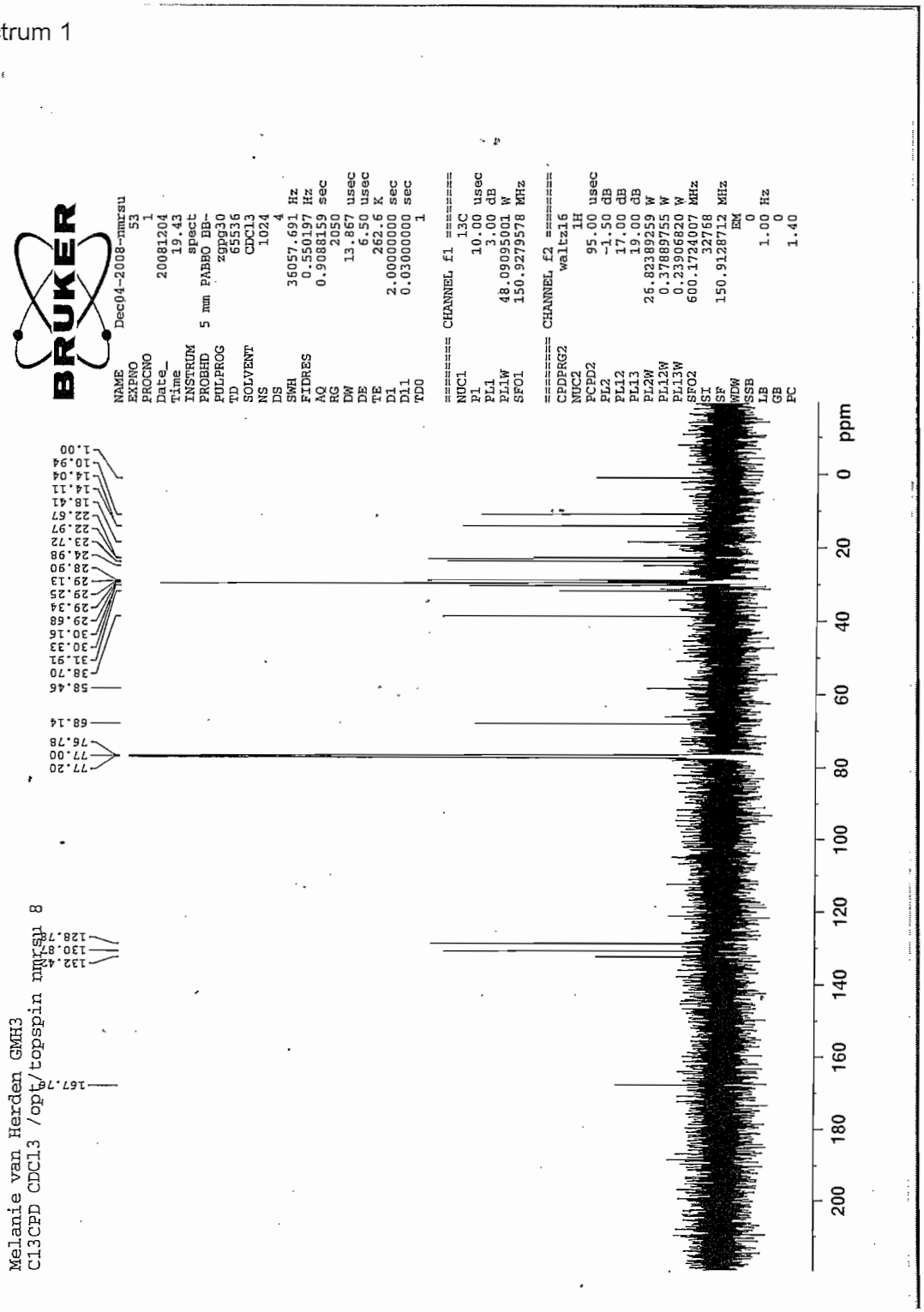
Spectrum 1


Melanie van Herden GMH3
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PROCNO 1
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Time 19.43
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PULPROG zgpg30
TD 65536
SOLVENT CDCl3
NS 1024
DS 4
SWH 36057.691 Hz
FIDRES 0.550197 Hz
AQ 0.90888159 sec
RG 2050
DW 13.867 usec
DE 6.50 usec
TE 262.6 K
D1 2.00000000 sec
D11 0.03000000 sec
TD0 1
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NUC1 13C
P1 10.00 usec
PL1 3.00 dB
PL1W 48.09095001 W
SFO1 150.9279578 MHz
======== CHANNEL f2 ========
CPDPRG2 waltz16
NUC2 1H
PCPD2 95.00 usec
PL2 -1.50 dB
PL12 17.00 dB
PL13 19.00 dB
PL2W 26.82389259 W
PL12W 0.37889755 W
PL13W 0.23906820 W
SFO2 600.1724007 MHz
SI 32768
SF 150.9128712 MHz
WDW EM
SSB 0
LB 1.00 Hz
GB 0
PC 1.40
167.79
132.47
130.87
128.78
77.20
77.00
76.78
68.14
58.46
38.70
31.91
30.33
30.16
29.68
29.34
29.25
29.13
28.90
24.98
23.72
22.97
22.67
18.41
14.11
14.04
10.94
1.00
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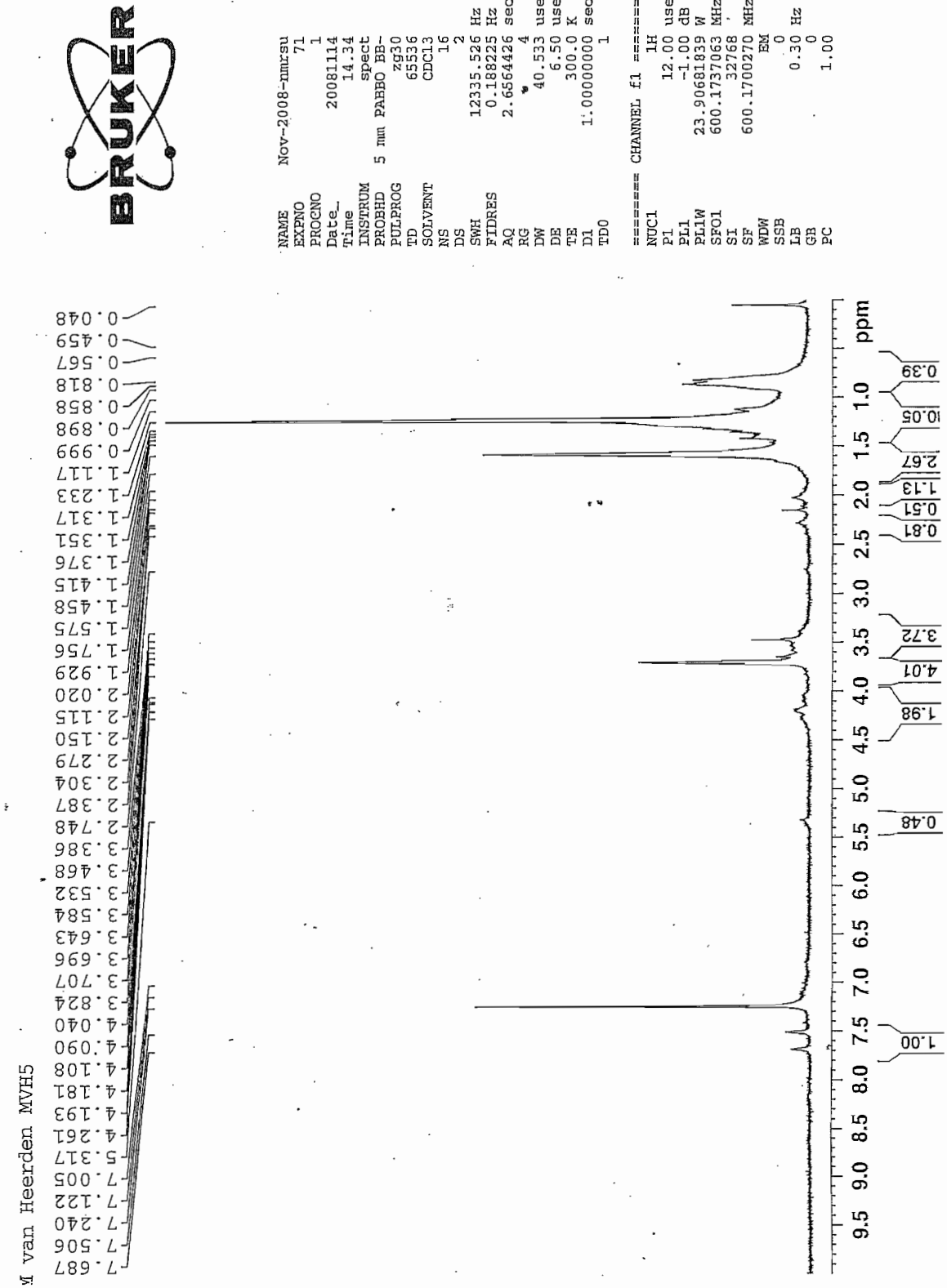
180
160
140
120
100
80
60
40
20
0
ppm

Spectrum 2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PROCNO 1
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Time 14.34
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PULPROG zg30
TD 65536
SOLVENT CDCl3
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FIDRES 0.188225 Hz
AQ 2.6564426 sec
RG 4
DW 40.533 usec
DE 6.50 usec
TE 300.0 K
D1 1.00000000 sec
TD0 1
======== CHANNEL f1 ========
NUC1 1H
P1 12.00 usec
PL1 -1.00 dB
PL1W 23.90681839 W
SFO1 600.1737063 MHz
SI 32768
SF 600.1700270 MHz
WDW EM
SSB 0
LB 0.30 Hz
GB 0
PC 1.00
M van Heerden MVH5
7.687
7.506
7.240
7.122
7.005
5.317
4.261
4.193
4.181
4.108
4.090
4.040
3.824
3.707
3.696
3.643
3.584
3.532
3.468
3.386
2.748
2.387
2.304
2.279
2.150
2.115
2.020
1.929
1.756
1.575
1.458
1.415
1.376
1.351
1.317
1.233
1.117
0.999
0.898
0.858
0.818
0.567
0.459
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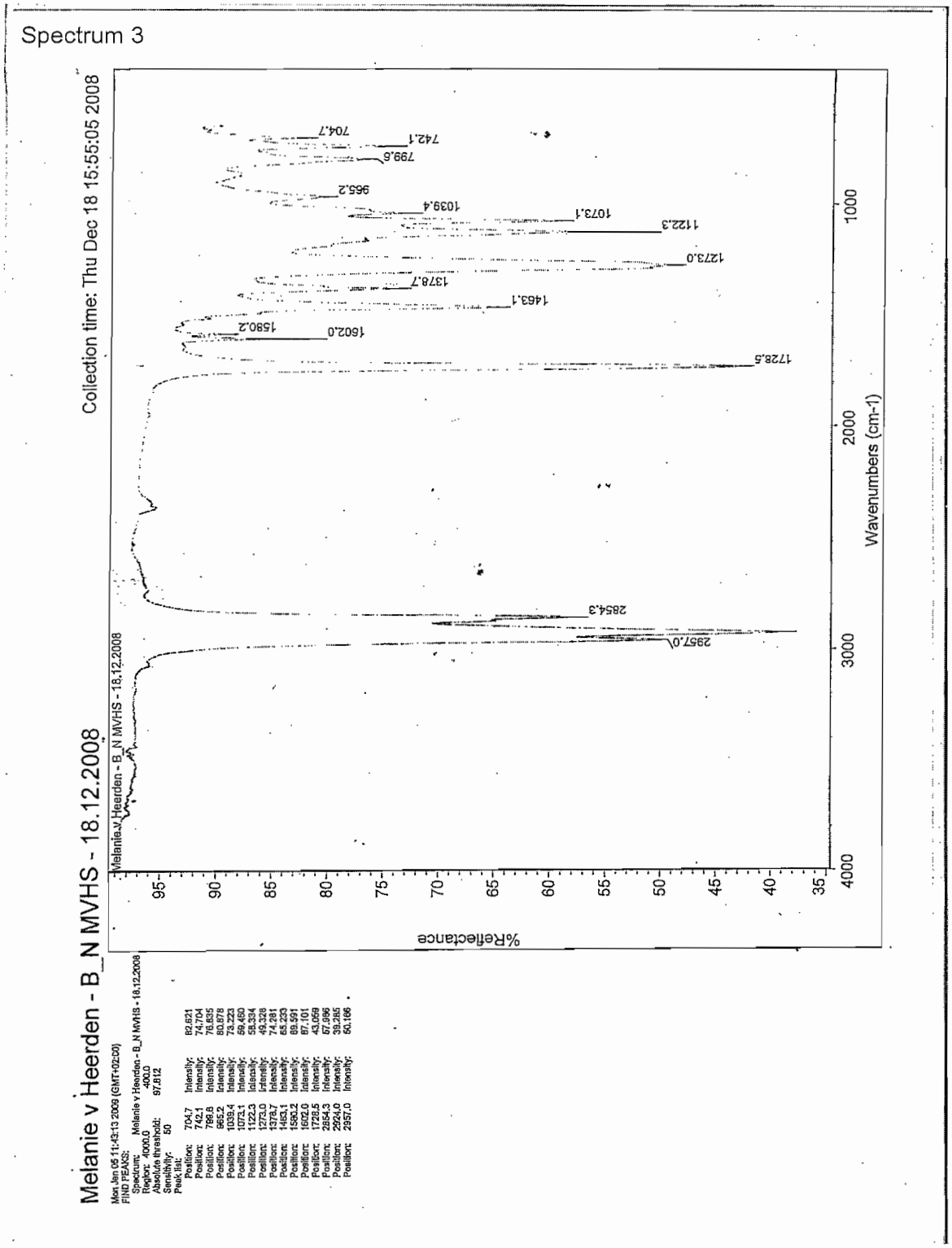

Mon Jan 05 11:43:13 2009 (GMT+02:00)
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Region: 4000.0
Absolute threshold: 97.812
Sensitivity: 60
Peak list:

Position:	704.7	Intensity: 82.821
Position:	742.1	Intensity: 74.704
Position:	786.8	Intensity: 76.835
Position:	865.2	Intensity: 80.878
Position:	1039.4	Intensity: 73.223
Position:	1073.1	Intensity: 59.460
Position:	1122.3	Intensity: 58.334
Position:	1273.0	Intensity: 49.328
Position:	1378.7	Intensity: 74.281
Position:	1463.1	Intensity: 65.223
Position:	1580.2	Intensity: 69.591
Position:	1602.0	Intensity: 67.101
Position:	1728.5	Intensity: 47.699
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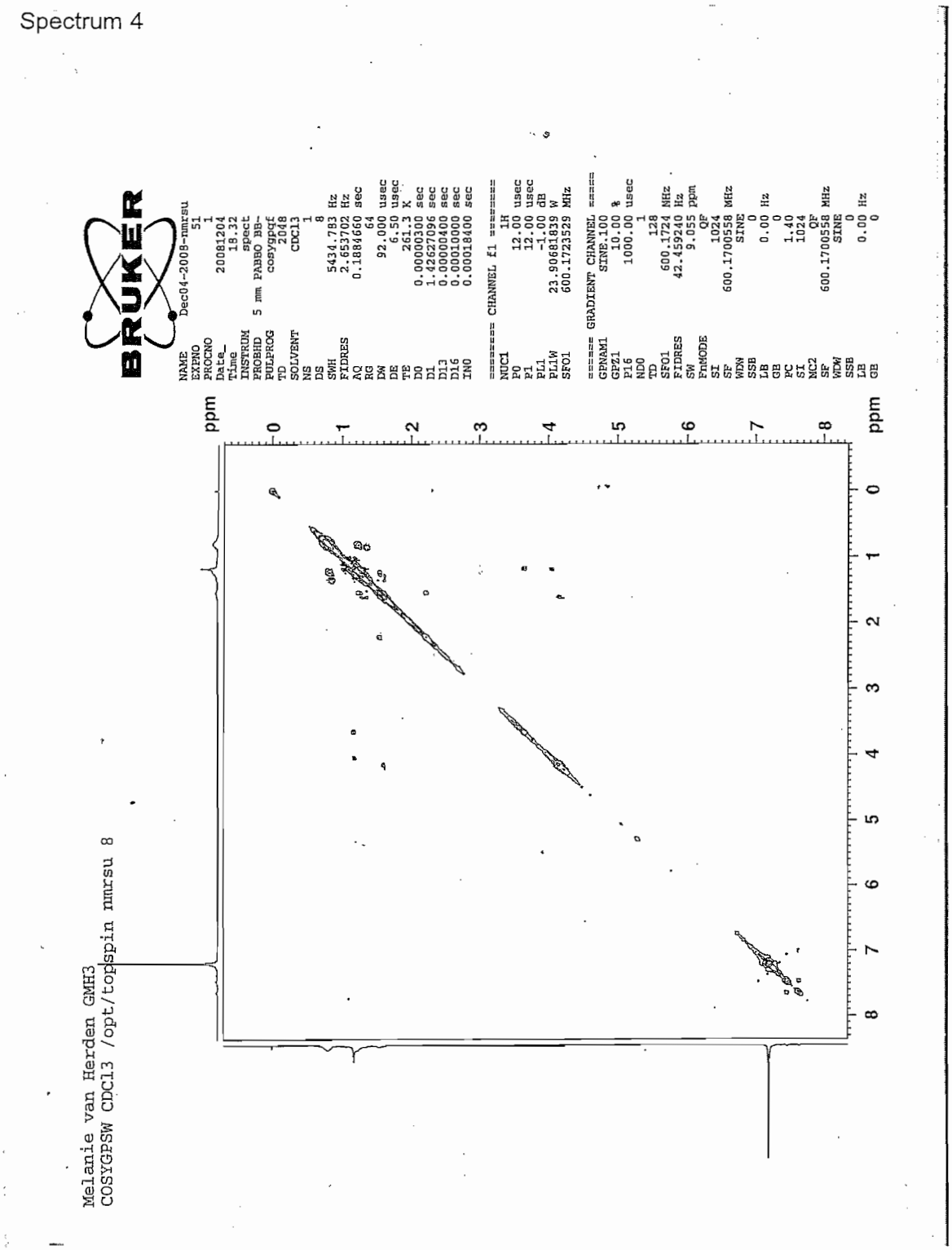
Position:	2924.0	Intensity: 39.285
Position:	2957.0	Intensity: 50.186

Spectrum 4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PULPROG cosygpqf
TD 2048
SOLVENT CDCl3
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FIDRES 2.653702 Hz
AQ 0.1884660 sec
RG 64
DW 92.000 usec
DE 6.50 usec
TE 261.3 K
D0 0.00000300 sec
D1 1.42627096 sec
D13 0.00000400 sec
D16 0.00010000 sec
IN0 0.00018400 sec
======== CHANNEL f1 ========
NUC1 1H
P0 12.00 usec
P1 12.00 usec
PL1 -1.00 dB
PL1W 23.90681839 W
SFO1 600.1723529 MHz
====== GRADIENT CHANNEL =====
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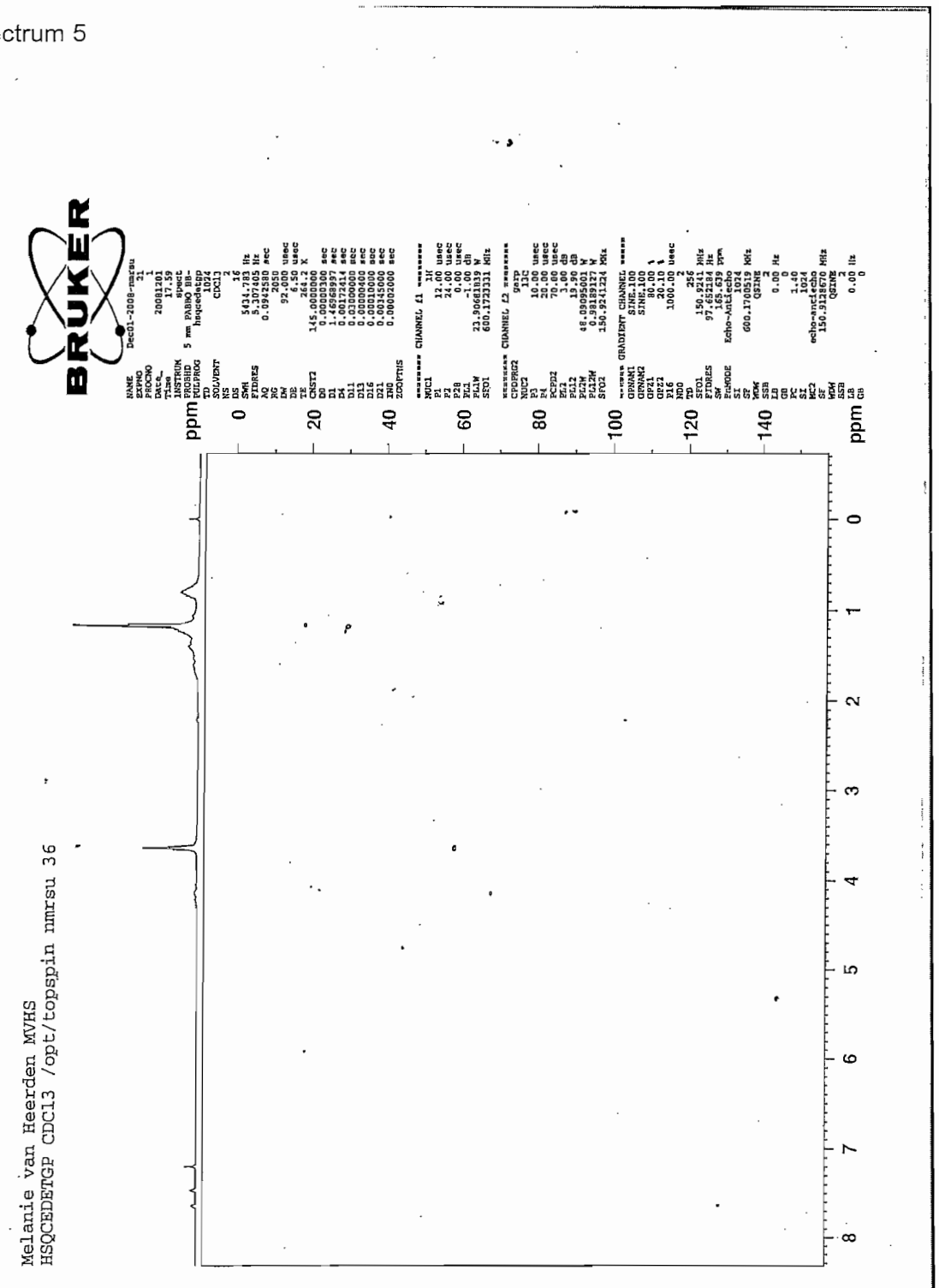
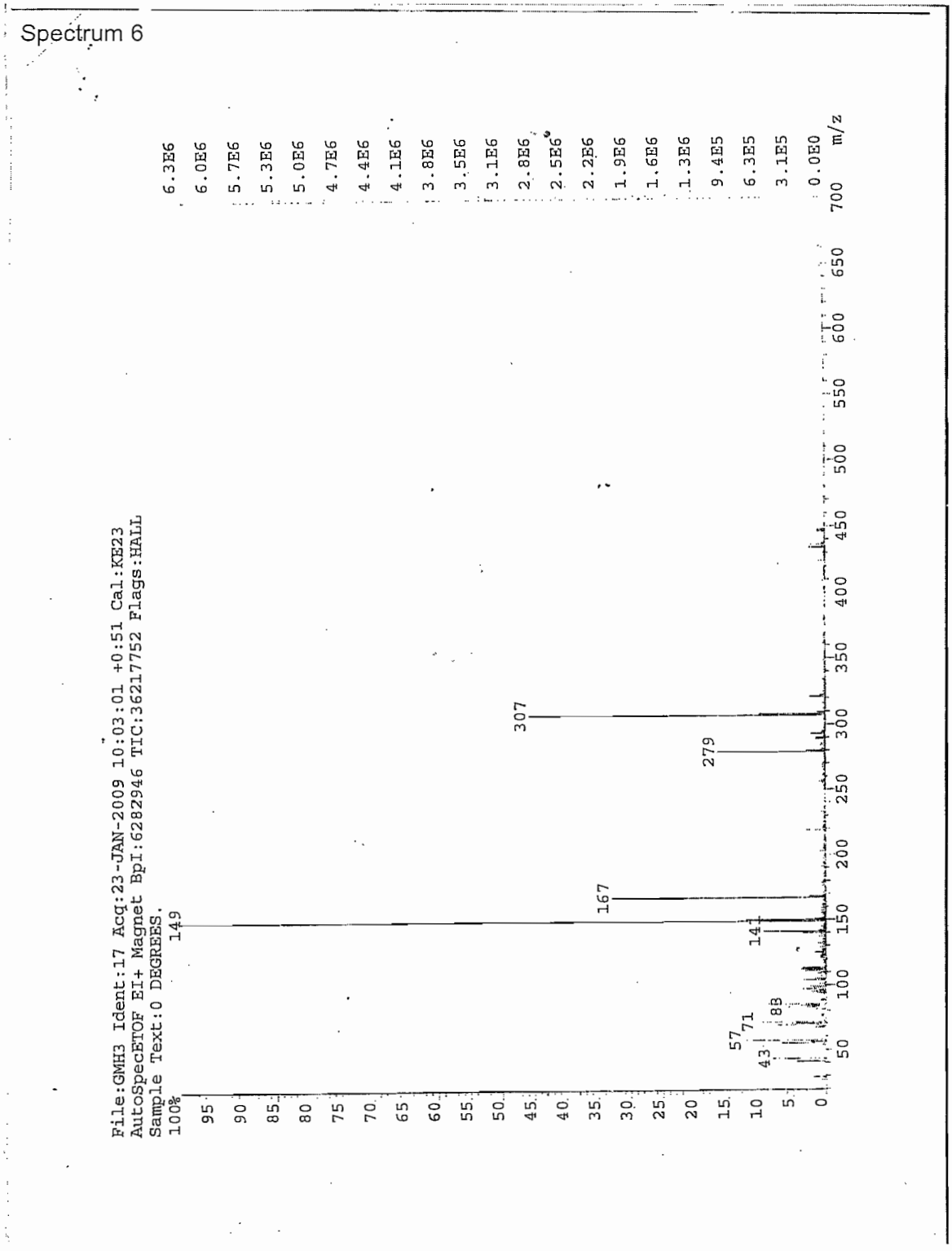
GPZ1 10.00 %
P16 1000.00 usec
ND0 1
TD 128
SFO1 600.1724 MHz
FIDRES 42.459240 Hz
SW 9.055 ppm
FnMODE QF
SI 1024
SF 600.1700558 MHz
WDW SINE
SSB 0
LB 0.00 Hz
GB 0
PC 1.40
SI 1024
MC2 QF
SF 600.1700558 MHz
WDW SINE
SSB 0
LB 0.00 Hz
GB 0
ppm
Melanie van Herden GMH3
COSYGPSW CDCl3 /opt/topspin nmrsu 8

Spectrum 5


Melanie van Heerden MVHS
HSQCEDETGP CDCl3 /opt/topspin nmrsu 36
BRUKER
ppm
ppm

Spectrum 6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