

# **Antioxidant properties of *Plumbago auriculata* Lam**

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**“If you see your path laid out in front of you -- Step one, Step two, Step three -- you only know one thing... it is not your path. Your path is created in the moment of action. If you can see it laid out in front of you, you can be sure it is someone else's path. That is why you see it so clearly.”**

***-- Joseph Campbell***

# ABSTRACT

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Parkinson's disease, a disease first described by James Parkinson two centuries ago is one of the most common neurodegenerative diseases. The prominent feature of this disease is the selective degeneration of dopaminergic neurons in the substantia nigra of the midbrain resulting in a decrease in dopamine levels in the brain. The substantia nigra appears to be an area of the brain that is highly susceptible to oxidative stress. Supplementation with antioxidants may protect the neurons from the damaging effects of oxidation by reacting with oxygen radicals and other reactive oxygen species (ROS).

The aim of this study was to investigate the antioxidant properties of the leaves of the plant *Plumbago auriculata* and to evaluate its antioxidant activity on rats. Four solvents; petroleum ether, dichloromethane, ethyl acetate and ethanol were used successively to extract substances from the leaves of the plant using the soxhlet apparatus. The Thiobarbituric Acid-Reactive Substances (TBARS) and the Nitro-Blue Tetrazolium (NBT) assays were performed to evaluate antioxidant activity. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was done to determine the relative toxicity of each extract. The results showed that the ethyl acetate and the ethanol crude extracts had significantly higher antioxidant activity than the petroleum ether and the dichloromethane extracts.

In the TBARS assay the ethanol and ethyl acetate extracts each at 2.5 mg/ml reduced malondialdehyde (MDA) levels significantly ( $p < 0.001$ ) compared to the toxin ( $H_2O_2 + FeCl_3 + Vit. C$ ). Ethanol and ethyl acetate extracts each had values of 0.0058 nm MDA/mg tissue and 0.0067 nm MDA/mg tissue respectively in comparison to the toxin's 0.0257 nm MDA/mg tissue. Results of the NBT assay results showed that at concentration ranges of 0.625 - 2.5 mg/ml, the ethyl acetate and ethanol extracts had the best ( $p < 0.001$ ) superoxide scavenging activity compared to the toxin (KCN). The ethyl acetate and petroleum ether extracts significantly inhibited the proliferation of HeLa cells by 11.52 % ( $p < 0.05$ ) and 27.3 % ( $p < 0.001$ ) respectively at 10 mg/mL, compared to the control when evaluated with the MTT assay. Although the MTT assay results showed toxicity with the 10 mg/ml concentration of the ethyl acetate extract, this extract is one of the two extracts that had the most promising antioxidant activity. It is possible that different compounds in each extract contributed to the antioxidant activity and toxicity. Therefore, the ethyl acetate extract was put through bioassay-guided fractionation using column chromatography to isolate antioxidant compounds.

Two compounds, PS and OS were isolated.  $^{13}C$  NMR, DEPT  $^{13}C$  NMR,  $^1H$  NMR and FT-IR were used to characterize the structures of the isolated compounds. PS was found to be  $\beta$ -sitosterol, while OS was proposed to be  $\beta$ -carotene. OS reduced MDA levels significantly at

## ***ABSTRACT***

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all concentrations. At 2.5 mg/ml, the reduction in MDA was almost to the level of the control. The isolated compounds are common in most plants and are known to have antioxidant activity. Further fractionation needs to be done to isolate less common compounds.

# OPSOMMING

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Parkinson se siekte is vir die eerste keer twee eeue terug beskryf deur James Parkinson en is een van die algemeenste neurodegeneratiewe siektes. Die siekte verlaag die dopamien vlakke in die brein deur middel van selektiewe degenerasie van dopamien neurone in die substantia nigra. Dit kom voor asof die gedeelte van die brein veral vatbaar is vir oksidatiewe stres. Die neurone kan beskerm word teen die vernietigende effekte van oksidasie deur aanvulling met antioksidante wat reageer met suurstofradikale en ander reaktiewe suurstofspesies.

Die doel van die studie was om die antioksidanteienskappe van die blare van *Plumbago auriculata* te ondersoek en hul antioksidantaktiwiteit op rotbreinhomogenaat te evalueer. Die blare is geëkstraheer deur Soxhlet ekstraksie met die hulp van vier oplosmiddels; petroleumeter, dichlorometaan, etielasetaat en etanol. Die antioksidant aktiwiteit is geëvalueer deur gebruik te maak van die tiobarbituursuur-reaktiewe substans (TBARS)- en die nitro-blou tetrasoliummetodes. Die 3-(4,5-dimetielthiasol-2-yl)-2,5-difenieltetrasoliumbromiedmetode (MTT) is gebruik om die relatiewe toksisiteit van elke ekstrak te toets. Die resultate het getoon dat die rou ekstrakte van etanol en etielasetaat hoër antioksidantaktiwiteit het as die ru ekstrakte van petroleumeter en dichlorometaan.

Die 2.5 mg/ml konsentrasie van die etanol- en etielasetaatekstrakte het die MDA vlakke betekenisvol ( $p < 0.001$ ) verlaag (0.0058 nm MDA/mg weefsel en 0.0067 nm MDA/mg weefsel onderskeidelik) in vergelyking met die toksien ( $H_2O_2 + FeCl_3 + Vit. C$ ) (0.0257 nm MDA/mg weefsel). Die resultate van die NBT-analise toon dat die etanol- en etielasetaatekstrakte by konsentrasies van 0.635 – 2.5 mg/ml die KCN-geïnduseerde stres betekenisvol ( $p < 0.001$ ) verlaag het. Tydens die evaluasie van MTT is die vermeedering van die HeLa selle betekenisvol verlaag deur die 10 mg/ml konsentrasies van etielasetaat (11.52 %,  $p < 0.05$ ) en petroleumeter (27.3 %,  $p < 0.001$ ) in vergelyking met die kontrole. Ten spyte daarvan dat die 10 mg/ml konsentrasie van etielasetaat toksisiteit getoon het in die MTT-analise, word hy nog steeds gesien as een van die belowende twee ekstrakte vir antioksidantaktiwiteit. Dit is moontlik dat verskillende komponente van die ekstrakte kan bydrae tot die antioksidantaktiwiteit en toksisiteit. Na aanleiding van die voorafgaande biologiese analises is die etielasetaatekstrak gefraksioneer en deur kolomchromatografie is die antioksidantkomponente geïsoleer.

Twee verbindings is geïsoleer, PS en OS.  $^{13}C$ ,  $^1H$  en FT-IR is gebruik om die struktuur van die geïsoleerde verbindings te karakteriseer. PS is 'n  $\beta$ -sitosterol en OS word voorgestel as 'n  $\beta$ -caroteen. Die  $\beta$ -caroteen het die MDA-vlakk betekenisvol verlaag by alle konsentrasies.

## **OPSOMMING**

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Die verlaging van die MDA in teenwoordigheid van die toksien by die 2.5 mg/ml konsentrasie was amper dieselfde as by die kontrole.

Beide geïsoleerde verbindings kom voor in meeste plante en is bekend vir antioksidantaktiwiteit. Verdere fraksionering is nodig om meer onbekende komponente te uit die plant te isoleer.

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## ABBREVIATIONS

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4-HNE	4-hydroxy-2-nonenal
6-OHDA	6-Hydroxydopamine
°C	Degrees Celsius
ADHD	Attention deficit hyperactivity disorder
AIDS	Acquired immune-deficiency syndrome
AMPA	$\alpha$ -amino-3-hydroxy-5methyl-4- isoxalopropionate
ANOVA	One way analysis of variance
ATP	Adenosine triphosphate
AR-JP	Autosomal juvenile parkinsonism
AUC	Area under curve
BBB	Blood brain barrier
BHT	Butylated hydroxytoluene
BSA	Bovine serum albumin
Ca <sup>2+</sup>	Calcium
COSY	Correlation spectroscopy
DAQ	Dopamine Quinone
DAT	Dopamine transporter
DCM	Dichloromethane
DEPT	Distortionless enhancement by polarization transfer
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulfoxide
EA	Ethyl acetate
EI	Electron Ionization
ETC	Electron transport chain
EtOH	Ethanol
FBS	Foetal Bovine Serum
Fe <sup>2+</sup>	Ferrous (iron II)
Fe <sup>3+</sup>	Ferrous (iron III)

## ABBREVIATIONS

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FBS	Foetal bovine serum
FRAP	Ferric reducing ability of plasma
GAA	Glacial acetic acid
H <sub>2</sub> O <sub>2</sub>	Hydrogen Peroxide
HeLa	Human epithelial
IR	Infrared
KCN	Pottasium cyanide
LMWA	Low molecular weight antioxidants
MDA	Malondialdehyde
MAO	Monoamine oxidase
MPP <sup>+</sup>	1-methyl-4-phenyl pridinium
MPTP	1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine
MS	Mass Spectrometry
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NBD	Nitro blue diformazan
NBT	Nitro blue tetrazolium
NMDA	<i>N</i> -methyl-D-aspartate
NMR	Nuclear Magnetic Resonance
NO <sup>•</sup>	Nitric oxide
NWU	North-west University
<sup>1</sup> O <sub>2</sub>	Singlet oxygen
O <sub>3</sub>	Ozone
O <sub>2</sub> <sup>•-</sup>	Superoxide anion/radical
OH <sup>•</sup>	Hydroxyl
ONOO <sup>-</sup>	Peroxynitrite
ORAC	Oxygen Radical Absorbance Capacity

## ABBREVIATIONS

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PBS	Phosphate buffer solution
PD	Parkinson's disease
PE	Petroleum ether
Ppm	parts per million
PUFA	Polyunsaturated fatty acid(s)
Rf	Retention factor
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SEM	Standard error of mean
SNpc	Substantia nigra pars compacta
SOD	Superoxide dismutase
TAC	Total antioxidant activity
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TCA	Trichloroacetic acid
TEP	1,1,3,3-Tetramethoxypropane
TLC	Thin layer chromatography
UBS	Ubiquitin-protease system
UV	Ultraviolet
WHO	World Health Organization

# CHAPTER ONE

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## Introduction

Plants are the oldest source of drugs known to the human race. History shows that the leaves, flowers, berries, barks and/or roots of plants were used as antibacterial, antioxidants, antimalarials, analgesics, and for several other ailments. Some plants are used for various ailments because of their broad medicinal properties. In the bible book of Ezekiel, in the last part of chapter 47 verse 12, the following is said regarding plant life: "...and the fruit thereof shall be for meat, and the leaf thereof for medicine" (Bible, 2007). This suggests that plants were used for medicinal purposes even before Christ was born.

The World Health Organization (WHO) recently estimated that about 80% of the world's population uses herbal medicine for primary health care (Herb Palace, 2003). Their use continues in the modern world as many conventional drugs are derived from plants. In South Africa, seventy-two percent of the black population is estimated to use traditional medicines (Mander *et al.*, 1998). This number grows daily as people now prefer to use more natural and less harmful products. Another reason why traditional medicines are still being used is that they are affordable.

Supplementation with antioxidants has received widespread attention in recent years. Health conscious consumers worldwide consume different herbal teas, for example green tea (Gadow *et al.*, 1997; Li *et al.*, 2008) for their antioxidant properties. There is no doubt that antioxidants are essential in maintaining a healthy body and preventing diseases. Recent evidence shows that antioxidants can be used topically to provide photoprotection for the skin (Murray *et al.*, 2008). Research in the past has established that antioxidants reduce the risk of chronic diseases like cancer and Parkinson's disease and also slow down the aging process (Inanami *et al.*, 1995). This they achieve as they prevent and repair damage caused by free radicals.

With respect to Parkinson's disease, it is one of the most common neurodegenerative diseases with the most prominent feature being the selective degeneration of dopaminergic neurons in the substantia nigra pars compacta of the midbrain therefore resulting in a decrease in dopamine levels in the striatum (Shimizu *et al.*, 2003). The substantia nigra appears to be an area of the brain that is highly susceptible to oxidative stress. Both external and internal stimuli can trigger damage to neurons in the brain. The brain is an ideal target for free radical damage because it is composed of large quantities of lipids which make an excellent target for free radical reactions (Foy *et al.*, 1999). Treatment of Parkinson's disease is aimed at maintaining dopamine at normal levels. This is achieved by drugs that replace dopamine (e.g. Levodopa),

drugs that stimulate dopamine receptors or by many other mechanisms that will be explained in chapter two. Another way to treat, or slow down the progression of this disease is by preventing damage caused by free radicals on the dopaminergic neurons. This is achieved by the use of antioxidants.

### 1.1 Research objectives

The aim of this study was to investigate the antioxidant properties and the toxicity of the leaves of *Plumbago auriculata*.

Twenty-one plants were screened for their total antioxidant capacities. From these twenty-one, *P. auriculata* was one of the plants with the highest activity (chapter 3) and was therefore selected for further analysis.

To achieve the aim of this study, the following objectives were met:

- Preparation of leaf extracts of the plant using organic solvents: petroleum ether, dichloromethane, ethyl acetate and ethanol in order of increasing polarity.
- Bioassay-guided fractionation of the most active fraction using the Thiobarbituric acid-Reactive Substances (TBARS) and the Nitro-Blue Tetrazolium (NBT) assays for antioxidant activity. The TBARS assay is used to assess lipid peroxidation while the NBT assay measures superoxide anion ( $O_2^{\bullet-}$ ) and possibly other free radicals.
- Assay for *in vitro* toxicity of each crude extract using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. This assay measures the metabolic activity of viable cells.
- Use of liquid-liquid extraction, column chromatography and preparative TLC for separation, isolation and purification.
- Determination of structures of pure compounds through Nuclear Magnetic Resonance (NMR), infrared spectroscopy (IR) and Mass spectrometry (MS).
- *In vitro* analysis of the antioxidant activity of the pure compounds using the TBARS and NBT assays.
- Assay for *in vitro* toxicity of the pure compounds using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay.

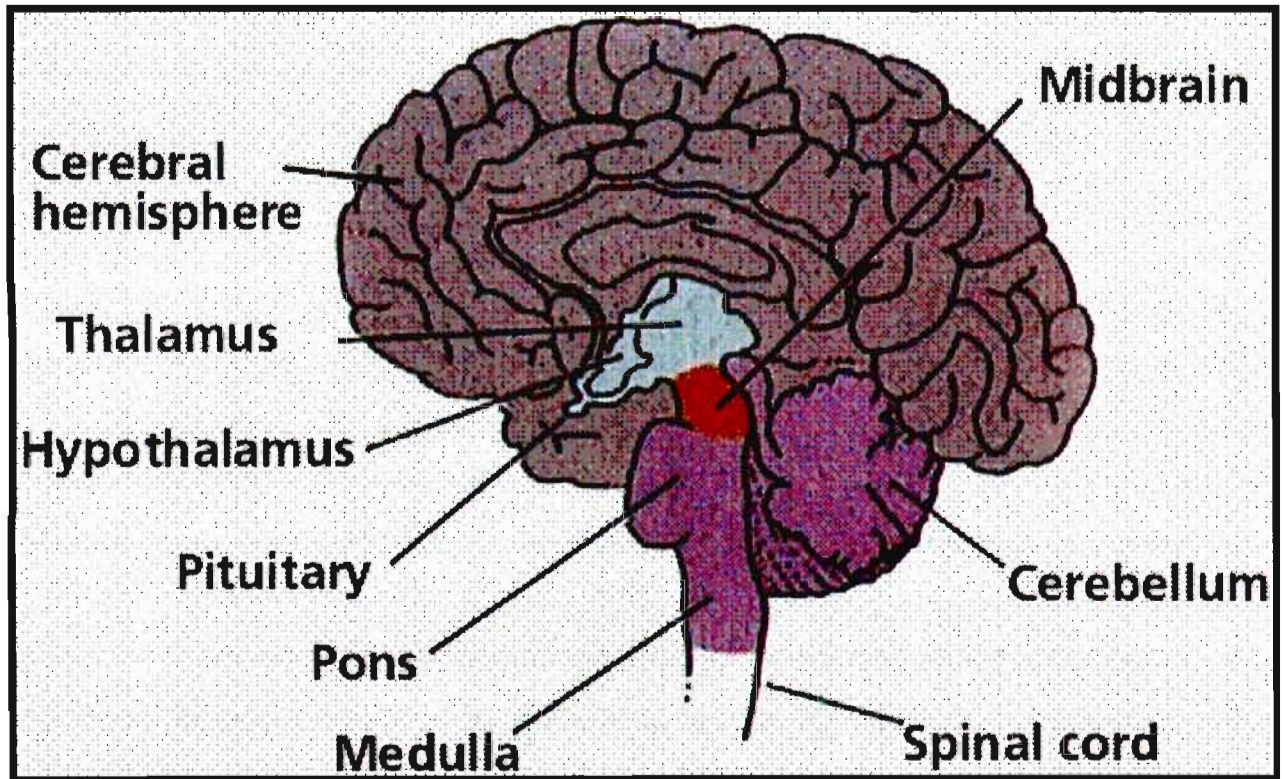
# CHAPTER TWO

## Literature review

### 2.1 Basic anatomy of the human brain

The brain and the spinal cord are the two main components of the central nervous system. The brain is the centre of thought and emotion (Online medical dictionary, 1997). Cells in different parts of the body after sensing anything send information to neurons that then send it to the brain for processing, and then signals are sent to the body for the appropriate action to be taken.

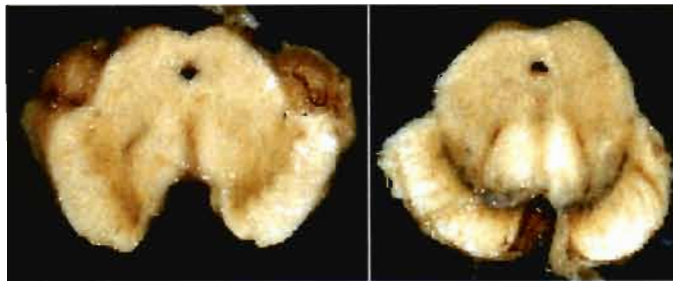
Three main parts make up the brain: the forebrain, midbrain, and hindbrain. The forebrain consists of the cerebrum, thalamus, and hypothalamus. The cerebral cortex is the most important structure in the forebrain. It is the part of the brain known as the gray matter. The cerebral cortex covers the outer part of the cerebrum and the cerebellum. The midbrain consists of the tectum, tegmentum and the cerebral aqueduct. The hindbrain is made of the cerebellum, pons and medulla. Often, the midbrain, pons, and medulla are collectively referred to as the brainstem.



*Figure 2.1 Midsagittal view of the human brain*

Perceptions, conscious awareness, cognition, and voluntary action are all controlled in the forebrain. The hypothalamus also controls the autonomic nervous system. Bodily functions are regulated in response to the needs of the organism (Bear *et al.*, 2001).

The midbrain controls many important functions such as the visual and auditory systems as well as eye and body movement. The substantia nigra is the largest nucleus of the human midbrain. It is divided anatomically into two parts, its dorsal region is called *pars compacta*, and its ventral region is called *pars reticulata*. The substantia nigra is responsible for controlling body movement. This darkly pigmented nucleus (figure 2.2 (b)) contains a large number of dopamine-producing neurons. The degeneration of the dopaminergic neurons in the substantia nigra leading to a substantial reduction in striatal dopamine is associated with Parkinson's disease.



(a)

(b)

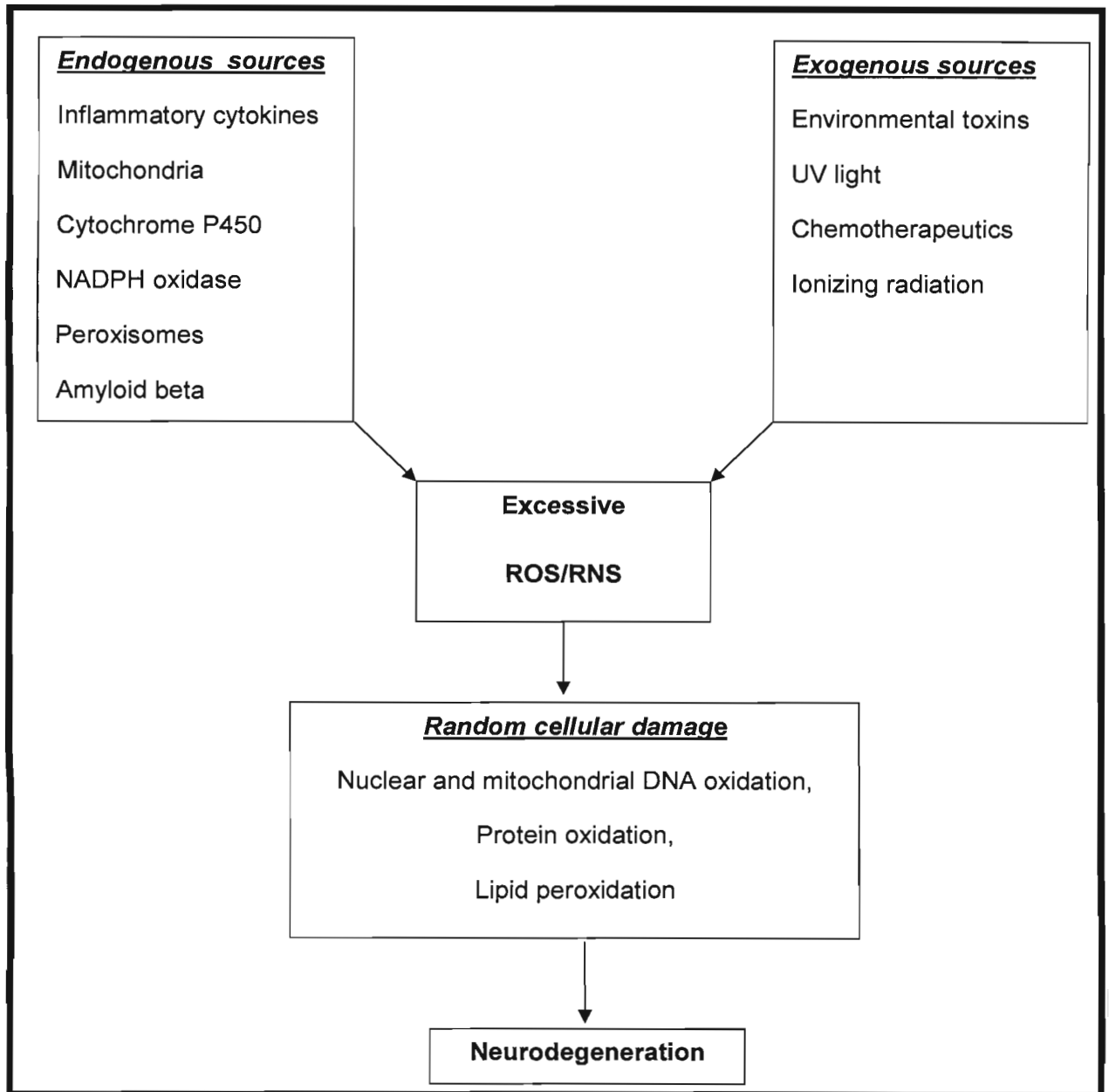
**Figure 2.2** (a) Substantia nigra without dopaminergic neurons (Parkinson's disease). (b) substantia nigra with dopaminergic neurons.

Neurons in the hindbrain contribute to the processing of sensory information, the control of voluntary information and regulation of the autonomic nervous system (Bear *et al.*, 2001). The cerebellum receives movement information from the pons and spinal cord and therefore its damage results in uncoordinated and inaccurate movement.

The human brain contains an average of 100 billion neurons. After their destruction, neurons in the brain cannot regenerate like other body cells, thus destruction of a huge number of these cells poses a problem to the transmission of signals in the brain. Damage to neurons in the brain can be due to oxidative stress that can occur during the normal aging process or due to external stimuli. Programmed cell death also damages neurons in a systematic way to regulate the number of neurons in the brain at a given time.

## 2.2 Causes of oxidative stress in the brain

The constant exposure of neurons to external and internal toxins leads to oxidative stress in the brain (Figure 2.3). This may be due to the excessive production of reactive oxygen species (ROS) and reactive nitrogen species (RNS).



**Figure 2.3** External and internal agents triggering reactive oxygen species (ROS), and cellular responses to ROS (Hajieva & Behl, 2006).

Reactive oxygen species (ROS) is an umbrella term that includes all highly reactive, oxygen-containing molecules, including free radicals. They normally exist in all aerobic cells together

with biochemical antioxidants (Gulam & Haseeb, 2006). The mitochondria are responsible for most of the ROS and the first produced superoxide anion ( $O_2^{\bullet-}$ ) radicals in human tissues (Andersen, 2004; Emerit *et al.*, 2004). The role of mitochondria is primarily the generation of oxidative phosphorylation and oxygen consumption. The enzymes responsible for oxidative phosphorylation are in the inner membrane of the mitochondria. Monoamine oxidase enzymes are bound to the outer membrane of mitochondria in most cell types of the body.

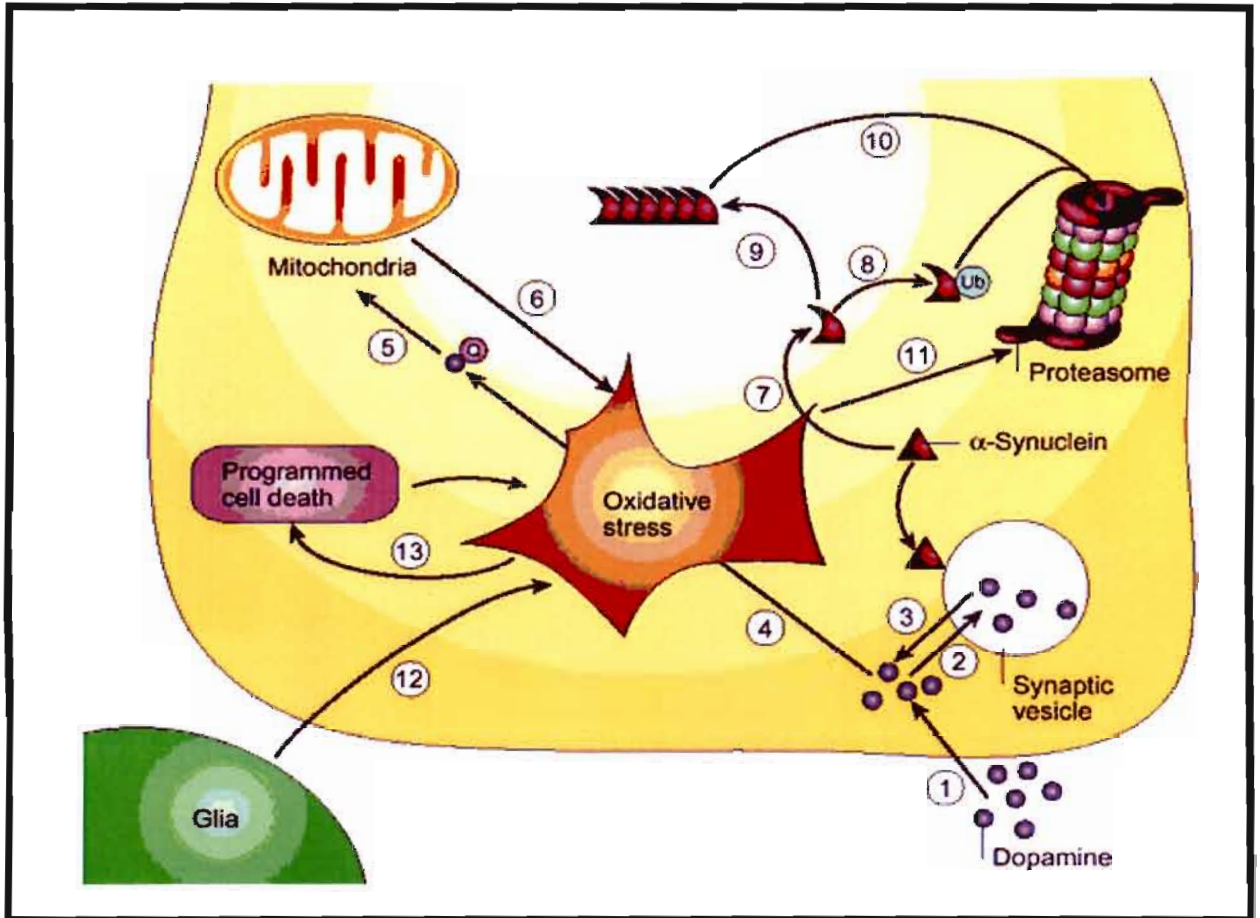
The neuronal mitochondria use oxygen taken up by the neuron to produce ATP. This ATP is produced through the flow of electrons along a series of molecular complexes in the inner mitochondrial membrane known as the electron transport chain (ETC) (Fariss *et al.*, 2005). Neurotoxins like rotenone and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) used to create Parkinson's disease models act by inhibiting the ETC at complex I of the mitochondria.

An excess in ROS and/or a reduction in antioxidants results in oxidative stress. The generation of ROS is a feature of normal cellular function like mitochondrial respiratory chain, phagocytosis and arachidonic acid metabolism. However, this normal production multiplies a lot during pathological conditions (Singh *et al.*, 2004).

Oxidative stress is implicated in neurodegenerative disorders including Parkinson's disease, Alzheimer's disease etc. The brain is an ideal target for free radical damage because it is composed of large quantities of lipids which make an excellent target for free radical reactions (Foy *et al.*, 1999). The brain also has low levels of the antioxidant enzyme catalase and is rich in iron. An assumption is made that free radicals cause point mutations and/or over expression of certain genes which may initiate degeneration and lead to death of dopaminergic neurons in idiopathic Parkinson's disease (Zigmond *et al.*, 1999).

Dopamine is a neurotransmitter that is important in the brain for motor skills and focus. Low levels of dopamine may lead to attention deficit hyperactivity disorders (ADHD), addictions, paranoia, and movement disorders like Parkinson's disease. This is a result of the damage to the dopaminergic neurons thus less production of dopamine. The formation of free radicals may be a result of the metabolism of dopamine, which gives rise to  $H_2O_2$  via monoamine oxidase enzymes (MAO), as well as dopamine auto-oxidation (Bahr, 2004). Monoamine oxidases are enzymes that catalyze the oxidation of monoamines hence they are associated with oxidative stress, and may promote aggregation and neuronal damage (Chua & Tang, 2006).

Figure 2.4 illustrates the possible ways in which dopaminergic neurons can be damaged.



**Figure 2.4** Diagram illustrating possible oxidative stress pathways in a dopaminergic neuron (Andersen, 2004).

1. Uptake into the dopaminergic neuron, of dopamine by the dopamine transporter (DAT)
2. Uptake of dopamine by the vesicular monoamine transporter VMAT2 into synaptic vesicles;
3. Dopamine is released from the synaptic vesicle by  $\alpha$ -synuclein;
4. Oxidation of dopamine to dopamine quinone (DAQ);
5. Production of potential mitochondrial inhibitors such as metabolites of 5cysDAQ conjugates by DAQ;
6. Production of oxidative stress by mitochondria;

7.  $\alpha$ -synuclein undergoes oxidation;
8.  $\alpha$ -synuclein is tagged by ubiquitin and subsequently degraded by the proteasome;
9. Oligomerization of  $\alpha$ -synuclein;
10. The interaction of  $\alpha$ -synuclein with the proteasome which is toxic;
11. Oxidative by-products such as 4-hydroxynonenol (4-HNE) interact with the proteasome;
12. Neighbouring glial cells produce oxidative stress and
13. Programmed cell death induction (Andersen, 2004).

Excitotoxicity is another way through which free radicals are produced.

### 2.2.1 Excitotoxicity

Most of the excitatory synaptic activity in the mammalian is accounted for by glutamate and related excitatory amino acids (Gilgun-Sherki & Offen, 2001). Glutamate acts primarily through activation of its ionotropic receptors (Olney, 1990; Gilgun-Sherki & Offen, 2001). There are three families of ionotropic receptors (Meldrum, 2000) which are involved in neurodegeneration and they all appear to be tetrameric (Laube *et al.*, 1998). These ionotropic receptors are divided into three major types based on their selective agonists: *N*-methyl-D-aspartate (NMDA),  $\alpha$ -amino-3-hydroxy-5methyl-4-isoxalopropionate (AMPA), and kainate.

The activation of glutamate-releasing neurons leads to neuronal death. Oxidative stress could lead to pathologic changes that result in the death of the neuron. The activation of glutamate's metabotropic receptors, leads to the opening of NMDA channels thus the entry of calcium in the neuron leading to depolarization. An overload of calcium is an essential factor in excitotoxicity (Rang *et al.*, 1999). Raised  $[Ca^{2+}]_i$  affects many processes. The ones that cause neurotoxicity include the following:

1. increased glutamate release,
2. activation of proteases (calpains) and lipases membrane damage,
3. activation of nitric oxide synthase (NOS), which together with ROS, generates peroxynitrite and hydroxyl free radicals, which react with several cellular molecules, including membrane lipids, proteins and DNA,

4. increased arachidonic acid release, which increases free radical production, and also inhibits glutamate uptake (Rang *et al.*, 1999).

Normally, glutamate is involved in energy metabolism, ammonia detoxification, protein synthesis and neurotransmission (Fonnum, 1985). It is responsible for many neurologic functions, including cognition, memory and sensation (Rang *et al.*, 1999).

### **2.2.2 Reactive oxygen species and free radicals**

ROS include superoxide anion radical ( $O_2^{\bullet-}$ ), singlet oxygen ( $^1O_2$ ), ozone ( $O_3$ ), hydrogen peroxide ( $H_2O_2$ ), the highly reactive hydroxyl radical ( $OH^{\bullet}$ ), nitric oxide radical ( $NO^{\bullet}$ ), and various lipid peroxides.

#### **2.2.2.1 Superoxide anion radical**

$O_2^{\bullet-}$  and  $H_2O_2$  can be produced as a result of UV irradiation, leading to the induction of apoptosis (Gorman *et al.*, 1997).  $O_2^{\bullet-}$  induces caspase activation and apoptosis in hepatocytes (Conde de la Rosa *et al.*, 2006).

#### **2.2.2.2 Singlet oxygen**

$^1O_2$  is a highly reactive non-radical molecule. It can induce oxidation of the DNA in cells (Ravanat *et al.*, 2000).

#### **2.2.2.3 Ozone**

Exposure to  $O_3$  induces changes to biomarkers of inflammation and oxidative stress in the lungs (Corradi *et al.*, 2002; Foucaud *et al.*, 2006). With respect to rat brains,  $O_3$  exposure caused a significant decrease in motor activity in rat brains. It also produced lipid peroxidation, loss of fibers and death of the dopaminergic neurons (Pereyra-Munoz *et al.*, 2006).

#### **2.2.2.4 Hydrogen peroxide**

$H_2O_2$  is not a free radical but one of the ROS that cause damage to cells in the body. It induces apoptosis and can therefore be used as a model for the degeneration of cells (Jiang *et al.*, 2003). It is a marker of oxidative stress in malignancies (Banerjee *et al.*, 2003).

$H_2O_2$  in the presence of metals is converted, via Fenton's reaction, into the highly reactive hydroxyl radical. Iron levels are significantly higher in the substantia nigra and the globus pallidus of patients with Parkinson's disease as compared to brains of people that are not

diseased (Griffiths *et al.*, 1999; Graham *et al.*, 2000). Elevated iron levels therefore contribute to the neurodegeneration in Parkinson's disease. An example is ferrous iron ( $\text{Fe}^{2+}$ ), a transition metal ion that reacts easily with  $\text{H}_2\text{O}_2$ . It reacts in the Fenton reaction ( $\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^\bullet + \text{OH}^-$ ) giving the highly reactive hydroxyl radical which causes damage to brain cells.

#### **2.2.2.5 Nitric oxide radical**

The biosynthesis of nitric oxide is controlled by nitric oxide synthase (NOS) enzymes. This free radical has both pro and anti-oxidant properties.  $\text{NO}^\bullet$  reacts with  $\text{O}_2^{\bullet-}$ , to form  $\text{ONOO}^-$ , a reactive nitrogen species. It has been shown to have antioxidant effects against  $\text{H}_2\text{O}_2$  and  $\text{O}_2^{\bullet-}$  (Svegliati-Baroni *et al.*, 2001; Wink *et al.*, 2001).

#### **2.2.2.6 Peroxynitrite**

$\text{NO}^\bullet$  can interact with superoxide anion to form peroxynitrite ( $\text{ONOO}^-$ ), a potent oxidant. Peroxynitrite is neurotoxic (Dawson *et al.*, 1991; Lipton *et al.*, 1993) and it causes apoptosis in leukemic cells (Lin *et al.*, 1995). It can initiate lipid peroxidation (Rice-Evans & Packer, 1998).

### **2.3 Effects of oxidative stress in the brain**

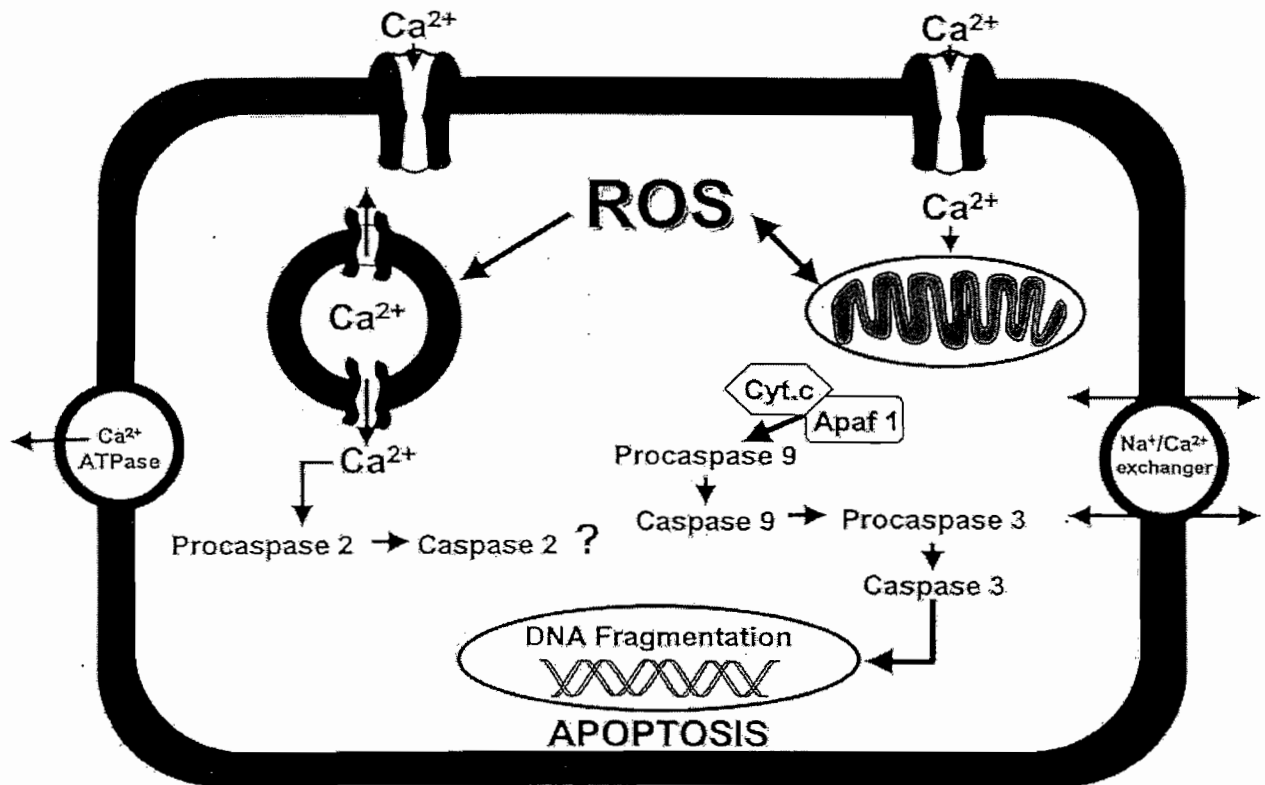
Oxidative stress induces a number of pathological processes including apoptosis, necrosis and the peroxidation of lipids. The induction of these processes can lead to a cycle that results in neuronal death thus less dopamine in the brain.

#### **2.3.1 Apoptosis**

Apoptosis is one of the types of programmed cell death that occurs during development of the nervous system to establish an optimized number of cells (Oppenheim, 1991). 20 - 80 % of neurons born are lost during naturally occurring cell death. Kerr & Colleagues (1972) proposed that apoptosis plays a 'complimentary but opposite role to mitosis in the regulation of animal cell populations'. It is induced to eliminate cells with irreparable damage to DNA that otherwise might become deleterious. According to Los *et al.* (2001), apoptosis is also induced when cell division has gone astray and cell cycle-progression is unscheduled.

Two signaling pathways of cell death are reported in apoptosis: the receptor mediated (extrinsic) and the mitochondrially mediated (intrinsic) pathway. The extrinsic pathway is

triggered by the activation of death receptors (Fas, TNF and TRAIL) residing on the cell membrane, while the intrinsic pathway involves the mitochondria and other organelles in the cell, such as the endoplasmic reticulum (Korhonen & Lindholm, 2004). Apoptosis is an active process which needs ATP (Zamaraeva *et al.*, 2005).



**Figure 2.5** Model of apoptosis induced by reactive oxygen species (Annunziato *et al.*, 2003).

Oxidative stress in neuronal cells leads to the production of ROS that trigger the release of cytochrome-c from the mitochondria and the activation of caspase-3 which then initiate apoptosis (figure. 2.5) (Annunziato *et al.*, 2003). Caspases or cysteine aspartases are a group of cysteine proteases that cleave target proteins at specific aspartate residues. They are the enzymes required for apoptosis and death of most cells.

When apoptosis is triggered by oxidative stress through the intrinsic pathway, the result is DNA damage, protein modifications and alteration in mitochondrial function (Francò *et al.*, 2009). There is evidence of apoptosis in the substantia nigra of Parkinson's disease patients (Mochizuki *et al.*, 1996).

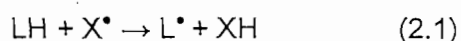
### 2.3.2 Lipid peroxidation

In a test by Agil *et al.* (2005) to see the role of Levodopa in plasma lipid peroxidation, it was found that Parkinson's disease patients had raised plasma lipid peroxidation concentrations compared to the controls. This suggests that they are chronically under oxidative stress. The results obtained also support the involvement of systemic oxidative stress in the pathogenesis of Parkinson's disease.

Lipid aldehydes like malondialdehyde and 4-hydroxy-2-nonenal (4-HNE) are the result of lipid peroxidation, an autocatalytic pathway that causes oxidative damage to cells (Walker *et al.*, 2001). These products of the break down of polyunsaturated fatty acid peroxides can be used as markers of lipid peroxidation and oxidative damage (Beal, 2002; Hashimoto *et al.*, 2003).

In general, *in vivo* lipid peroxidation proceeds via a radical chain reaction, which consists of a chain initiation reaction, a chain propagation reaction and termination. The chain initiation reaction is a feature of the reaction of free radicals with non-radicals: one radical begets another (Halliwell & Chirico, 1993). The hydroxyl radical ( $\text{OH}^\bullet$ ), and peroxynitrite ( $\text{ONOO}^-$ ) are possible ROS responsible for the initiation reaction (Rice-Evans & Packer, 1998). The highly reactive  $\text{OH}^\bullet$  reacts with hydrogens from any nearby C-H to form  $\text{H}_2\text{O}$ .

A highly energetic one electron oxidant ( $\text{X}^\bullet$ ), such as a hydroxyl radical, extracts a hydrogen atom from a lipid fatty acid chain, producing a carbon-centered radical,  $\text{L}^\bullet$ .

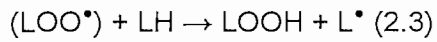


Once a radical is generated, propagation chain reactions result in the oxidation of polyunsaturated fatty acids (PUFA) to fatty acid hydroperoxides. Propagation allows a reaction with oxygen.

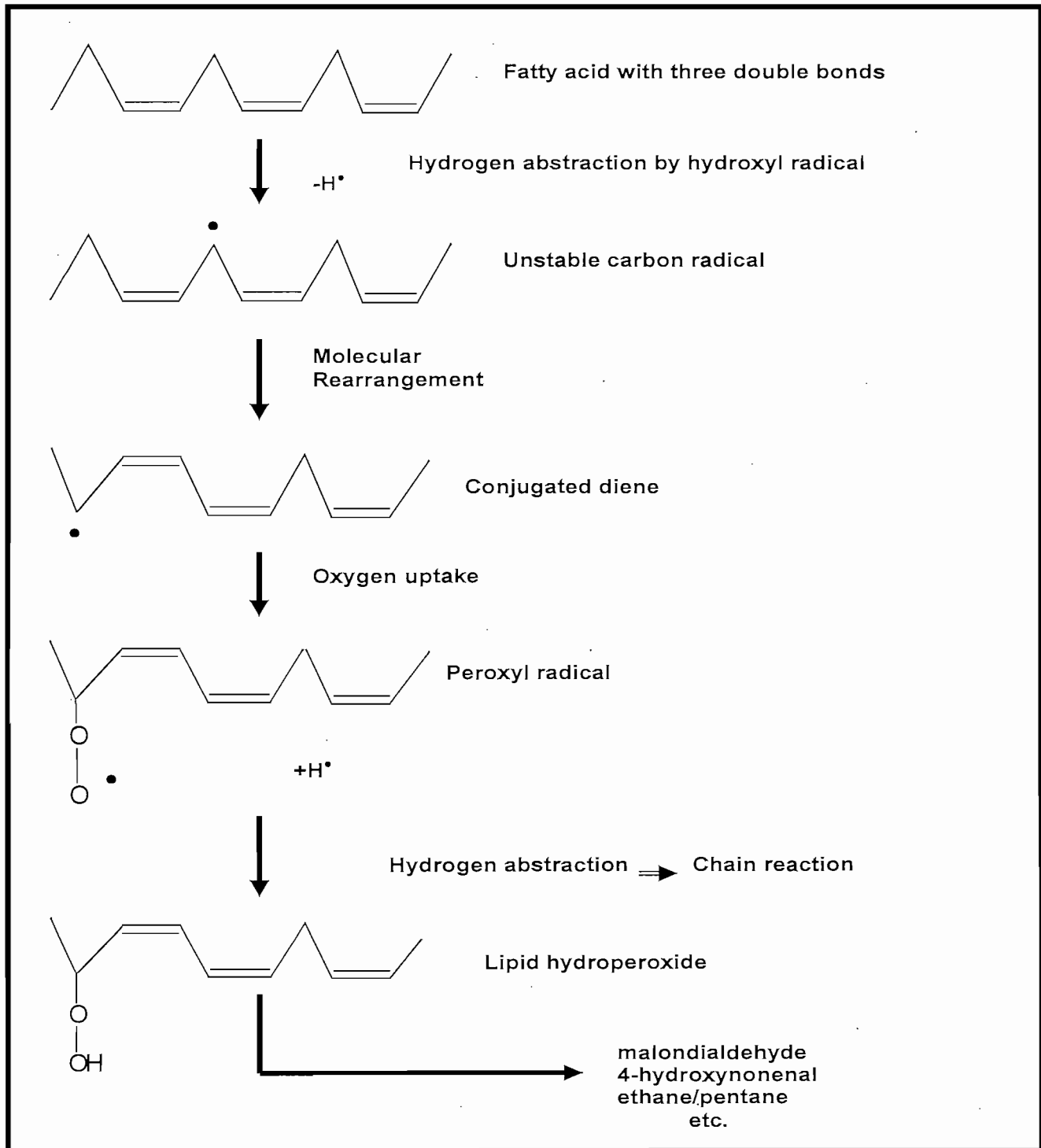


The length of the propagation chain depends on many factors including, the lipid-protein ratio in a membrane, the fatty acid composition, the presence of chain breaking antioxidants within the membrane and the oxygen concentration (Aikens & Dix, 1991).

The peroxide radical ( $\text{LOO}^\bullet$ ) formed from propagation can then react with the original substrate:



Thus reactions 2.2 and 2.3 form the basis of a chain reaction process (Gurr & Harwood, 1991).



**Figure 2.6** Basic reaction sequence of lipid peroxidation (Young & McEneny, 2001)

Tests by Aikens & Dix (1991) demonstrated that  $\bullet\text{OOH}$  and not  $\text{O}_2^{\bullet-}$  is active in initiating lipid peroxidation in chemically defined fatty acid dispersions.

### 2.3.3 Necrosis

Necrosis, like apoptosis can also be a type of programmed cell death (Proskuryakov *et al.*, 2003). A number of receptors are implicated in triggering necrosis. It can be induced when antioxidant defences like Vitamin E are reduced (Mutaku *et al.*, 2002) and by severe environmental changes.

Necrosis starts with the swelling of cells, followed by the collapse of the plasma membrane and finally the lysing of the cells. It however has different consequences from apoptosis where the cells die by shrinking. The activation of certain proteases (caspases) and DNA fragmentation are absent from necrosis as compared to apoptosis (Proskuryakov *et al.*, 2003).

When neurons in the brain have been subjected to oxidative stress this leads to apoptosis, the peroxidation of lipids and necrosis. Neurodegenerative diseases are a consequence of this oxidative stress. Of main interest is Parkinson's disease, which is a consequence of the damage of dopaminergic neurons therefore leading to low levels of the neurotransmitter dopamine in the brain.

## 2.4 Parkinson's disease

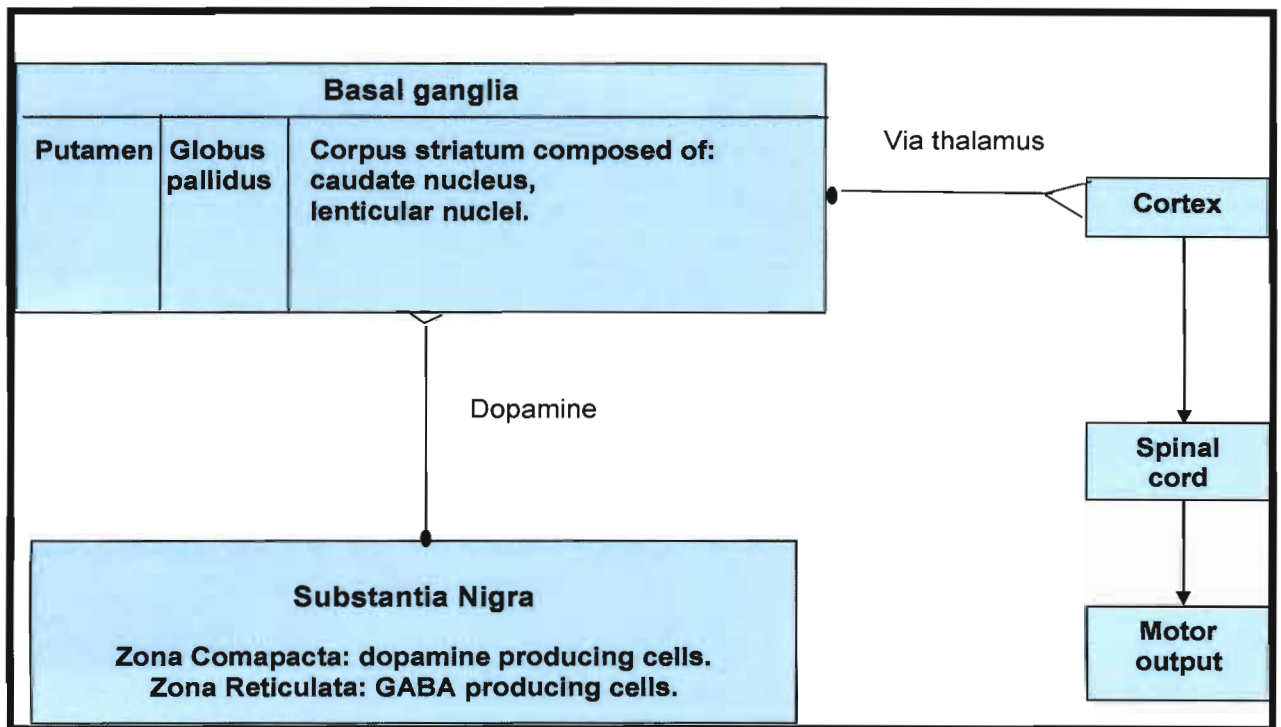
Parkinson's disease was first described by James Parkinson (1755-1824) two centuries ago. It is one of the most common neurodegenerative diseases with the most prominent feature being the selective degeneration of dopaminergic neurons in the substantia nigra pars compacta of the midbrain therefore resulting in a decrease in dopamine levels in the striatum (Shimizu *et al.*, 2003). The dopamine receptors are not found only in the midbrain. A study was performed on brain tissue from 16 patients who died with a clinical diagnosis of idiopathic Parkinson's disease and 14 controls. The study showed that the dopamine  $\text{D}_1$  receptors are also expressed in neurons in the globus pallidus and the substantia nigra and not only in the striatal efferent neurons. It was also found that the expression of dopamine  $\text{D}_1$  receptors would be affected by drug-treated end stage Parkinson's disease (Hurley *et al.*, 2001).

The original description of the disease by James Parkinson was published in 1817 as a short monograph. The essay by James Parkinson describes the course of the illness in six

different cases. Not much attention was paid to this publication for the next five decades. In 1861, Charcot and colleagues were the first to use the term 'Parkinson's disease'. In each of the cases by James Parkinson, the person observed was over fifty and almost all of them thought the condition they now had was due to old age. Old age does account for the loss of dopaminergic neurons but cases of Parkinson's disease in young people have been reported. As humans increase in age, there is a great decrease in the number of dopaminergic neurons in the pars compacta of the substantia nigra, whether they have neurological disease or not. At the time of death even mildly affected Parkinson's disease patients have lost about 60% of their dopaminergic neurons, and it is this loss, in addition to possible dysfunction of the remaining neurons, that accounts for the approximately 80% loss of dopamine in the corpus striatum (Zigmond & Burke, 1999).

After extensive research and study on Parkinson's disease, the real cause of this disease still remains unknown. Parkinson's disease mainly affects reaction time and speed of performance. It is normal for reaction time to increase with age but the change in Parkinson's disease is great (Latash, 1998). The absence of any toxic or other underlying etiology makes the treatment not to arrest the progression of the disease but to slow it down.

The extrapyramidal system (figure 2.7) is part of the motor system involved in the coordination of movement. It helps regulate movements such as walking and to maintain balance. Damage to any parts of this system leads to movement disorders like Parkinson's disease.



**Figure 2.7** Extrapyramidal motor systems in the brain, responsible for the coordination of movement (Rang *et al.*, 1999).

#### 2.4.1 Signs and symptoms

1. Tremor at rest
2. Rigidity
3. Bradykinesia
4. Postural instability(Uitti *et al.*, 2005)

#### 2.4.2 Etiology

In the past, the exact cause of Parkinson's disease was not known. Recent studies however have suggested oxidative stress as being one of the causes of the disease. An abundance of free radicals leads to the destruction of dopaminergic neurons in the substantia nigra (Akaneya *et al.*, 1995). The factors below explain how the dopaminergic neurons may be destroyed.

#### 2.4.2.1 Age

As individuals grow older, the total numbers of neurons in the brain decrease. This however is not in a uniform pattern. Parkinson's disease is one of the most common neurodegenerative diseases of the elderly. A hypothesis was made that oxidative injury might directly cause the aging process and this was supported by the finding of oxidative damage to macromolecules (DNA, lipids and proteins) (Gilgun-Sherki & Offen, 2001). The major role aging itself plays in the pathogenesis of Parkinson's disease remains unclear. However it has been proved that with increase in age, striatal dopamine is lost (Gilgun-Sherki & Offen, 2001). Additional links between the two focus on the mitochondria.

#### 2.4.2.2 Genetic factors

For many years, genetic factors were considered unlikely to play an important role in the pathogenesis of Parkinson's disease. This concept was based largely on twin studies conducted in the early 1980s that demonstrated a very low rate of concordance for the disease among identical twins. Nevertheless, it has been recognized that Parkinson's disease could occasionally be identified in families. Specific disease-causing mutations were identified thus exploration of pathogenesis at a molecular level is now possible. A study by Tanner *et al.* (1999) provides very clear evidence that the common, sporadic forms of late-onset Parkinson's disease are highly influenced by environmental factors, whereas the early-onset forms of Parkinson's disease have a strong genetic basis.

Two genes are important in the study of Parkinson's disease. These are parkin and  $\alpha$ -synuclein.

#### Parkin

Mutations of the gene parkin are associated with early onset Parkinson's disease (Lucking *et al.*, 2000). The older a person grows, the lesser the likelihood of the mutation of this gene. It may be as high as 50% percent for people younger than twenty-five. Examples of features that distinguish parkin-linked Parkinsonism from sporadic Parkinson's disease include: wide ranges of age at onset, frequent dystonia and slow progression (Ishikawa & Takahashi, 1998; Lucking *et al.*, 2000).

The identification of parkin as a component of the ubiquitylation cycle strengthens the theory that ubiquitin-proteasome system (UPS) dysfunction is central to Parkinson's disease pathogenesis (Mata *et al.*, 2004). The UPS plays a key role in cellular quality control and in defence mechanisms. The logical link between the UPS and Parkinson's disease

pathogenesis is the finding that the gene parkin is involved in protein degradation as an ubiquitin ligase collaborating with an ubiquitin-conjugating enzyme. However, parkins that have mutated in AR-JP have a loss of the ubiquitin-protein ligase activity (Shimura *et al.*, 2000).

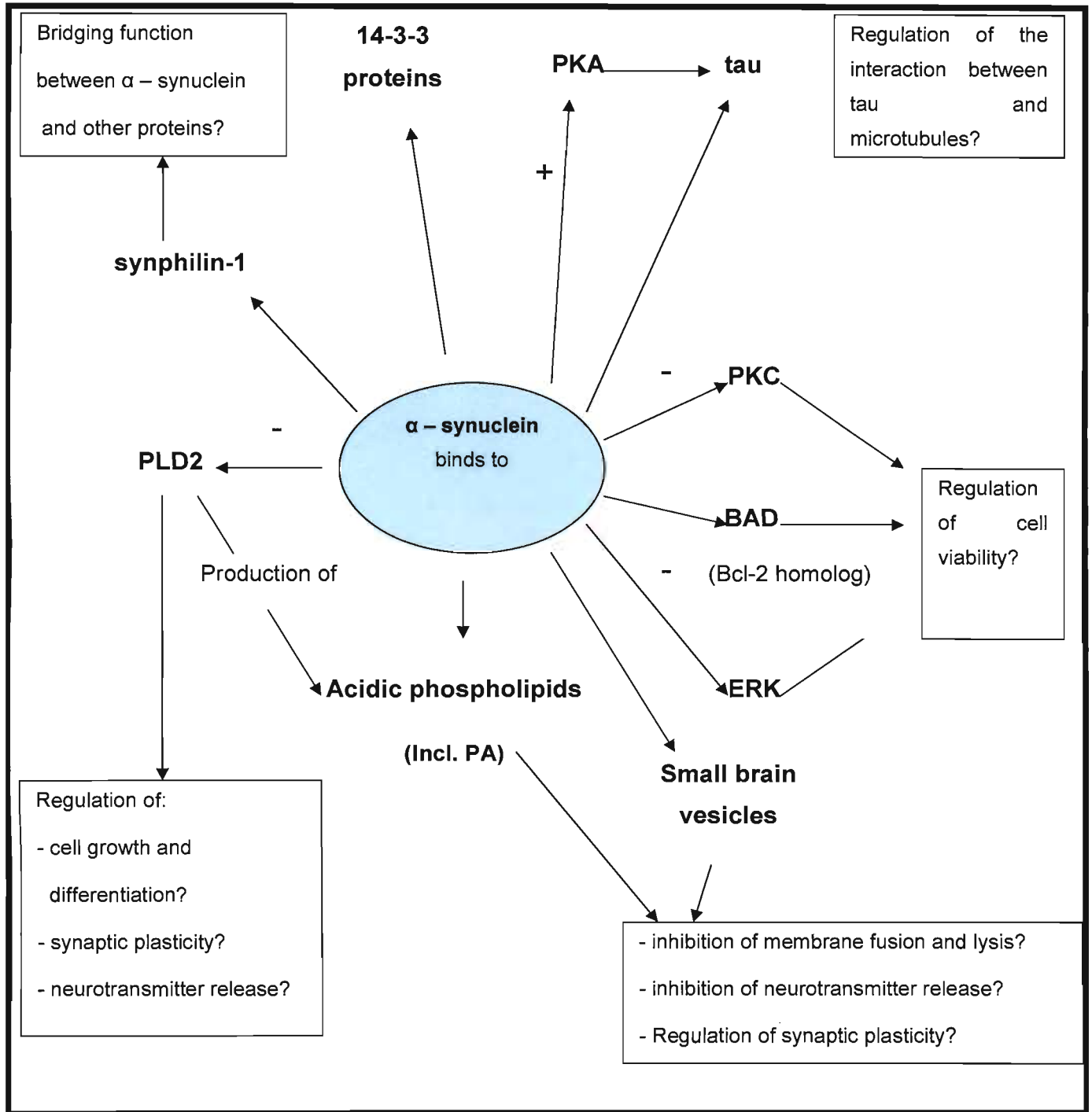
In 1998, the first parkin mutations were identified and they were described as rare autosomal juvenile Parkinsonism (AR-JP) (Kitada *et al.*, 1998). The levels and activity of parkin have been found to be either low or absent in AR-JP, thus suggesting that the neurodegeneration is probably from loss of function (Romero-Ramos, 2004).

### **$\alpha$ -Synuclein**

$\alpha$ -synuclein belongs to a family of highly conserved, small proteins that include beta and gamma synuclein. This type of protein is seen in various tissue types, it is mostly expressed in the CNS, where it is located in synaptic terminals, in close proximity to vesicles. The name synuclein was chosen because it is located in both synapses and the nuclear envelope (Maroteaux *et al.*, 1988).

Before discussing  $\alpha$ -synuclein any further it will be more beneficial to understand its normal functioning (figure 2.8). One of the most interesting potential roles of synuclein is to coordinate nuclear and synaptic events. This protein may be involved in signal transduction. It could also be a molecular monitor of cellular conditions, responding to changes of the physiological state of the cell both in the nucleus and the nerve terminal (Maroteaux *et al.*, 1988).

Putative normal functions of  $\alpha$ -synuclein



**Figure 2.8** Normal functions of  $\alpha$ -synuclein. The binding partners of  $\alpha$ -synuclein are indicated by arrows. ‘-’ and ‘+’ indicate enzyme inhibition or activation by  $\alpha$ -synuclein respectively. The boxes describe potential functions of  $\alpha$ -synuclein interacting with the respective partner.

1. PLD2, phospholipase D2

Localizes primarily to plasma membrane

2. PKC, protein kinase C

Promotes colon carcinogenesis

3. PKA, protein kinase A

Phosphorylates a variety of substrates and regulates many important processes such as cell growth, fibrillation, differentiation and flow of ions across cell membrane.

4. 14-3-3 proteins

Found in all organisms and cell types observed except for the prokaryote kingdom. They were found to be key regulators of mitosis and apoptosis in animals during the past few years (Rosenquist, 2003).

5. Synphilin 1

Linked to the pathogenesis of PD based on its identification as  $\alpha$  – synuclein and parkin interacting protein. Component of Lewy bodies in brains of sporadic PD patients.

6. BAD

It is localized in the mitochondrial membrane. Induces pore formation in this membrane and blocks cytochrome C release. It interacts with mitochondrial membrane in a manner that either promotes or prevents movements across mitochondrial membranes.

$\alpha$ -synuclein interacts with a number of molecules including monoamines. It is a major component of Lewy bodies in all Parkinson's disease patients. Lewy bodies are usually present in the brain stem, basal forebrain and the autonomic ganglia and are mostly abundant in the substantia nigra, and the locus coeruleus (Mezey *et al.*, 1998). They are found in the remaining dopaminergic neurons in the substantia nigra and other nuclei. Lewy bodies were first described in 1912 by Frederick H. Lewy who observed them from brains of patients with Parkinson's disease (Who named it? 2009). A number of proteins are thought to take part in the formation of the Lewy bodies. The possible role played by protein aggregation in Parkinson's disease was suggested by the presence of these Lewy bodies in diseased brains. More support was given by the discovery of the mutations in  $\alpha$ -synuclein (Prasad *et*

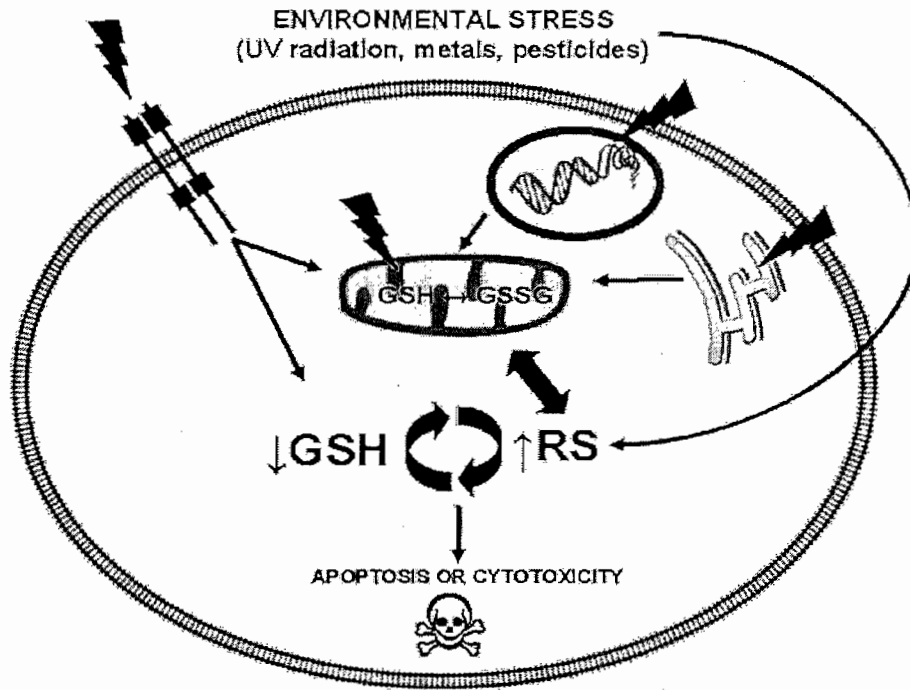
*al.*, 1999). In a test performed by Mezey *et al.* (1998), it was proven that  $\alpha$ -synuclein is indeed present in Lewy bodies.

In a recent test by Chu & Kordower (2006), the data obtained after testing monkey and human brains illustrated an increase in  $\alpha$ -synuclein protein as a function of human aging and this change is strongly associated with decreases in nigro-striatal activity. The age-related increase in  $\alpha$ -synuclein puts a burden on the already challenged lysosome, leading to formation of inclusion bodies in Parkinson's disease nigral neurons and thus driving the dopaminergic levels past a symptomatic level (Chu & Kordower, 2006).

#### **2.4.2.3 Environmental factors**

Since the real cause of Parkinson's disease has not yet been discovered, it is postulated that the environment acting through oxidative stress also has an effect on the onset of this disease. The discovery of MPTP an environmental agent gave credence to the concept that environmental factors could be a common cause of Parkinson's disease (Parker & Swerdlow, 1998). Parkinson's disease symptoms were observed in some drug users who had taken synthetic heroin contaminated with MPTP. After administration of levodopa, the symptoms were reversed (Landrigan *et al.*, 2005)

Rural residence in North America and Europe appear to be associated with early onset Parkinson's disease. Vegetable farming, well water drinking, wood pulp, paper and steel industries are some of the factors associated with this early onset of the disease (figure 2.9). In China, living in industrialized urban areas increases the risk of developing Parkinson's disease (Tanner *et al.*, 1999). Helen Petrovitch and colleagues (2002) did a study regarding plantation work in Hawaii and their results supported that exposure to pesticides increases the risk of Parkinson's disease.



**Figure 2.9** Environmental stress leads to oxidative stress and consequently apoptosis (Franco *et al.*, 2009).

### 2.4.3 Treatment options for Parkinson's disease

Parkinson's disease is said to be less common in Africa than anywhere else in the world. About 1 in 300 people in South Africa have Parkinson's disease (The Parkinson's disease and related movement disorders association of South Africa, 2009).

Surgery is available for the treatment of this disease. It ranges from about R70 000 to R80 000 per session and thus its use will be limited because of the great cost of the procedure (Health and Fitness, 2009). Drugs are a much cheaper mode of treatment. Drugs with both anti-muscarinic and anti-nicotinic activity are used for treatment of Parkinson's disease (Cousins *et al.*, 1997; Gao *et al.*, 1998). The following being the classes of the drugs used:

1. Dopaminergic agents e.g. Levodopa

This remains the treatment of choice for Parkinson's disease, but is not effective in drug induced Parkinsonism.

2. Dopamine agonists e.g. Bromocriptine and Pramipexole.

These stimulate dopamine receptors directly. They are formerly reserved as second line therapy.

3. COMT inhibitors e.g. Entacapone

These reduce the metabolism of levodopa. They are indicated in late stage Parkinsonism where they are used together with levodopa to reduce motor fluctuations.

4. MAO-B Inhibitors e.g. Selegeline

They are used as an adjunct to levodopa in the management of Parkinson's disease. They may improve the control of the on-off effect.

5. Anticholinergics e.g. Benztropine

They are less effective than levodopa in Parkinson's disease. However, they are still useful in the mild or early stages of disease and in those unable to tolerate levodopa or who do not benefit from it

#### **2.4.4 Parkinson's Disease in Africa**

It is said that Parkinson's disease is less common in Africa than any other part of the world. There is a shortage of health workers and resources in most of the African countries. The population in Africa is ageing just like the one in Europe. This is due to the strong and economically active being wiped in large numbers by HIV/AIDS or a result of a loss of trained staff to more developed parts of the world where there are better living conditions and better salaries. This makes the elderly in these communities very important. Sadly however, treatments for the neurodegenerative diseases they will suffer are not affordable for them (Pearce & Wilson, 2007). Furthermore, there is a short continuous supply of medication and most are treated with benzhexol with only a few receiving Levodopa (Dotchin *et al.*, 2008).

When one gets sick in some places in Africa, they are believed to be bewitched and instead of getting medical attention early, they go to traditional healers who are more affordable (Pearce & Wilson, 2007). This is because knowledge of neurological diseases and Parkinson's disease in particular is limited. Many patients only seek medical help 2-5 years after the first symptoms of the disease (Dotchin *et al.*, 2008).

#### **2.5 Induction of neurodegeneration**

Several toxins in the past after being ingested, gave symptoms similar to Parkinson's disease. These neurotoxins as cited in literature include 6-hydroxydopamine, paraquat, rotenone and MPTP.

### 2.5.1 6-Hydroxydopamine

6-hydroxydopamine is the chemical isomer of 5-hydroxydopamine (Malmfors & Thoenen, 1971). Ambani and colleagues suggested that 6-hydroxydopamine has an ability to form  $H_2O_2$  by auto-oxidation in the neurons hence its cytotoxic activity (Ambani *et al.*, 1975). In the brains of Parkinson's disease patients, there is a decrease in catalase and peroxidase activity thereby facilitating the accumulation of  $H_2O_2$  (Ambani *et al.*, 1975).  $H_2O_2$  breaks down into  $H_2O$  and  $O_2$ . Gas embolism may be the likely cause of injury (Ashdown *et al.*, 1998) in the brain because of the break down. Another consequence of  $H_2O_2$  toxicity that has been reported is a generalized chemical sympathectomy in anaesthetised dogs (Gauthier *et al.*, 1972).

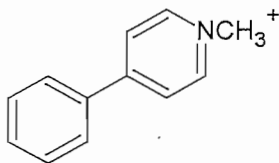
In a study by Palazzo and colleagues (1978), 6-hydroxydopamine caused degeneration in the neuronal terminals, preterminals and processes of monkeys.

### 2.5.2 Paraquat

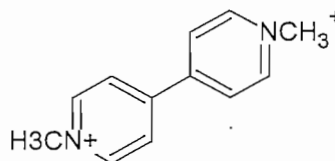
Paraquat is the third most widely used herbicide in the world (Pesticide Action Network, 2003). It is a non-selective herbicide that destroys plant tissue by disrupting photosynthesis. It is mainly used for maize, orchards, soybeans, vegetables and rice. It can be used to kill grasses and weeds in no-till agriculture.

The chemical name for paraquat is 1,1'-dimethyl-4,4'-bipyridinium. It has been a potential risk factor for Parkinson's disease due to its structural similarity to  $MPP^+$ , the active metabolite of MPTP (Javitch *et al.*, 1985).

MPP<sup>+</sup>



Paraquat cation



**Figure 2.10**  $MPP^+$  and Paraquat cation

Paraquat is charged like  $MPP^+$  whereas MPTP is non-charged and lipophilic (Hart, 1987). It was thought that it would not readily cross the blood brain barrier and therefore would not affect the substantia nigra.  $MPP^+$  could however accumulate in specific brain cells through the monoamine transport system. Paraquat is a dicationary compound and is thus not able to use this system; therefore it does not accumulate in the brain cells indicated in the

development of Parkinson's disease (Perry *et al.*, 1986). In 2001 however, Shimizu and colleagues suggested that a possibility for paraquat uptake through the blood-brain-barrier was via the neutral amino acid transporter. McCormack and colleagues (2005) also made the same suggestion. They further showed that levodopa, which is transported across the BBB through the amino acid carrier, protected the neurons against the toxicity of paraquat (McCormack *et al.*, 2003).

Recent tests by Richardson *et al.* (2005) have demonstrated that paraquat requires the dopamine transporter (DAT) to be taken up into the dopaminergic neurons. The results obtained showed that paraquat is neither an inhibitor nor substrate of DAT and will therefore not affect DAT expression. Complex 1 inhibition is not required for its toxicity (Richardson *et al.*, 2005).

Shimizu *et al.* (2003) hypothesized that paraquat must be accumulated in dopaminergic terminals via the DAT to induce dopaminergic toxicity. They treated rat brains with an inhibitor (GBR-12909) which resulted in significantly reduced paraquat uptake into the striatal tissue, indicating that paraquat was taken into the dopaminergic terminals by the DAT. The dose of paraquat used in this experiment was quite high although not fatal. Decreased dopamine levels were also observed in the cortex and the nigrostriatum (Shimizu *et al.*, 2003).

However, the mechanism by which paraquat kills dopamine neurons was still not clear after the experiments done by Richardson and colleagues (2005). Experiments by McCormack *et al.* (2005) showed that there is a two fold increase in the counts of (4-hydroxy-2-nonenal) 4-HNE after a single injection of paraquat in the midbrain section of mice. 4-HNE is a product of the decomposition of polyunsaturated fatty acid peroxides and can be used as a marker of lipid peroxidation and oxidative damage (Beal, 2002; Hashimoto *et al.*, 2003). Paraquat is therefore neurodegenerative.

### **2.5.3 Rotenone**

Rotenone is a botanical derived from roots of certain tropical plants found primarily in Malaysia, East Africa and South America. This pesticide has been registered under the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) since 1947.

Rotenone is an inhibitor of complex 1 of the mitochondrial electron transport chain (ETC) (Sherer *et al.*, 2003). For rotenone to be neurodegenerative, it must cross the blood brain barrier. It is an isoflavonoid derivative that inhibits mitochondrial NADH-oxidase. Tests by Radad *et al.* (2006) on embryonic mouse mesencephala to investigate in detail the potential

molecular mechanisms underlying the degeneration of dopaminergic neurons induced by rotenone indicated that rotenone destroyed tyrosine hydroxylase neurons. Tyrosine hydroxylase immunohistochemistry is used to identify dopaminergic neurons (Shimuzu *et al.*, 2003) in primary mesencephalic culture in a dose and time dependant manner. It enhanced superoxide production in primary mesencephalic cultures, increased ROS formation, induced apoptotic features, decreased the mitochondrial membrane potential and finally, it increased LDH and lactate release into the culture medium.

Results from *in vitro* experiments by Sherer *et al.* (2003) showed that rotenone exposure in rats reproduced many features of Parkinson's disease. Dose - dependant neuroblastoma cell death occurred after a 48 hour period of exposure. It was also found that the toxicity of rotenone is caused by complex 1 inhibition in the mitochondrial ETC and oxidative damage *in vitro*. Complex 1 inhibition enhances the production of reactive oxygen species thus initiating apoptosis (Li *et al.*, 2003). Dose – dependant elevations in oxidative damage in this case indicated by increased protein carbonyl levels in midbrain slices and damaged midbrain dopaminergic neurons were seen (Sherer *et al.*, 2003; Testa *et al.*, 2005). Total cellular glutathione levels were also reduced by the treatment. Observed also was the fact that the toxicity of rotenone does not result solely from the depletion of ATP because rotenone mildly depleted cellular ATP levels. Rotenone-induced death was reduced by pre-treatment with  $\alpha$ -tocopherol in a dose dependant manner (Sherer *et al.*, 2003; Testa *et al.*, 2005).

#### **2.5.4 MPTP**

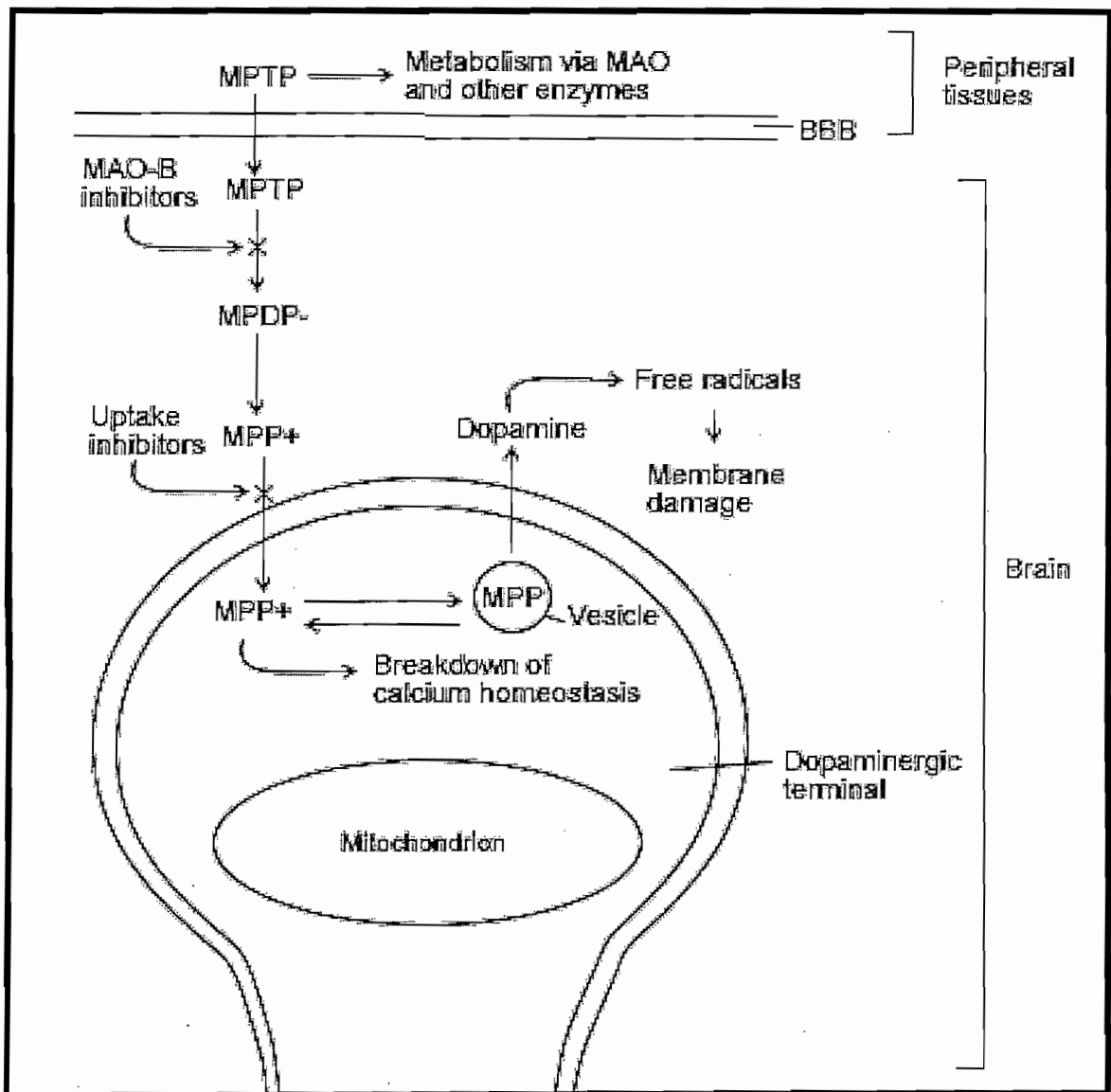
1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP), a meperidine analogue is a false narcotic that was first tested for its possible therapeutic use in 1947. The primates that were tested became rigid and died.

In 1976, a 23-year old pethidine addict took a synthetic shortcut as he manufactured two related byproducts. One of the byproducts was later found to be MPTP. He injected himself with these byproducts and on the third day presented with Parkinson's disease symptoms. He responded well to levodopa with some of the symptoms reversing in no time. An autopsy 18 months later, after he committed suicide revealed destruction of the dopaminergic neurons in the substantia nigra of his brain (Williams, 1984).

When ingested, MPTP produces an irreversible and severe Parkinsonian syndrome characterized by all the symptoms of Parkinson's disease including tremor, rigidity, slowness of movement, postural instability and freezing (Kucheryants *et al.*, 1989). Only the symptoms that are similar to those of Parkinson's disease are seen in the rat but not the disease itself.

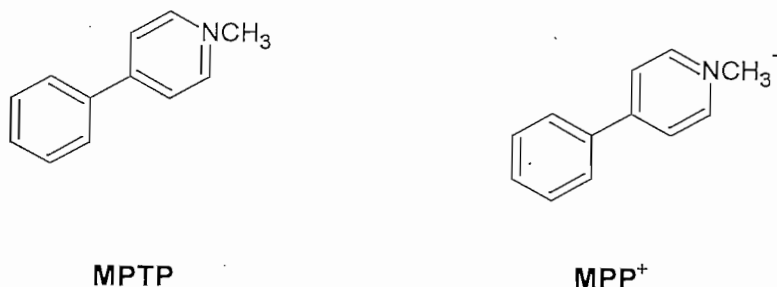
Just as in Parkinson's disease, susceptibility to MPTP increases with age in both monkeys and mice.

Once MPTP has accumulated in the brain, it is metabolized to the lipophilic species, 1-methyl-4-phenyl pyridinium ( $MPP^+$ ) a complex 1 inhibitor that is concentrated in the mitochondria (Parker *et al.*, 1998). The enzyme monoamine oxidase B (MAOB) metabolizes MPTP to  $MPP^+$ , which is the active form of the toxin. Figure 2.11 illustrates what happens to MPTP from the time it crosses the blood brain barrier until it causes damage to the membrane.



**Figure 2.11** The Mechanism of MPTP Neurotoxicity. Adapted from work by Prof. C. Marsden, University of Nottingham Medical School.

A monoamine transport system determines the neurotoxicity of MPP<sup>+</sup> by transporting it to the brain and allowing it to accumulate in specific brain cells (Javitch *et al.*, 1985). The use of monoamine oxidase B inhibitors (e.g. selegiline) can prevent MPTP induced neurotoxicity by inhibiting its conversion to MPP<sup>+</sup>.



**Figure 2.12** Structures of MPTP AND MPP<sup>+</sup>

The inhibition of complex 1 by MPTP can lead to increased oxidative stress particularly through the production of O<sub>2</sub><sup>-•</sup>. A reduction in mitochondrial function and decreased ATP production is also observed (Andersen, 2004).

Kucheryants *et al.* (1989) proved that lipid peroxidation products increase in the striatum during development of the Parkinsonian syndrome induced by injection of MPP<sup>+</sup>. The results obtained indicated that the changes in the concentration of the lipid peroxidation products were in proportion with the severity of the Parkinsonian syndrome in the animals.

Thus, MPTP and the other toxins explained above are toxins that cause neurodegeneration. To slow down the progression of this degeneration in the brain, neuroprotectors may prove to be very useful. Neuroprotectors prevent degeneration by protecting the neurons from apoptosis or any other damage to them. Antioxidants are an example of a mechanism of neuroprotection to the neurons.

## 2.6 Antioxidants

Antioxidants are any substances that, when present at low concentrations compared to those of oxidisable compounds, can delay or prevent the oxidation of that compound (Halliwell, 1996). They protect the body cells from the damaging effects of oxidation by reacting with free radicals and other reactive oxygen species (ROS) thus preventing and repairing the damage caused.

Aerobic cells have their own antioxidant defence mechanisms that counteract the toxicity of too much oxygen.

One major antioxidant system is the chain-breaking antioxidants. These inhibit free-radical mediated chain reactions like lipid peroxidation. Other mechanisms include the removal of O<sub>2</sub>, the scavenging of ROS/RNS species or their precursors, inhibition of ROS formation, binding of metal ions needed for the catalysis of ROS generation and up-regulation of endogenous antioxidant defenses (Gilgun-Sherki *et al.*, 2001).

Antioxidants are classified into two major groups: enzymes and low molecular weight antioxidants (LMWA). A number of proteins are included in the enzymes: superoxide dismutase (SOD), catalase and glutathione peroxidase (GPX), as well as some supporting enzymes (Gilgun-Sherki *et al.*, 2001). Superoxide dismutase provides modest protection against ultraviolet irradiation thus preventing the production of free radicals (Gorman *et al.*, 1997).

The LMWA group is further classified into direct-acting (e.g., scavengers and chain-breaking antioxidants) and indirect acting antioxidants (e.g., chelating agents). The direct-acting ones are very important in combating against oxidative stress (Gilgun-Sherki *et al.*, 2001).

### **2.6.1 Antioxidant compounds in plants**

Some plants that are eaten in South Africa were shown to have antioxidant activity (Fennell *et al.*, 2004).

The secondary compounds from plants have significant biological activity. Secondary compounds in plants are defined as compounds that have no recognisable role in the maintenance of fundamental life processes in the organisms that synthesize them (Bell, 1981). Intermediates and products of primary metabolic pathways such as photosynthetic pigments of green plants are excluded from the definition. The secondary products in plants are not inert and are further metabolized and even degraded. These secondary compounds are found in very small quantities and are chemically more diverse than other compounds like proteins, nucleic acids and carbohydrates that are relatively homogenous (Cannell, 1998).

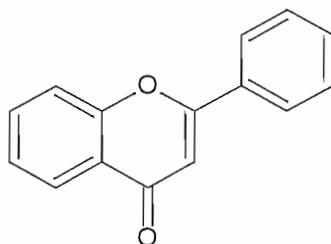
#### **2.6.1.1 Phenols**

Phenolic compounds are widely occurring phytochemicals. Chemically, phenols are compounds containing a cyclic benzene ring and one or more hydroxyl groups. One important aspect about the phenols is their tendency to be oxidized therefore they make

good antioxidants. Phenols are subdivided into two major groups: flavonoids and non-flavonoids. The simple phenols are colourless solids when pure, but become dark when exposed to air due to oxidation. Since phenols are developed as a defence mechanism for plants, the more stressed the plants are, the more phenols the plants will produce.

### Flavonoids

The flavonoids are a class of polyphenolic secondary metabolites formed in plants from aromatic amino acids (phenylalanine and tyrosine) and malonate (Cody *et al.*, 1986). They contribute to the brilliant shades of blue, scarlet, and orange, in leaves, flowers and fruits (Brouillard, 1988). Many of the compounds from this group are water-soluble and those that are only slightly water-soluble are sufficiently polar to be well extracted with ethanol or acetone.



**Figure 2.13** Molecular structure of the flavone backbone (2-phenyl-1,4-benzopyrone)

Phytomedicines containing flavonoids are most commonly known for their antioxidant, anti-inflammatory, antispasmodic and antiviral properties (Rice-Evans & Packer, 1998).

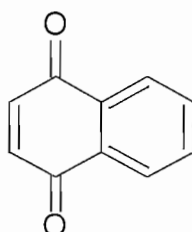
Experimental data support radical scavenging as the main method of the antioxidative function of the flavonoids. They are able to function as metal chelators and inhibit lipid peroxidation (Rice-Evans & Packer, 1998). Classes of flavonoids include flavones, chalcones, aurones, anthocyanins, flavonols, isoflavones, flavanones, flavanonols, catechins and proanthocyanidins. Anthocyanins are antioxidants, which can prevent cancers and possibly other diseases. They give colors to many fruits, vegetables and flowers and are located in vacuoles.

Quercetin is the most active flavone (Foti *et al.*, 1996). It has antioxidant and antihistamine properties. In an experimental model of Parkinson's disease, Dajas and colleagues (2001) administered recognised antioxidants, including quercetin to test their ability to cross the

blood brain barrier (BBB). The results demonstrated that flavonoids and some metabolites were indeed able to cross the BBB. Quercetin is therefore a useful neuroprotective agent.

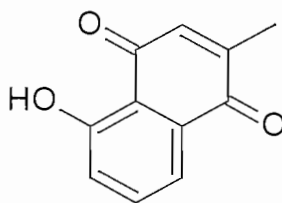
### Naphthoquinones

The biological uses of Plumbaginaceae are due to the presence of several of these naphthoquinones. Naphthoquinone, or more precisely 1,4-naphthoquinone, is an organic compound. The variable capacity of quinones to accept electrons is due to the electron-attracting or electron-donating substituents at the quinone moiety, which modulate the redox properties responsible for the resulting oxidative stress (Dos Santos *et al.*, 2004).



**Figure 2.14** Basic Naphthoquinone structure

*Plumbago* species contain naphthoquinones, of which plumbagin (figure 2.15) is one of the major compounds. Plumbagin is a naturally-occurring yellow pigment. It has antitumor (Devi *et al.*, 1999; Nguyen *et al.*, 2004), bactericidal (Wang & Huang, 2005) and radiomodifying properties (Devi *et al.*, 1999).



**Figure 2.15** Chemical structure of plumbagin

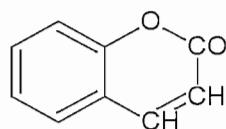
### Tannins

Tannins are traditionally used for converting animal hides to leather ('tanning'). They have the ability to precipitate proteins. There are two types of tannins that occur in plants: the hydrolysable and the condensed tannins. They occur as secondary metabolites in plants. Experiments by Zhang & Lin (2008) showed that condensed tannins from a certain plant consisted mainly of procyanidins and prodelphinidins with 2,3-*cis* stereochemistry. The tannins showed very good radical scavenging activity and ferric reducing power. High

molecular weight tannins have stronger antioxidant activity than low molecular weight tannins (Gu *et al.*, 2008).

### Coumarins

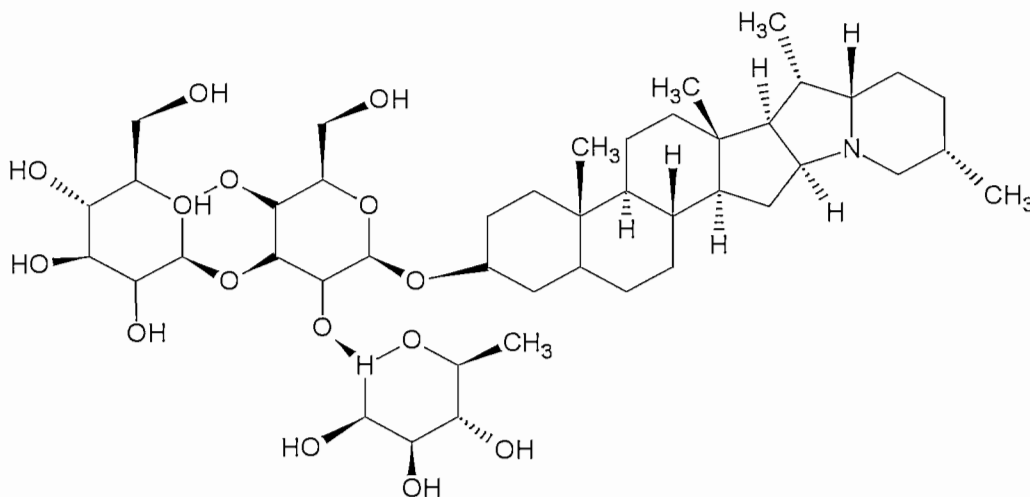
Coumarins represent the phenol type natural antioxidants. They are a combination of a benzene nucleus and a pyrone ring hence their name: benzo- $\alpha$ -pyrones.



**Figure 2.16** *benzo- $\alpha$ -pyrone*

Coumarins are found in plants in the free or combined condition (Sethna & Sha, 1945). Coumarins have low antioxidant activity (Foti *et al.*, 1996).

#### 2.6.1.2 Saponins



**Figure 2.17** *Chemical structure of the saponin  $\alpha$ -solanin*

Saponins are amphipathic glycosides, containing a hydrophobic and a hydrophilic moiety, which make them powerful surface active agents (Robinson, 1983). They have a distinct foaming characteristic. The formation of foam during the extraction of the plant is the evidence of their presence (Haborme, 1984).

Saponins occur in the roots of many plants, notably the genus *Saponaria*, whose name derives from the Latin *sapo*, meaning soap. Glycosides of both triterpenes and steroids have

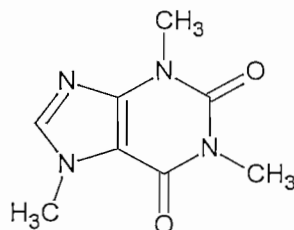
been detected in over 70 families of plants (Manato *et al.*, 1982; Robinson, 1983). They cause haemolysis by destroying the membranes of the erythrocytes (Haborne, 1984).

Plants rich in saponins have been found to have antioxidant activity due to their antiradical properties (Dini *et al.*, 2009) and their ability to inhibit lipid peroxidation (Miaomiao *et al.*, 2008).

### 2.6.1.3 Alkaloids

The alkaloids all contain nitrogen, frequently in a heterocyclic ring (figure 2.18). They are mostly basic and exist in plants as salts. They are the easiest plant secondary metabolites to isolate. These features of the alkaloids distinguish them from other plant components.

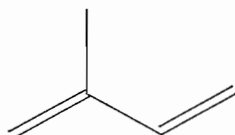
Plant fractions containing alkaloids have the ability to scavenge free radicals in the initiation or propagation phases of a free-radical oxidative chain reaction (Quezada *et al.*, 2004).



**Figure 2.18** Chemical structure of caffeine, a xanthine alkaloid.

### 2.6.1.4 Terpenes

Terpenes are produced by a wide variety of plants. Most terpenes are hydrocarbons. Isoprene units form the basic core of terpenes, thus they are also known as isoprenoids.



**Figure 2.19** Isoprene unit.

Terpenes are classified according to the number of isoprene units in their structure. Table 1.1 summarises the classes of terpenes and their examples.

Table 2.1 Classification of terpenes

Class name	Carbon number	Isoprene units	Example (Molecular formula)
Monoterpene	10	2	Menthol (C <sub>10</sub> H <sub>20</sub> O)
Sesquiterpene	15	3	Bulgarene (C <sub>15</sub> H <sub>24</sub> )
Diterpene	20	4	Cafestol (C <sub>20</sub> H <sub>28</sub> O <sub>3</sub> )
Triterpene	30	6	Campesterol (C <sub>28</sub> H <sub>48</sub> O)
Tetraterpene	40	8	Lycopene (C <sub>40</sub> H <sub>56</sub> )

Lycopene and other carotenoids have antioxidant activity and are located in plastids, either chloroplasts or chromoplasts. Carotenoids are natural pigments synthesized by most plants. Carotenoids are lipophilic in nature (Oshima *et al.*, 1993). they physically quench the oxidative stress caused by <sup>1</sup>O<sub>2</sub> and possibly other free radicals (Cantrell *et al.*, 2003). β-carotene, a metabolic precursor of Vitamin A, is the most studied carotenoid. It has a potent antioxidant activity *in vivo* (Nagakawa *et al.*, 1996). It can be used as a chemopreventative agent against cancer (Naves *et al.*, 1998).

### 2.6.1.5 Vitamins

Vitamin E is an example of antioxidant vitamins. In a study by Shirpoor & colleagues (2008), Vitamin E alleviates oxidative stress via decreasing protein oxidation and lipid peroxidation. A deficiency in Vitamin E, results in an increase in MDA concentration (Mutaku *et al.*, 2002). MDA is a product of lipid peroxidation, thus meaning there is an increase in oxidative stress.

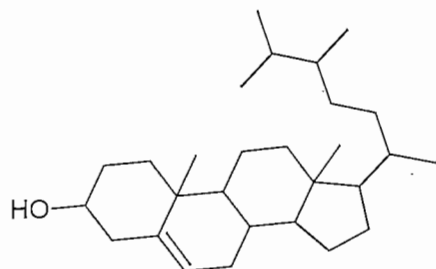
α-tocopherol is one of the E vitamins that possess extensive biological properties (Ingold *et al.*, 1986) and is found mostly in mammalian tissues. Results from a study by Terrasa and colleagues (2009) show that α-tocopherol suppresses the ascorbate-Fe<sup>2+</sup> induced lipid peroxidation processes. It also reduces rotenone-induced cell death in a dose dependant manner (Sherer *et al.*, 2003; Testa *et al.*, 2005) thus it is neuroprotective.

Vitamin C is another strong antioxidant. Its depletion increases superoxide generation in a model of the living brain (Kondo *et al.*, 2008). Vitamin C can however lead to lipid

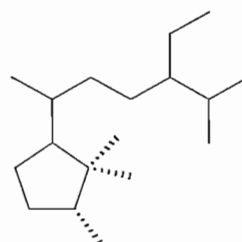
peroxidation when it reduces  $\text{FeCl}_3$  to  $\text{Fe}^{2+}$  and  $\text{Cl}_3$ ,  $\text{Fe}^{2+}$  which then reacts in the Fenton reaction ( $\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^\bullet + \text{OH}^-$ ) giving the highly reactive hydroxyl radical.

### 2.6.1.6 Sterols

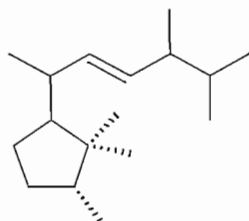
Sterols are white solids that are hydroxylated steroids. They are found in most plants and food. Plant sterols are known to have a hypocholesterolemic function and are considered safe (Deng, 2009). They are triterpenes resembling cholesterol, with a side chain at carbon 17, composed of 9 or 10 carbon atoms whereas the cholesterol side chain only has 8 carbons. The most abundant phytosterols reported from the plant species are sitosterol, stigmasterol and campesterol (figure 2.20).



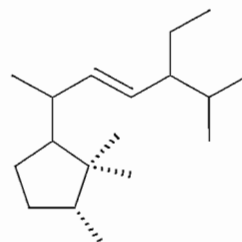
campesterol



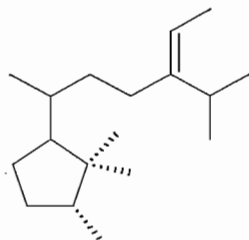
sitosterol



brassicasterol



stigmasterol



avenasterol

**Figure 2.20** The most common plant sterols (Christie, 2009).

Results from research by Yasukazū & Etsuo (2003) showed that the three phytosterols  $\beta$ -sitosterol, stigmasterol, and campesterol taken together, chemically act as an antioxidant, and a modest radical scavenger. Free phytosterols also lower plasma and liver cholesterol, and are effective at blocking cholesterol absorption (Hayes *et al.*, 2002).

## 2.7 Plants of the genus *Plumbago*

Plants of the genus *Plumbago* have been used traditionally for various types of ailments. Studies on the different *Plumbago* species have been done to test for their different medicinal properties.

Use in the past has shown that the *Plumbago* species is cytotoxic, antibacterial, anti-inflammatory, and antifungal and can be used in the removal of warts and the treatment of fractures (Watt & Breyer-Brandwijk, 1962). *Plumbago scandens* was shown to have activity on tremorine-induced tremor suggesting some anticholinergic activity in the plant (Morais *et al.*, 2004). The *Plumbago* species are shown to contain antioxidants (Tilak *et al.*, 2004). This property of the plant makes it suitable for slowing down the progression of neurodegenerative diseases like Parkinson's, Alzheimer's and Huntington's disease. This is because oxidative stress plays a role in the pathogenesis of these diseases. Examples of antioxidants in this plant are naphthoquinones, flavonoids and saponins.

Most of the biological uses of these species have been attributed to the presence of naphthoquinones. The major naphthoquinones in the *Plumbago* species is plumbagin (5-hydroxyl-2-methyl-1, 4-naphthoquinone). Plumbagin was previously isolated from the roots of *P. zeylanica* (Lin *et al.*, 2003; Nguyen *et al.*, 2004), the roots of *P. scandens* (De Paiva *et al.*, 2004), and the roots of *P. rosea* (Devi *et al.*, 1999; Kapadia *et al.*, 2005).

Experiments with animals show that a tincture containing plumbagin is spasmodic (Martindale, 1993). Studies by Devi & colleagues (1999) showed that plumbagin has antitumor and radiomodifying properties. Sankar & colleagues (1987) demonstrated that plumbagin is capable of inhibiting lipid peroxidation levels *in vitro*. This is because of the powerful antioxidant capacity of plumbagin.

In Northeastern Brazil, goats that ingested fresh parts of *P. scandens* died in approximately 3 weeks. Tests were then done on four goats to see if *P. scandens* was indeed responsible for the toxicity. The goats that ingested this plant presented with depression, anorexia, bruxism and foamy salivation (Medeiros *et al.*, 2001).

In this study, the plant *Plumbago auriculata* was tested for its antioxidant properties and an unknown compound was isolated, purified and tested for its antioxidant properties and toxicity to HeLa cells.

### 2.7.1 *Plumbago auriculata* Lam.

The scientific classification Of *P. auriculata* is as follows (Wikipedia, 2009):

Kingdom: Plantae

Division: Magnoliophyta

Class: Magnoliopsida

Order: Caryophyllales

Family: Plumbaginaceae

Genus: *Plumbago*

Species: *Plumbago auriculata* Lam.



**Figure 2.21** *P. auriculata* flower

Common names: *Plumbago capensis*, *Cape plumbago*, *Cape leadwort*, *blue Plumbago*.



**Figure 2.22** *P. auriculata* bush

*Plumbago auriculata* is a small scandent shrub which grows up to 2 meters tall, with leaves up to 5 cm long. It is indigenous to South Africa and is a lesser-known species in ethnopharmacognosy. A recent study showed that a hydroalcoholic extract of the roots of *Plumbago auriculata* had anti-inflammatory activity in rat models of carrageenan-induced inflammation (Dorni *et al.*, 2006).

The naphthoquinones plumbagin and *epi-isoshinanolone*, the steroids sitosterol and 3-O-glucosylsitosterol, plumbagic and palmitic acids have been isolated from *P. auriculata* (De Paiva *et al.*, 2005).

Not much research into the antioxidant activity of this plant has been done.

### Plant selection, screening and extraction

#### 3.1 Introduction

Most of the medicines used today are plant derivatives. Traditional medicines are affordable in developing countries. As compared to pure active constituents, galenical products can be grown easily in almost any country and a tincture is manufactured at minimum costs compared to isolating pure compounds (Farnsworth *et al.*, 1985). Farnsworth and colleagues (1985), analysed 119 prescription drugs, identified their plant sources and their uses in therapy. Of the 119 plants, 74% were discovered because of their use as traditional medicine.

The use of traditional medicines however has its shortfalls. Each plant has many compounds, of which each compound has different pharmacological properties, some of which may be toxic. A lot of experience is needed in selecting the plants and using them for the right ailment. Despite the affordability of traditional medicines, it is safer to use pure active components that have been tested for their pharmacological properties.

It is important to select the plant for research carefully, because inappropriate selection can result in wasting of time and resources (Souza-Brito, 1996). Examining the uses of traditional preparations can help in selecting a suitable plant for the development of a new drug (Farnsworth *et al.*, 1985; Rates, 2000). Studying the environment where the plant grows can give important information about its possible biological activity. An example is the finding that plants growing in conditions of decreased water or fertility have increased antioxidant activity (McCune & Jones, 2007). The same plant picked in different seasons, can have varying activity as the composition of compounds differs from season to season.

After a number of suitable plants are selected, activity guided fractionation with biological assays helps to identify the most suitable plants and then the most active fractions in that plant until active compounds are isolated. This method of fractionation also helps to identify synergistic effects of compounds in the plant (Eloff, 2004).

#### 3.2 Plant selection

After an extensive literature search, twenty-one plants were selected due to their antioxidant activity reported. The leaves of the plants were collected in such a way that the plant would continue growing and the supply of the leaves would be sustainable and the population was not reduced. The leaves of these plants were then assayed for their total antioxidant

properties. The following species were selected:

1. *Acacia karoo*
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. *Solenostemo latifolia*
18. *Solenostemon rotundifolius*
19. *Tarchonathus camphorates*
20. *Vagueria infausta*
21. *Vernonia Oligocephala*

Soxhlet extraction was employed to extract substances from the leaves of the twenty-one plants. Four solvents: PE, DCM, EA and EtOH were used in order of increasing polarity. Based on the results from the Oxygen radical absorbance capacity (ORAC) and the Ferric reducing

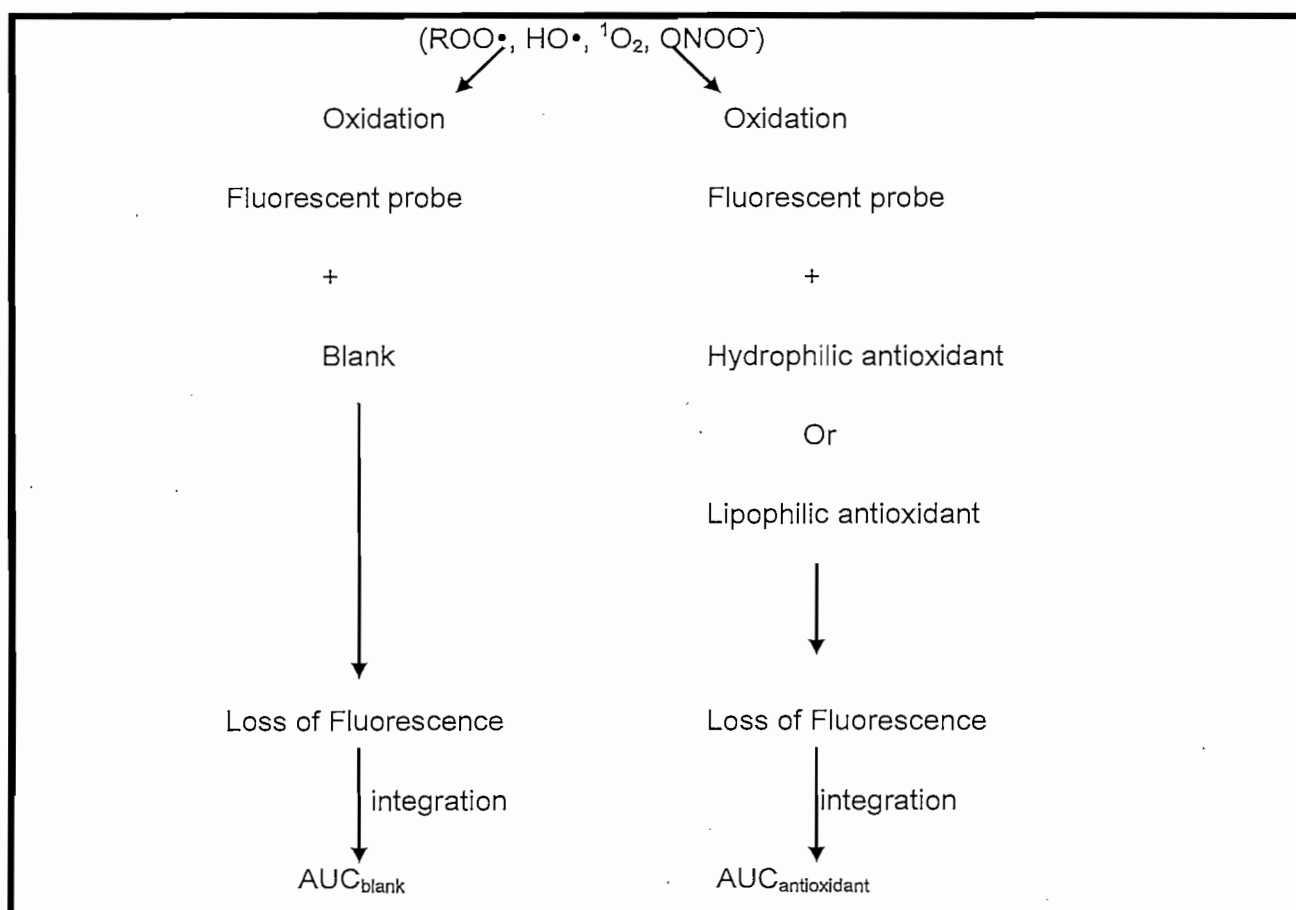
antioxidant (FRAP) assays obtained from my fellow co-workers, *P. auriculata* was selected for further research.

### 3.3 ORAC Assay

#### 3.3.1 Background

The ORAC assay measures antioxidant inhibition of peroxy radical-induced oxidations. Its mechanism depends on the free radical damage to a fluorescent probe through the change in its fluorescent intensity. This change in the fluorescent intensity then shows the degree of free radical damage (Huang *et al.*, 2002). Figure 3.1 shows the principle behind the ORAC assay.

#### ROS/RNS



$$\text{Antioxidant Capacity} = \text{AUC}_{\text{antioxidant}} - \text{AUC}_{\text{blank}}$$

**Figure 3.1** Schematic illustration of the principle of the ORAC assay (Huang *et al.*, 2002). The antioxidant activity of the tested sample is expressed as the net area under the curve (AUC).

In a test used to compare the antioxidant capacities of different antioxidants in human serum, the ORAC assay is shown to have high specificity. This is because it measures the capacity of

an antioxidant to directly quench free radicals (Cao & Prior, 1998). Both inhibition time and the degree of inhibition are combined into a single quantity in the ORAC assay (Cao & Prior, 1999).

The original ORAC assay was developed using a hydrophilic environment (Cao *et al.*, 1993, 1995; Ou *et al.*, 2001). Modifications were made for the ORAC assay to measure the antioxidant capacity of lipophilic antioxidants such as tocopherols, by addition of methylated  $\beta$ -cyclodextrin to enhance solubility of the lipid-soluble antioxidants.

### 3.3.2 Results

As seen, the ethanol extract of *P. auriculata* has the fourth highest antioxidant capacity (Table 3.1; Figure 3.2). The ethanol extract from most of the plants showed the best antioxidant activity as seen in the ORAC values when compared to the other three extracts (PE, DCM and EA).

**Table 3.1** ORAC values for all extracts of the 21 plants that were selected.

	PE	DCM	EA	EtOH
<i>Acacia karroo</i>	4732.4	3837.9	4753.9	23382.7
<i>Berula erecta</i>	4371.2	6804.4	8404.8	20768.6
<i>Clematis brachiata</i>	7814.5	12276.4	27462.3	19368.8
<i>Elephantorrhiza elephantine</i>	11388.7	9923.4	10413.5	11111.1
<i>Erythrina zeyheri</i>	5729.4	26486.6	11144.7	67362.9
<i>Gymnosporia buxifolia</i>	11045.2	21091.8	26974.7	64318.8
<i>Heteromorpha arborescens</i>	4745.8	6433.8	13246.7	11698.7
<i>Leonotis leonurus</i>	4480.4	9504.5	13742.8	13750.6
<i>Lippia javanica</i>	14189.2	49147.8	75908.1	NUM
<i>Physalis peruviana</i>	3048.3	2208.6	13271.2	14423.5
<i>Plectranthus ecklonii</i>	5003.9	7079.8	9818.1	11863.1
<i>Plectranthus rehmanii</i>	15534.1	7302	8293.7	15288.5
<i>Plectranthus ventricillatus</i>	8268.1	13539.5	13068.3	8438.7
<i>Plumbago auriculata</i>	3185.3	6801.9	5406.8	35586.7
<i>Salvia auritia</i>	-442.3	8834.7	1757.6	5902.1
<i>Salvia rincinata</i>	31944.5	14914.9	12415.5	28229.6
<i>Solenostemon latifolia</i>	5352.4	9853.7	7014.9	10141.4
<i>Solenostemon rotundifolius</i>	-1491.8	-444.8	4305.5	14542.5
<i>Tarchonanthus camphorates</i>	6285.4	25822.6	18347.8	32077
<i>Vageria infausta</i>	6614.9	10469.2	11581.2	13629.4
<i>Vernonia Oligocephala</i>	6513.6	19838.9	24994	19601.1

Figure 3.2 represents the extracts from twenty-one plants with the highest ORAC values as obtained from the research of co-workers (unpublished data).

The green star represents the *P. auriculata* ORAC values. The highest value represents the extract (i.e. PE, DCM, EA or EtOH) with the best antioxidant capacity.

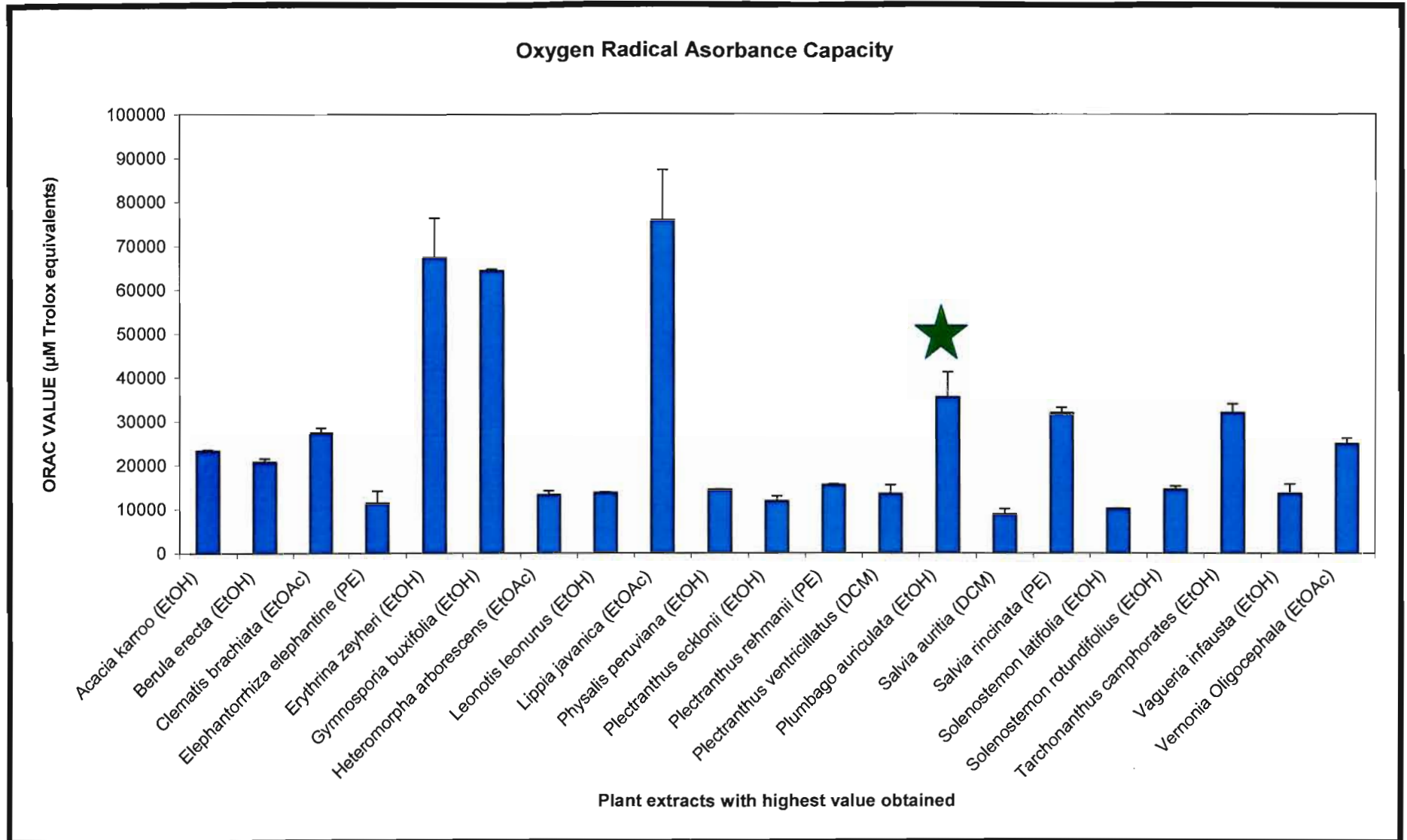


Figure 3.2 ORAC values of the twenty-one plants that were screened. Each bar represents the extract with the highest ORAC value from each plant.

### 3.4 FRAP Assay

#### 3.4.1 Background

The FRAP assay measures the ferric reducing antioxidant power of a sample. The ability to reduce ferric (III) iron to ferrous (II) iron is used as a basis to determine the antioxidant activity (Benzie & Strain, 1996). The principle of this assay is based on the reducing potential of the antioxidants to react with a ferric tripyridyltriazine (FeIII-TPTZ) complex and produce a colored ferrous tripyridyltriazine (FeII-TPTZ) form which can be read at 593 nm on a spectrophotometer. To get the FRAP values; these readings are compared to those containing ferrous ions in known concentrations (Benzie & Strain, 1996).

This assay is totally different from the ORAC assay because there are no free radicals or oxidants applied in the sample (Cao & Prior, 1998). The FRAP assay gives fast, reproducible results that are straightforward and is a relatively inexpensive method (Benzie & Strain, 1996; Schlesier *et al.*, 2002).

#### 3.4.2 Results

*P. auriculata* has the seventh best FRAP value (Table 3.2; Figure 3.3). The starred bar represents the FRAP values of *P. auriculata*.

**Table 3.2** FRAP values for the 21 plants that were selected

	PE	DCM	EA	EtOH
<i>Acacia karroo</i>	0	0	21.0878	4421.69
<i>Berula erecta</i>	39.0351	81.9089	131.762	1454.99
<i>Clematis brachiata</i>	138.297	139.686	1955.92	1324.32
<i>Elephantorrhiza elephantine</i>	30.1001	27.3258	62.4674	331.114
<i>Erythrina zeyheri</i>	7.36174	490.817	71.4402	1057.37
<i>Gymnosporia buxifolia</i>	163.419	434.979	435.977	3310.74
<i>Heteromorpha arborescens</i>	31.8739	48.9404	719.694	618.849
<i>Leonotis leonurus</i>	321.483	212.878	712.974	893.464
<i>Lippia javanica</i>	238.552	698.434	6692.87	9009.32
<i>Physalis peruviana</i>	121.791	112.815	744.463	1060.14
<i>Plectranthus ecklonii</i>	97.6089	171.591	44.01	916.962
<i>Plectranthus rehmanii</i>	2954.72	175.644	274.941	3235.99
<i>Plectranthus ventricillatus</i>	128.064	222.192	1043.97	1066.7
<i>Plumbago auriculata</i>	28.6617	86.1759	43.7684	1982.16
<i>Salvia auritia</i>	133.215	1522.69	189.163	92.0596
<i>Salvia rincinata</i>	618.64	0	652.236	2387.2
<i>Solenostemon latifolia</i>	3.21199	678.322	277.528	800.891
<i>Solenostemon rotundifolius</i>	131.687	1091.53	110.998	1496.74
<i>Tarhonanthus camphorates</i>	111.479	1283.63	861.936	4384.31
<i>Vagueria infausta</i>	70.7901	82.3502	298.288	583.579
<i>Vernonia Oligocephala</i>	64.3171	378.277	1404.78	1523.15

Figure 3.3 represents the FRAP values from the twenty-one plants. The bar for each plant is the extract (PE, DCM, EA, EtOH) with best FRAP values as obtained from the results of co-workers (unpublished data). The green star represents the ethanol extract of *P. auriculata*.

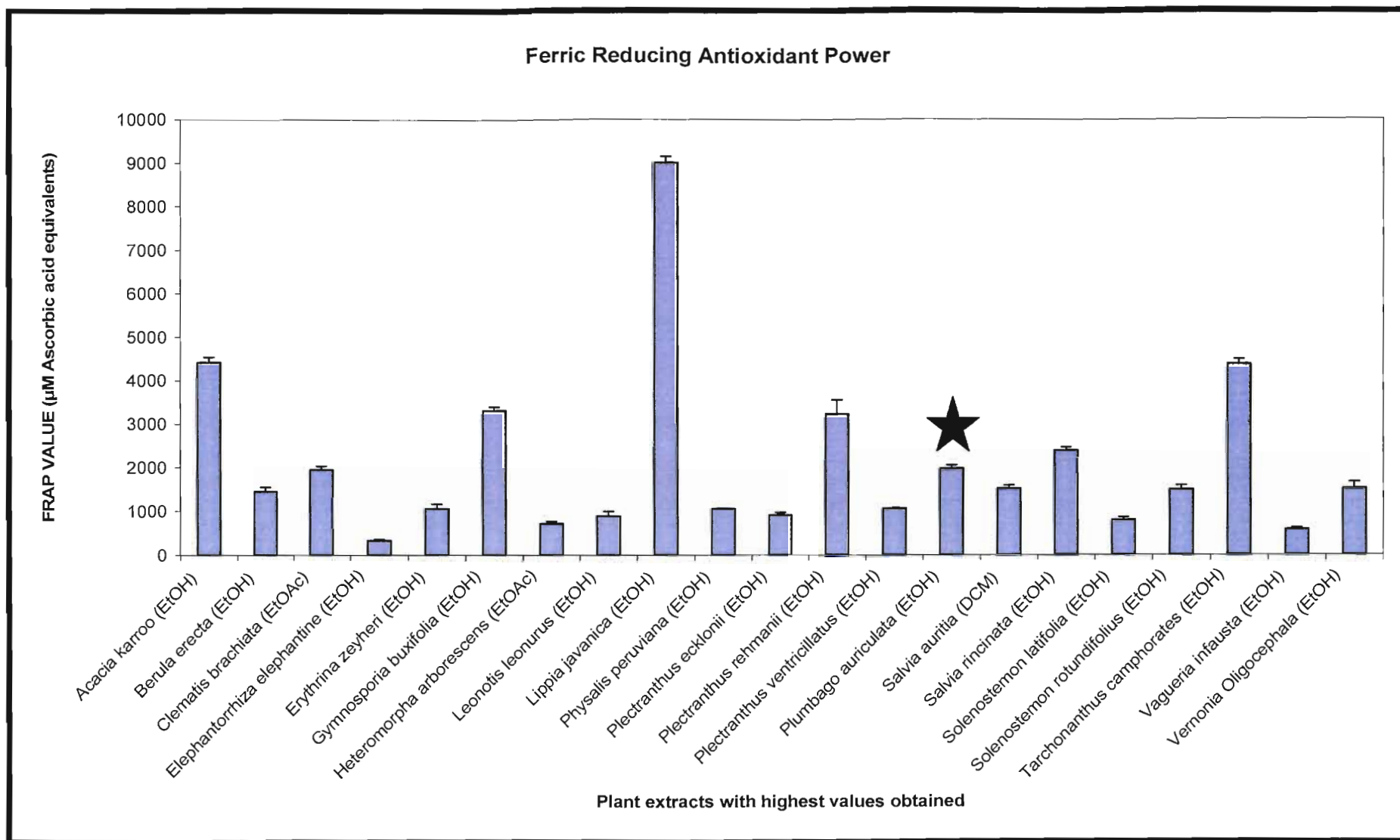


Figure 3.3 FRAP values of the twenty-one plants that were screened. Each bar represents the extract with the highest FRAP value from each plant

*Acacia karroo*, *Lippia javanica*, *Gymnosporia buxifolia*, *Tarchonanthus camphorates* also had good antioxidant values as seen in both the ORAC and FRAP assays. *Lippia javanica*, *Gymnosporia buxifolia* and *Tarchonanthus camphorates* were studied by co-workers in the same research group.

Taking the above into consideration, *P. auriculata* was selected for further investigation.

### 3.5 Collection, storage and extraction of *P. auriculata* Lam.

The leaves of *P. auriculata* were collected from the North-West University (Potchefstroom) botanical garden between January and March, 2008. Plants were identified with the help of Mr. Martin Smit, curator of the botanical gardens. Voucher specimens were prepared and are kept at the A.P. Goossens Herbarium (PUC), North-West University, Potchefstroom. Accession number: PUC 9764.

The leaves were selected and left to dry in the laboratory for a week. They were then ground to a fine powder using an ordinary kitchen blender. About 6 kg of the powder was extracted using the soxhlet method. Soxhlet extraction was chosen because in the past, when different techniques were compared by De Paiva & colleagues (2004), it was found to be the most efficient with the highest yield of extract in a short time.

The efficiency of soxhlet extraction is a result of the use of a small volume of solvent which is renewed in contact with the plant material thus ensuring more interaction between them. This study also confirmed that the solvent should be changed frequently (approximately every 24 hours), in order to maintain a better yield, as long exposure to high temperatures leads to the degradation of the extract (De Paiva *et al.*, 2004).

Four solvents: petroleum ether, dichloromethane and ethyl acetate were used in order of increasing polarity. The crude extract was left to dry and stored in a fume hood.

***In vitro* antioxidant and toxicity assays**

**4.1 Introduction**

Several methods are used to measure the total antioxidant capacity (TAC) in samples. After finding out what the TAC of each different plant is (Chapter 3), it is easier to screen them according to their activity and select the most active plant for further research. The method used to measure TAC depends on the properties of the plant. Components in plants are generally divided into two fractions, lipophilic and hydrophilic. Most popular *in vitro* antioxidant measurement methods are designed primarily for hydrophilic components and may not be suitable for lipophilic measurements (Wu *et al.*, 2004). It may thus be beneficial to separate the lipophilic components from hydrophilic components to obtain a good measure of total antioxidant capacity (Cano *et al.*, 2000).

Table 4.1 gives a summary of some of the methods used to measure the total antioxidant capacity of plants.

**Table 4.1** *Methods used to measure total antioxidant capacity in vitro (summary from article by Schlesier *et al.*, 2001)*

<u>Assay</u>	<u>Principle</u>	
Total Radical-trapping antioxidant Parameter Assay (TRAP)	<ul style="list-style-type: none"> <li>• Uses organic radical producers</li> <li>• Determines the delay of radical generation as well as the ability to scavenge the radical.</li> </ul>	
Trolox equivalent Antioxidant Capacity Assay (TEAC I - III)	I	<ul style="list-style-type: none"> <li>• Determines the delay of radical generation as well as the ability to scavenge the radical.</li> <li>• Uses organic radical producers</li> </ul>
	II	<ul style="list-style-type: none"> <li>• Uses organic radical producer</li> </ul>

		radical cation
	III	<ul style="list-style-type: none"> <li>• Uses organic radical producers</li> <li>• Analyzes the ability of the radical cation</li> </ul>
N,N-dimethyl- <i>p</i> -phenyldiamine Assay (DMPD)		<ul style="list-style-type: none"> <li>• Uses organic radical producers</li> <li>• Analyzes the ability of the radical cation</li> </ul>
Ferric Reducing Ability of Plasma Assay (FRAP)		<ul style="list-style-type: none"> <li>• Uses metal ions for oxidation</li> <li>• Analyzes the ability of the ferric ion</li> </ul>
Oxygen Radical Absorbance Capacity Assay (ORAC)		<ul style="list-style-type: none"> <li>• Depends on the free radical damage to a fluorescent probe.</li> </ul>
Photochemiluminescence assay (PCL)		<ul style="list-style-type: none"> <li>• Uses organic radical producers</li> <li>• Determines the delay of radical generation as well as the ability to scavenge the radical.</li> </ul>

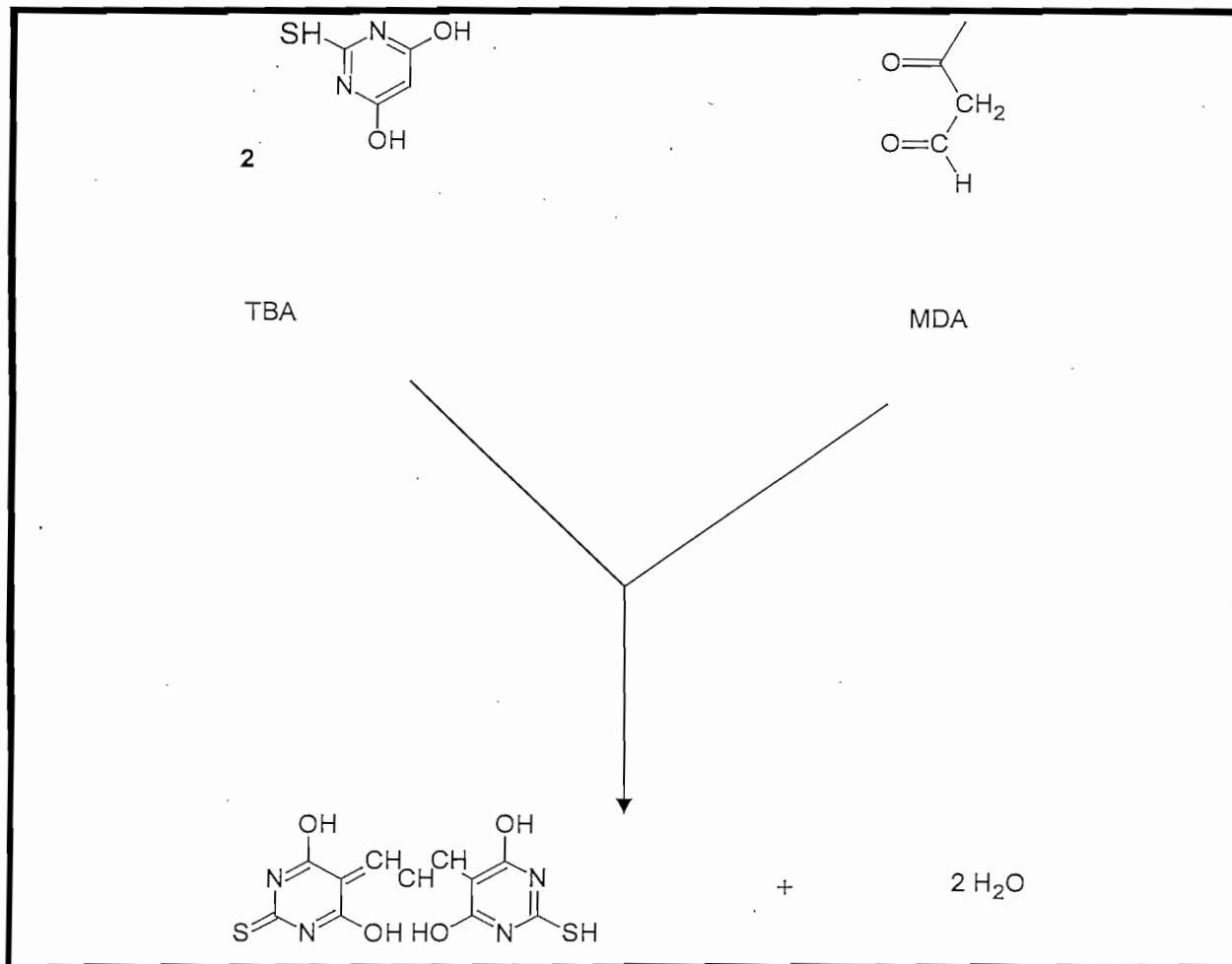
Bioassay-guided fractionation was used to further separate fractions of each crude extract of *P. auriculata*. The Thiobarbituric Acid-Reactive Substances (TBARS) and the Nitro-blue Tetrazolium (NBT) assays were used to assay for antioxidant activity. The MTT assay was used to evaluate the toxicity of each crude extract on HeLa cells.

## 4.2 Thiobarbituric Acid-Reactive Substances (TBARS) Assay

### 4.2.1 Background

Lipid peroxidation is one of the consequences of oxidative stress. The Thiobarbituric Acid-Reactive Substances (TBARS) assay is the most commonly used method to assess lipid peroxidation. This assay measures the ability of an extract to scavenge the hydroxyl free radical ( $\text{HO}^{\bullet}$ ). The consequence of peroxidation by this free radical is the production of malondialdehyde (MDA) and other reactive aldehydes (Esterbauer *et al.*, 1991; Luo *et al.*, 1995). The detection of MDA shows the extent of lipid peroxidation in the brain. In this assay, malondialdehyde (MDA) and the malondialdehyde equivalents react with thiobarbituric acid (TBA) to form a pink coloured TBA-MDA complex (Ottino & Duncan, 1997). The chemical reaction of the TBA-MDA adduct is shown in Figure 4.1.

This method however is not specific for MDA only. MDA is formed in some tissues by enzymatic processes with prostaglandin precursors as substrates and the bulk of the TBARS is not MDA (Liu *et al.*, 1997). The acid heating step also results in the formation of derivatives that also react with TBA. Other aldehydes that are not results of the peroxidation of lipids by free radicals are also measured. However, this method was still used as a simple test to show the attenuation of any lipid peroxidation products regardless of the cause of their formation.



**Figure 4.1** The chemical reaction between TBA and MDA to yield the pink TBA-MDA adduct as discussed in the passage above (Williamson et al., 2003).

The TBARS assay was used due to its simplicity and affordability. It was used to assess lipid peroxidation using the method of Ottino & Duncan (1997). Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), in combination with ascorbic acid (Vit. C) and FeCl<sub>3</sub> were used to induce lipid peroxidation in the rat brain. Vit. C, the reducing agent leads to a cycle, which increases the damage to biological molecules. Vit. C reacts with FeCl<sub>3</sub> to give Fe<sup>2+</sup> (ferrous) and Cl<sub>3</sub>. Fe<sup>2+</sup> then reacts in the Fenton reaction (Fe<sup>2+</sup> + H<sub>2</sub>O<sub>2</sub> → Fe<sup>3+</sup> + OH• + OH<sup>-</sup>) giving the highly reactive hydroxyl radical. Fe<sup>3+</sup> reacts slowly with H<sub>2</sub>O<sub>2</sub> thus the reducing agent stimulates the Fenton reaction in the following reaction:



Butylated hydroxytoluene (BHT), a powerful chain-breaking antioxidant was added to the experiment before adding TBA to stop any further peroxidation of lipids in the brain.

Trichloro-acetic acid (TCA) was added to precipitate macromolecules such as proteins, DNA and RNA.

#### 4.2.2 Reagents and Chemicals

All the chemicals used were of the highest available purity and were purchased from Merck, Darmstadt, Germany unless otherwise stated. Vit. C was purchased from Saarchem (PTY) Ltd., Krugersdorp, South Africa. 2-Thiobarbituric Acid (98 %) (TBA), butylated hydroxytoluene (BHT), and 1,1,3,3-tetramethoxypropane (TEP), trichloroacetic acid (TCA) were purchased from Sigma Chemical Co., St Louis, MO, USA. Hydrogen peroxide (5 mM H<sub>2</sub>O<sub>2</sub>) was purchased at a local pharmacy.

Dimethyl sulfoxide (DMSO) and iron (III) chloride (Fe<sub>3</sub>Cl) were purchased from Merck-Chemicals (Saarchem, South Africa).

Phosphate buffer solution (PBS) at pH 7.4 consisted of 137 mM NaCl, 2.7 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub> and 2 mM KH<sub>2</sub>PO<sub>4</sub> in 1 L Mill-Q water.

Trolox was used as a positive control throughout the experiments.

#### 4.2.3 Extract preparation

The dry crude extracts used were each dissolved in 10 % DMSO. The concentrations used for each extract were 0.625 mg/ml, 1.25 mg/ml and 2.5 mg/ml in 10 % DMSO (Merck).

#### 4.2.4 Animal tissue preparation

Adult male Sprague-Dawley rats weighing between 200 - 250 g were used after the North-West University Ethics Committee approved the experimental protocol. The rats were sacrificed by decapitation. The whole brain was then homogenized in 0.1M PBS, pH 7.4 giving a final concentration of 10 % w/v.

#### 4.2.5 Method

Rat brain homogenate was added to each of the test tubes. To each test tube the control, the toxin (H<sub>2</sub>O<sub>2</sub>) and different concentrations of each extract were added and then vortexed. The test tubes were incubated for 1 hour at 37° C in an oscillating water bath. They were then centrifuged for 20 min at 2000 x g to remove insoluble protein (supernatant). Following centrifugation, the supernatant was removed and put in new test tubes. 0.5 ml BHT, 1 ml 10% TCA and 0.5 ml TBA were added to the test tubes and then vortexed. This was incubated for 1 hour at 60° C in a water bath and later cooled on ice. Butanol (2 ml) was

added to each test tube to extract the pink colored TBA-MDA complex and subsequently vortexed. This was then centrifuged for 10 min at 2000 x g. The top layer was removed and put in a cuvette. Absorbance readings were taken at 532 nm with butanol as the blank. The absorbance values obtained were converted to MDA levels (nmole MDA) from the calibration curve generated with TEP (table 4.2; figure 4.2).

#### 4.2.6 Statistical analysis

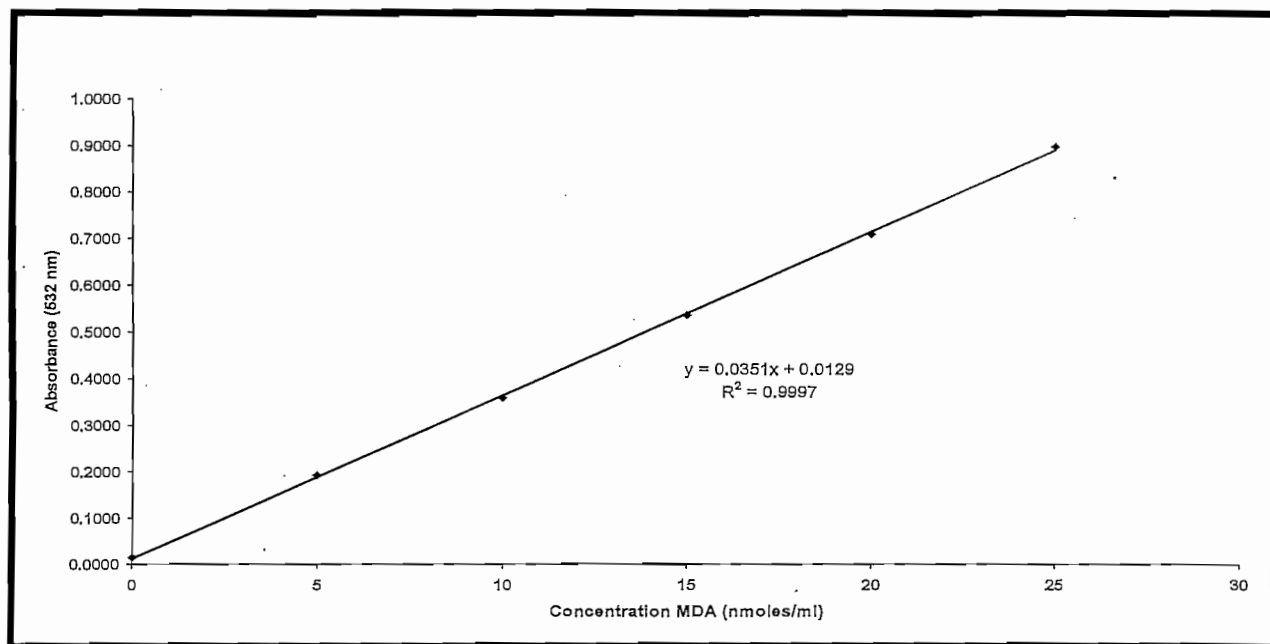
The Graphpad Instat program was used for statistical analysis. A one-way analysis of variance (ANOVA) method followed by the Student-Newman-Keuls Multiple range test was used to analyze the results obtained. The level of significance was accepted at  $p < 0.05$ . The data represented for these experiments is the mean  $\pm$  S.E.M of five determinations ( $n = 5$ ).

#### 4.2.7 Standard curve

A calibration curve was drawn to show the absorbance of MDA before running the assay. 1,1,3,3-Tetramethoxypropane (TEP) was used as a standard. This was achieved by preparing five different concentrations (between 5 and 25 nmol/L) of TEP in PBS. This curve was set as a standard for the results obtained in the assay.

**Table 4.2** Standard curve values for TBARS assay.

Concentration (nmol/L)	ABS 1	ABS 2	ABS 3	ABS 4	ABS 5	ABS 6	MEAN	STDEV
0	0.0000	0.0040	0.0150	0.0620	0.0050	0.0040	0.0150	0.0236
5	0.2020	0.1820	0.1870	0.2050	0.1850	0.1970	0.1930	0.0096
10	0.3580	0.3440	0.3320	0.3760	0.3570	0.3860	0.3588	0.0199
15	0.5350	0.5240	0.4750	0.5220	0.5500	0.6100	0.5360	0.0441
20	0.8340	0.6630	0.6000	0.7640	0.6590	0.7420	0.7103	0.0851
25	1.1080	0.9020	0.8240	0.8460	0.8150	0.8970	0.8987	0.1088



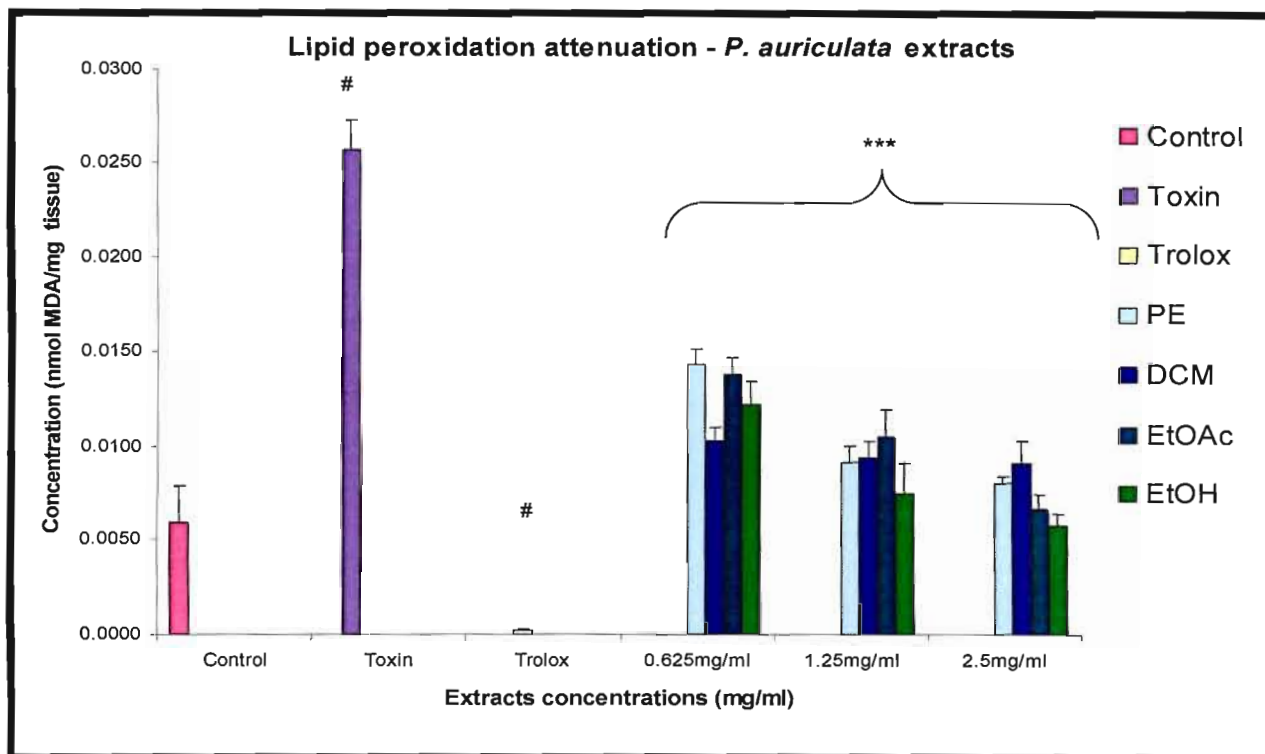
**Figure 4.2** Calibration curve of MDA generated from TEP.

#### 4.2.8 Results

The *in vitro* exposure of brain homogenate to the varying concentrations of *P. auriculata* caused a significant decrease in MDA as compared to the toxin (table 4.3; figure 4.3).

**Table 4.3** Inhibition of lipid peroxidation by *P. auriculata* extracts

Extract	Concentration nmol MDA/mg tissue n = 5	± S.E.M
Control	0.006	0.0003
Toxin 5 mM H <sub>2</sub> O <sub>2</sub> , 4.44 mM Vit. C 1.68 mM Fe <sub>3</sub> Cl	0.027	0.0005
Trolox	0.0002	0.00004
PE 0.625 mg/ml	0.014	0.0003
1.25 mg/ml	0.009	0.0004
2.5 mg/ml	0.008	0.0001
DCM 0.625 mg/ml	0.010	0.0004
1.25 mg/ml	0.009	0.0004
2.5 mg/ml	0.009	0.0006
EA 0.625 mg/ml	0.014	0.0004
1.25 mg/ml	0.011	0.0007
2.5 mg/ml	0.007	0.0003
EtOH 0.625 mg/ml	0.012	0.0006
1.25 mg/ml	0.008	0.0007
2.5 mg/ml	0.006	0.0003



**Figure 4.3** Lipid peroxidation graph obtained after exposure of rat brains to the four crude extracts (PE, DCM, EA and EtOH) at concentrations of 0.625 mg/ml, 1.25 mg/ml and 2.5 mg/ml for each extract. Each bar represents the mean  $\pm$  S.E.M,  $n=5$ ; \*\*\*  $p < 0.001$  vs. toxin(#).

#### 4.2.9 Discussion

Since an antioxidant compound is to be isolated from the four crude extracts (petroleum ether, dichloromethane, ethyl acetate and ethanol) of *P. auriculata*, it was important to find out which of these four extracts had the best antioxidant capacity.

The TBARS measured the ability of each extract to scavenge  $\text{HO}^\bullet$ . Figure 4.3 shows that all the plant extracts had antioxidant activity. The ethanol and ethyl acetate extracts had the best lipid peroxidation attenuation when compared to the toxin. It was therefore concluded that the crude ethyl acetate extract and the ethanol extract had the most promising antioxidant activity and they were therefore selected for isolation of the active compounds.

### 4.3 Nitroblue tetrazolium (NBT) assay

#### 4.3.1 Background

Oxygen free radicals are implicated in a number of diseases including Parkinson's disease. Superoxide anion is one of the reactive oxygen species that contribute to the neurodegeneration in the brain. The NBT assay is used to assay  $O_2^{\bullet-}$  and possibly other free radicals.  $O_2^{\bullet-}$  reduces the membrane permeable, water-soluble, yellow-coloured, nitro blue tetrazolium to blue or black diformazan crystals. The cells containing blue NBT formazan deposits are counted. The rat brain, on addition of the crude extract generates less or no superoxide anions and thus less nitro blue diformazan is detected in the cells. The less diformazan containing cells counted, the less  $O_2^{\bullet-}$  present and therefore the greater the antioxidant capacity of the extract. This method however has the possibility of biased results dependent on the counting of the cells containing blue NBT formazan deposits by the observer. The method of Ottino *et al.* (1997) was used for this assay.

KCN is a neurotoxin that results in mitochondrial dysfunction and stimulates intracellular generation of reactive oxygen species, which initiate apoptosis (Jones *et al.*, 2003). This toxin was used to induce the formation of  $O_2^{\bullet-}$  in the rat brain.

#### 4.3.2 Reagents and Chemicals

Bovine serum albumin (BSA), Trolox (Vit. E), nitro-blue tetrazolium (NBT), and nitro-blue diformazan (NBD) were purchased from Sigma Chemical Co., St Louis, MO, USA. All other chemicals were purchased from Merck, Darmstadt, Germany and were of highest chemical purity.

Copper reagent solution (Biret reagent) consisted of 2 g of 2 % disodium carbonate in 100 ml 0.1M NaOH. To this, 1 ml  $CuSO_4$ , 1 ml sodium tartrate and 98 ml disodium carbonate were added and the solution was mixed.

1 % NBT solution was prepared by dissolving NBT in ethanol and then diluting to the required volume with Milli-Q water. Fresh solutions were prepared daily and were protected from light by covering with foil. The final concentration of NBT in each test tube was less than 0.5 %.

Potassium cyanide (KCN) dissolved in water was added as stock solution for KCN. KCN was tested at the following concentrations; 0.25, 0.5, and 1 mM, to determine whether KCN induced  $O_2^{\bullet-}$  would be generated.

#### 4.3.3 Extract preparation

The crude extracts were prepared according to the method specified in 4.2.3. The concentrations used for each extract were 0.625 mg/ml, 1.25 mg/ml and 2.5 mg/ml.

#### 4.3.4 Animal tissue preparation

Adult male Sprague-Dawley rats weighing between 200 - 250 g were used after the North-West University Ethics Committee approved the experimental protocol. The animals were prepared in the manner described in 4.2.4.

#### 4.3.5 Method

The rats were decapitated; the brain removed, put in 10 % w/v PBS and left on ice. Test tubes each with a total volume of 1 ml of the homogenated brain were prepared. Each extract was prepared separately and the control and toxin were also included. Each test tube was then vortexed and 0.4 ml NBT (0.05 g NBT + 1 ml EtOH + 49 ml distilled water to make 50 ml) was added and this was closed with foil as NBT is light sensitive. This was vortexed once again. It was then incubated in an oscillating water bath for 1 hr. after which it was centrifuged at 3000 x g for 10 min. The supernatant was thrown away. To the 'pellet' left in test tubes, 2 ml glacial acetic acid (GAA) was added and then the contents were vortexed. The test tube contents were centrifuged at 4000 g for 5 min. Absorbance readings were taken at 560 nm with GAA as the blank.

#### Protein assay

Protein content of each brain was estimated prior to the NBT assay.

0.1 ml of the brain was homogenated in 4.9 ml PBS. This was then vortexed and 1ml of homogenate was added to a new set of test tubes in duplicate. 6 ml of Biret reagent was added to each test tube and then vortexed. This was left standing for 10 min after which 10 ml of Folin--Ciocalteau's phenol reagent was added and then vortexed. The tubes were left to stand in the dark for 30 minutes after which absorbance readings were taken at 500 nm with PBS as the blank. Each brain's protein reading was measured in duplicate.

The absorbance values obtained from the the protein assay were converted to mg protein, using the calibration curve of BSA shown in figure 4.4. These values were used in expressing the superoxide anion scavenging results.

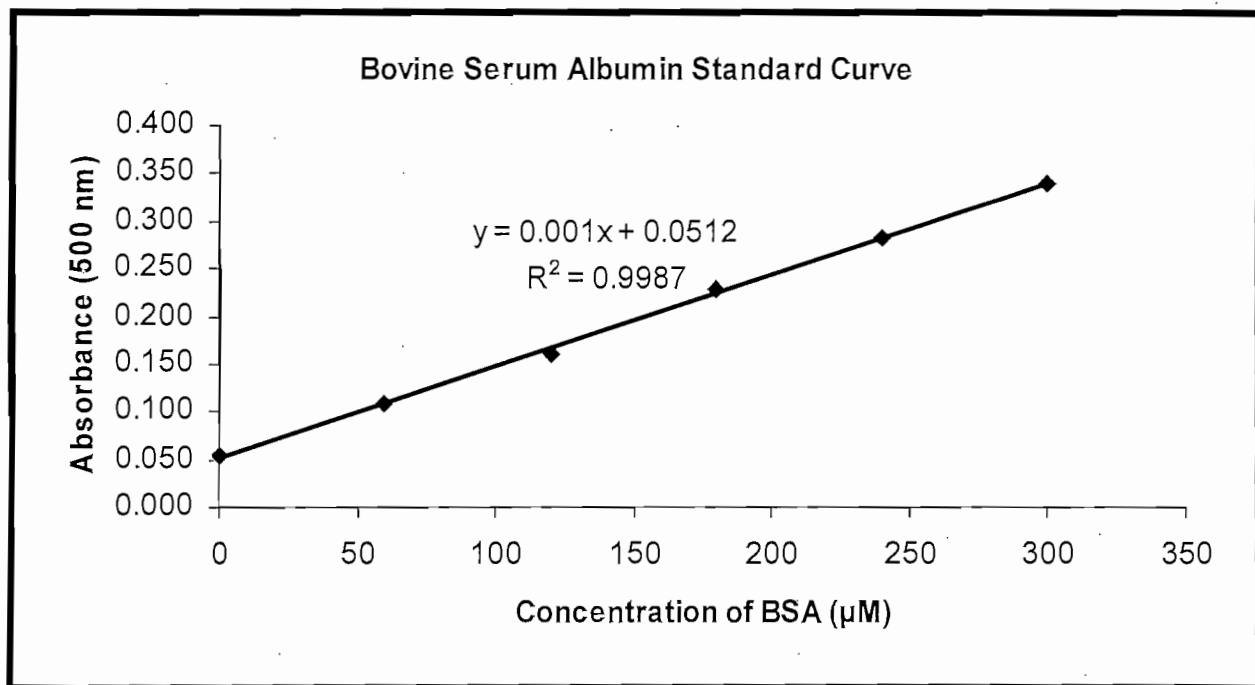
The standard curve generated from increasing concentrations of NBD (figure 4.5) was used to convert absorbance values of the NBT assay to  $\mu$ moles diformazan produced. The results were expressed as  $\mu$ moles diformazan/mg protein.

#### 4.3.6 Statistical Analysis

The results were analyzed using the methods specified in 4.1.5.

#### 4.3.7 NBT Assay Standard curves

To generate the protein standard curve, increasing concentration of bovine serum albumin (BSA) were used as standard to determine the protein content in the brain.



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

To measure the level of scavenged  $O_2^{\cdot-}$  in the assay, NBD was used as a standard. NBD dissolved in acetic acid was put in a series of aliquots. The standard curve was generated by measuring the absorbance at 560 nm in 100 µmole/ml increments in the range of 0 - 400 µM (figure 4.5)

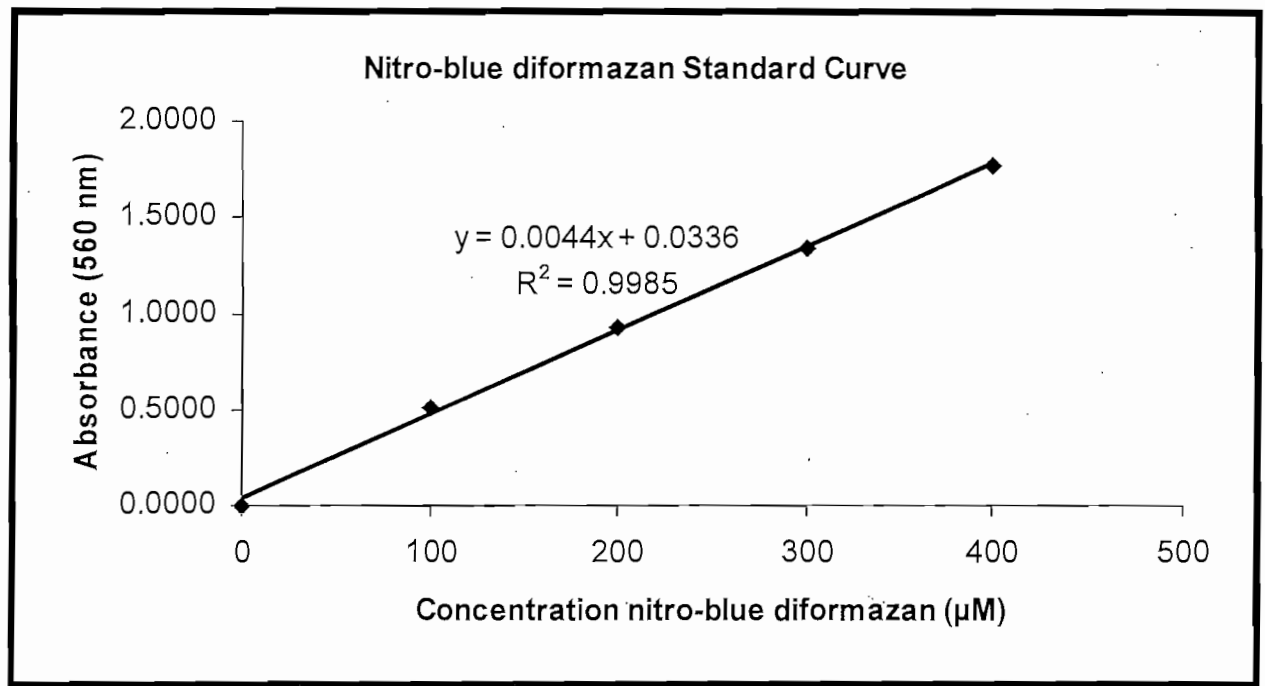
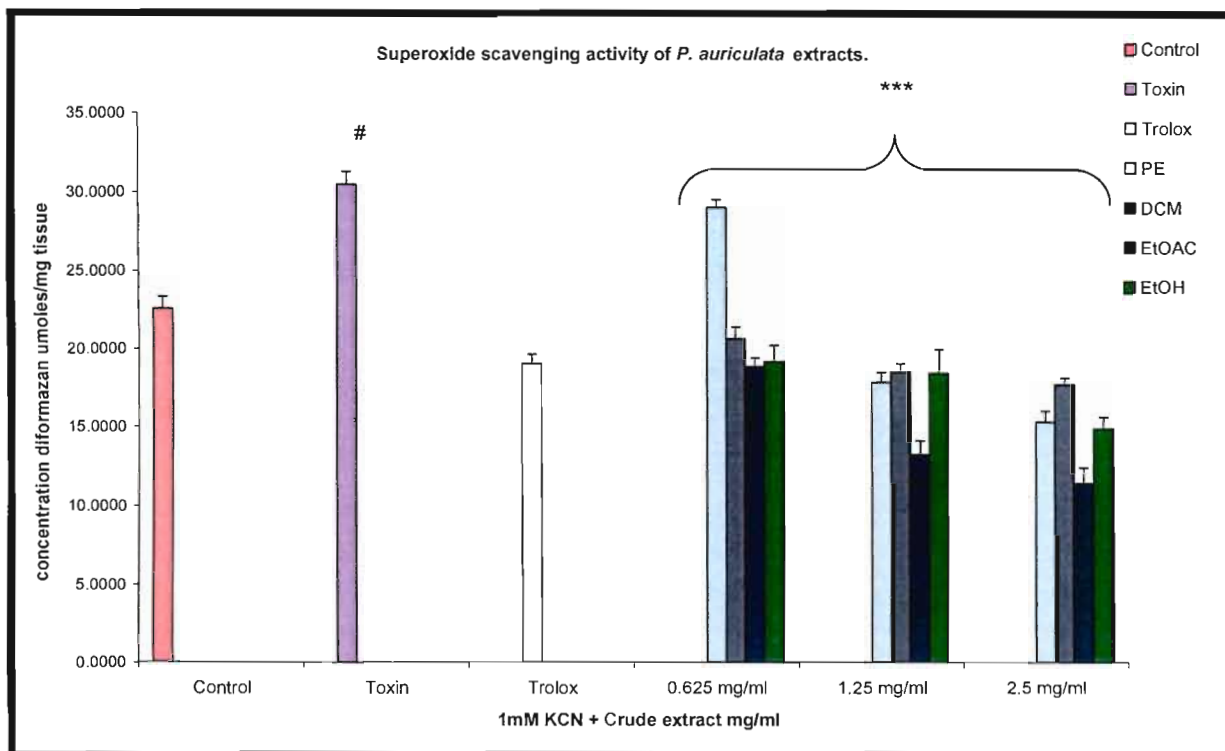


Figure 4.5 NBT standard curve.

## 4.3.8 Results

Table 4.4 NBT results

Extract	Concentration diformazan $\mu\text{mol/mg protein}$  n = 5	$\pm$ S.E.M
Control	22.554	0.765
Toxin 1 mM KCN	30.501	0.781
Trolox	19.051	0.585
PE 0.625 mg/ml	29.019	0.516
1.25 mg/ml	17.843	0.636
2.5 mg/ml	15.317	0.706
DCM 0.625 mg/ml	20.648	0.730
1.25 mg/ml	18.515	0.547
2.5 mg/ml	17.738	0.380
EA 0.625 mg/ml	18.868	0.536
1.25 mg/ml	13.240	0.876
2.5 mg/ml	11.443	0.973
EtOH 0.625 mg/ml	19.173	1.039
1.25 mg/ml	18.4213	1.522
2.5 mg/ml	14.895	0.730



**Figure 4.6** Graph obtained after exposure of rat brains to the four crude extracts of *P. auriculata* (PE, DCM, EA and EtOH) at concentrations of 0.625 mg/ml, 1.25 mg/ml, and 2.5 mg/ml. Each bar represents the mean  $\pm$  SD, n=5; \*\*\* p < 0.001 vs. toxin (#).

#### 4.3.9 Discussion

In this assay, the ethyl acetate extract showed (table 4.4, figure 4.6) the most promising antioxidant activity. Ethanol did not have as much activity as the ethyl acetate extract. The ethyl acetate extract reduced the quantity of  $O_2^{\bullet-}$  produced in the rat brain. The concentration of diformazan of the ethyl acetate extract at 2.5 mg/ml was 11.443 compared to that of the toxin which was 30.501. This could be a result of the reduction of the amount of  $O_2^{\bullet-}$  by the ethyl acetate extract indicating that it had high antioxidant activity. A reduction is also seen for the other two concentrations of the ethyl acetate extract (table 4.4).

### 4.4 MTT Assay

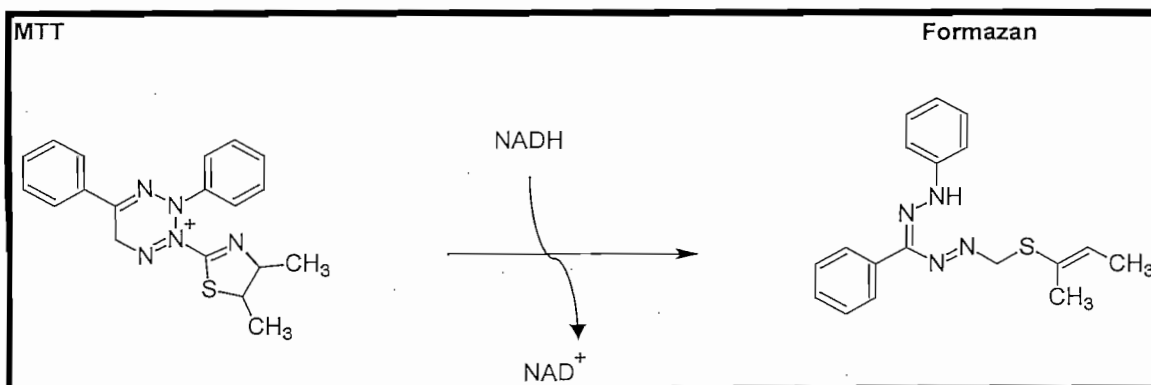
#### 4.4.1 Background

In Northeastern Brazil, goats that ingested parts of the plant *Plumbago scandens* presented with depression, anorexia, bruxism and foamy salivation and died in approximately 3 weeks. Tests were then done with parts of *P. scandens*, on four goats, which proved that *Plumbago*

*scandens* was indeed responsible for the toxicity (Medeiros *et al.*, 2001). Taylor *et al.* (2003) also did tests to screen South African plants for genotoxic effects. Dichloromethane extracts of the foliage of *P. auriculata* were found to have bacterial toxicity rather than mutagenicity. Based on past experimental work on the toxicity of plants of the *Plumbago* genus and especially the toxicity of the foliage of *P. auriculata*, the need to test for the toxicity of this plant was seen as the active compound found could probably be used in *in vivo* experiments.

The toxicity of each crude extract was determined using the MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] assay. The MTT assay was first described in 1983 by Mosmann. This assay measures the metabolic activity of viable cells.

MTT was observed to have the ability to readily cross the plasma membranes of intact cells. Thus its reduction is catalyzed by both plasma membrane reductases and intracellular reducing enzyme and species (Bernas & Dobrucki, 2000). The mitochondria have dehydrogenase enzymes, which have the ability to cleave the tetrazolium rings of the pale yellow MTT and form dark blue formazan crystals that are largely impermeable to cell membranes, thus resulting in their accumulation within healthy cells. The number of surviving cells is directly proportional to the level of formazan product created. The surviving cells can then be quantified using a simple colorimetric assay.



**Figure 4.7** Reduction of MTT to formazan.

#### 4.4.2 Materials and Reagents

All the chemicals used were of highest available purity. DMEM (Dulbecco's Modified Eagle's Medium), trypsin, foetal bovine serum (FBS), corning flasks (150 cm<sup>3</sup>), 24 well plates and 96 well plates were purchased from the Scientific Group (Midrand, South Africa). MTT and isopropanol (2-propanol) were purchased from Sigma Chemical Co (St Louis, MO, USA). Phosphate buffer solution (PBS), consisted of 8 g NaCl, 0.2 g KCl, 1.44 g Na<sub>2</sub>PO<sub>4</sub> and 0.24 g KH<sub>2</sub>PO<sub>4</sub> added to 800 ml distilled water. The pH of this solution was adjusted to 7.4 using

HCl and NaOH. After the pH was satisfactory, the solution was made up to 1 L with more distilled water. Crude plant extracts (PE, DCM, EA and EtOH) were dried in a fume hood and dissolved to the desired concentrations.

#### **4.4.3 Cell culture preparation**

Human epithelial (HeLa) cells were obtained from the Scientific Group (Midrand, South Africa). The HeLa cells were cultured in DMEM supplemented with 10 % FBS, 100 IU/ml penicillin, 0.1 mg/ml streptomycin and 0.25 µg/ml fungizone. The cell cultures were incubated at 37 °C in a humidified atmosphere of 10 % carbon dioxide. The growth medium was changed twice a week so as to maintain the highest levels of sterility and to avoid infecting the cells. Cells were examined daily. As soon as the flask was confluent, the cells were trypsinised and split into two corning flasks and left once again to multiply. Two corning flasks were used for each experiment. Each experiment was done in triplicate.

#### **4.4.4 Extract preparation**

The four dry crude extracts were each dissolved in 1 % Dimethyl Sulfoxide (Merck) in distilled water. They were then filter sterilised before they were used. Concentrations made for each extract were 0.08 mg/ml, 0.4 mg/ml, 2 mg/ml, and 10 mg/ml.

#### **4.4.5 Assay protocol**

On the first day, cells were harvested from the corning flask using 3 ml of trypsin. The cells were then cultured at 0.75 million cells per well in 24-well plates after which they were incubated at 37°C in 10 % CO<sub>2</sub> for 24 hours. Thus after 24 hours the cell density was 1,5 x 10<sup>6</sup> cells/ml. The cell culture was aspirated before pre-treatment. 400 µL of DMEM media and 100 µL of the extract were added to each well. A cell-free media was included for each experiment as the blank and an extract-free media control was also included. The blank served as an indicator of contamination with 0 % growth, while the control served as 100 % cellular growth with no contamination. On the third day, a stock solution (5 mg/ml) of MTT was prepared and stored in the dark until it was required for use. DMEM was then aspirated from each well and 200 µL MTT was added to each well. This was again left to incubate for 2 hours to terminate the cell growth after which the MTT was aspirated from each well. 250 µL isopropanol was added to each well and left for 5 minutes to dissolve the formazan crystals completely. 100 µL of the contents of each well was transferred to a 96-well plate and the absorbance was measured at 560 nm and 650 nm using a multi-well reader (Labsystems multiskan RC reader). The results were expressed as a percentage cellular viability of the controls using equation 4.1.

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

$$\Delta \text{ Control} - \Delta \text{ Blank}$$

#### Equation 4.1

Where:  $\Delta \text{ Control (mean cell control)} = \text{Cell control}_{560} - \text{Cell control}_{650}$

$$\Delta \text{ Blank} = \text{Mean blank}_{560} - \text{Mean blank}_{650}$$

$$\Delta \text{ Absorbance} = \text{Absorbance}_{560} - \text{Absorbance}_{650}$$

#### 4.4.6 Statistical analysis

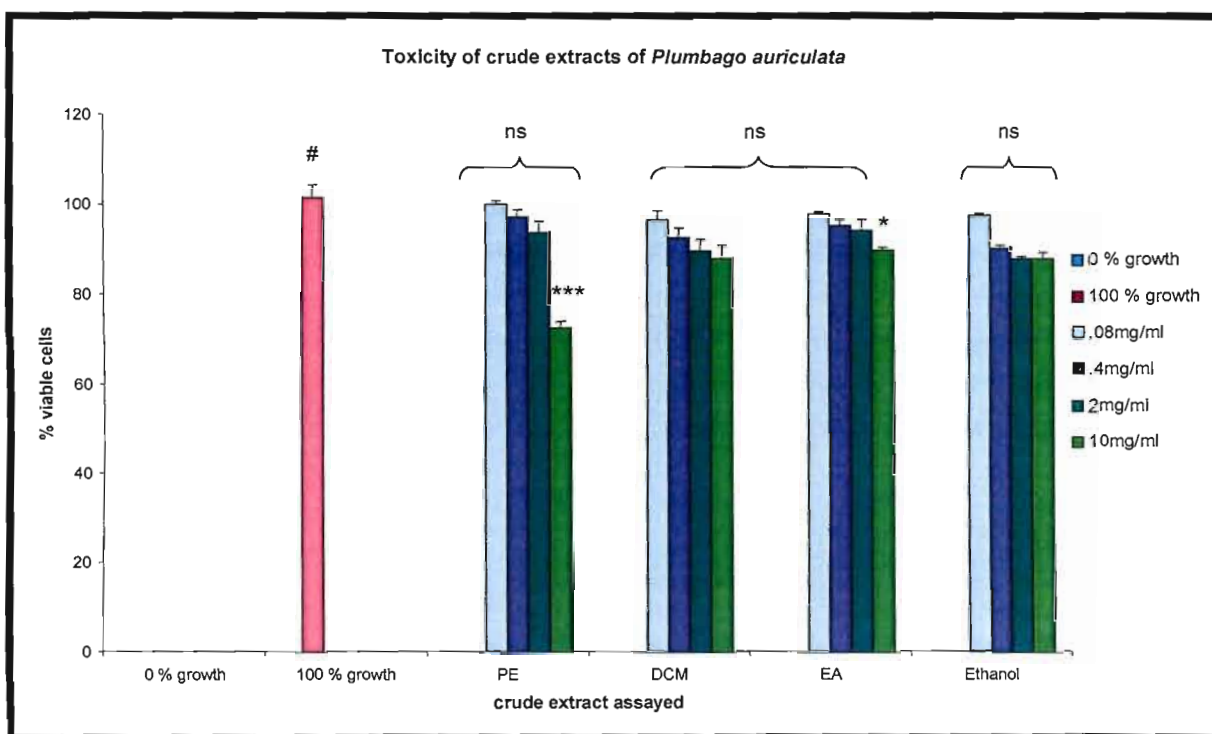
Each data point is an average of triplicate measurements, with each individual experiment performed in triplicate. Statistical analysis was done using one way analysis of variance (ANOVA) method, followed where appropriate, by the Student-Newman-Keuls Multiple range test with a significance of  $p < 0.05$  where appropriate. The different extracts at the different concentrations were each compared to the control. The results given below were all in comparison to the control.

#### 4.4.7 Results

The different extracts at the different concentrations were each compared to the control which had 100 % cell growth. The results were read on a multiwell scanning spectrophotometer. The results (Table 4.5 & Figure 4.8) are all in comparison to the control.

**Table 4.5** Percent viable HeLa cells after exposure to extracts from leaves of *P. auriculata* in the MTT assay.

Extract	Concentration (mg/ ml)	Percent viable cells n = 9	± S.E.M
PE	0.08	99.97	0.7796
	0.4	97.28	1.496
	2	93.77	2.443
	10	72.69	1.358
DCM	0.08	96.47	2.039
	0.4	92.68	2.034
	2	89.77	2.506
	10	88.08	2.838
EA	0.08	97.76	0.544
	0.4	95.43	1.431
	2	94.35	2.249
	10	89.95	0.6779
EtOH	0.08	97.54	0.4187
	0.4	90.46	0.767
	2	88.13	0.5746
	10	88.15	1.387



**Figure 4.8** Graphs obtained after 24-hour exposure of HeLa cells in DMEM to 0.08 mg/ml, 0.4 mg/ml, 2 mg/ml and 10 mg/ml concentrations of each of the four crude extracts PE, DCM, EA and EtOH of *P. auriculata*. Each bar represents the mean  $\pm$  S.E.M,  $n=9$ ; \*\*\*  $p < 0.001$  vs. control (100 % growth (#)).

#### 4.4.8 Discussion

The control used did not have any of the extract but just medium and the cells. Most growth (100 %) was therefore seen in the control compared to all the extracts. The blank did not have any cells at all but plant extract and the medium.

The ethyl acetate and petroleum ether, each at 10 mg/ml, significantly inhibited the proliferation of HeLa cells by 11.52 % ( $p < 0.05$ ) and 27.3 % ( $p < 0.001$ ) respectively (table 4.5, figure 4.8). The rest of the extracts at each of the concentrations used had no significant difference from the control. The possibility of false positive results was considered because in the past Rollino *et al.* (1995), proved that it was possible to get positive results due to interferences of different substances on the MTT.

#### 4.5 Conclusion

Results from both the TBARS and NBT assays showed that the ethyl acetate and ethanol extract had the best antioxidant activity. Results from the MTT assay showed that the ethyl acetate and petroleum ether, each at 10 mg/ml, significantly inhibited the proliferation of HeLa cells.

The ethyl acetate extract was chosen for further evaluation that would lead to the isolation of pure antioxidant compounds. This is because it showed more promising antioxidant properties than the ethanol extract in both the TBARS and the NBT assays. Although the ethanol extract did not show any toxicity towards the HeLa cells, its attenuation of lipid peroxidation was not as good as that of the ethyl acetate extract, thus the decision to investigate the ethyl acetate extract further. The ethanol extract were not evaluated due to time limitations. After isolating the compounds from this extract, the MTT assay was again done on these compounds to determine their toxicity.

### Isolation and characterization of compounds from *P. auriculata* leaves

#### 5.1 Background

From the results in the previous chapters, the ethyl acetate extract of *Plumbago auriculata* was selected for further investigation. Bioassay-guided fractionation and isolation methods were employed to isolate pure antioxidant compounds.

#### 5.2 Analytical techniques

Silica gel aluminium backed TLC sheets (Merck®, TLC silica gel 60 F<sub>254</sub>) were used for the selection of the best mobile phase to separate compounds. The plates were viewed under UV light. They were also sprayed with 5 % H<sub>2</sub>SO<sub>4</sub> in ethanol to detect organic compounds.

Columns of different sizes were used to perform column chromatography. Silica gel (Macherey-Nagel®, Germany; 0.063-0.2 mm) in the mobile phase of choice was used as the stationary phase. The dry extracts were dissolved in the mobile phase, filtered and then applied to the packed column using a pasteur pipette.

Merck® TLC silica gel 60 F<sub>254</sub>, 2 mm preparative TLC plates was used for further purification. To remove any contaminants from the silica gel the preparative TLC plates were developed in ethanol and dried in an oven at 90 °C before use. The plates were then developed in Chloroform, ethyl acetate; 3:1 as the mobile phase. The plate was then visualized under UV light and the band of choice marked and scraped out with a spatula. Chloroform was used to wash out the compound from the silica. The solution was then filtered and concentrated using a vacuum evaporator.

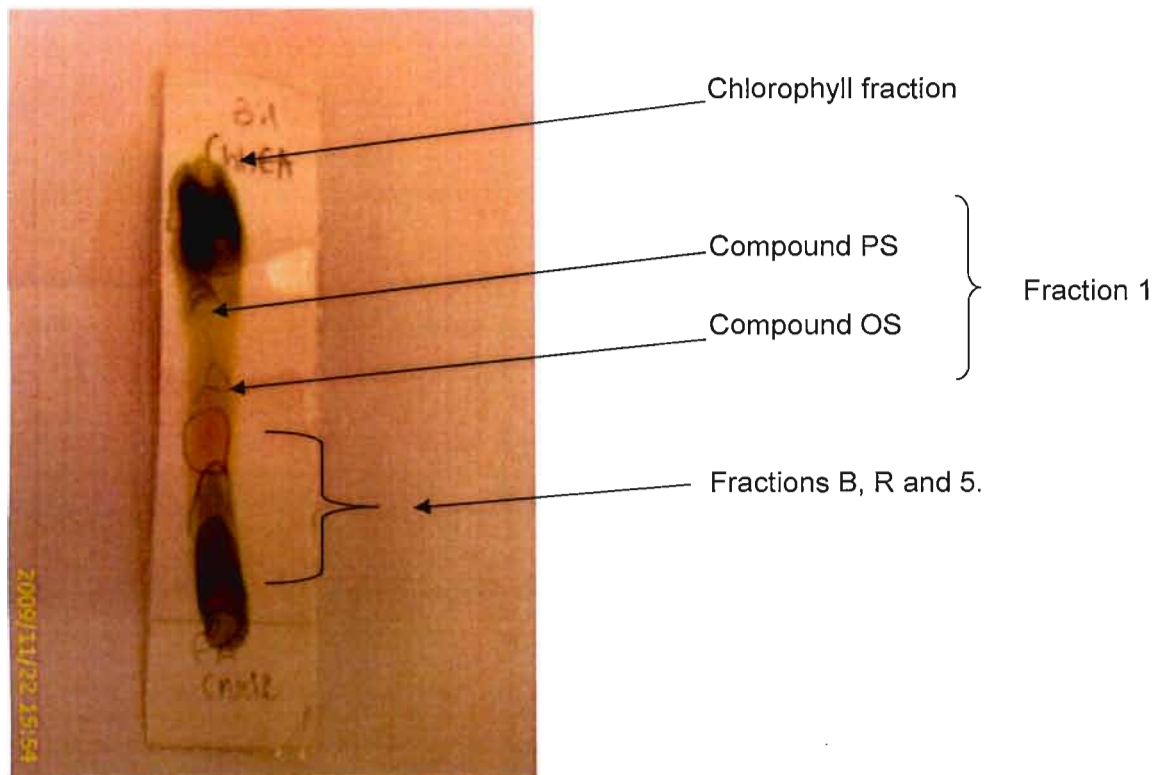
#### 5.3 Extract preparation

The plant was collected from the botanical garden of the NWU. The leaves were dried and then ground before the plant was extracted using the soxhlet method (Chapter 3).

#### 5.4 Isolation of compounds

The crude extract was divided into different fractions using the bioassay-guided approach. The ethyl acetate extract of *P. auriculata* showed the greatest antioxidant activity in the TBARS assay. Figure 5.1 shows the TLC plate of the crude ethyl acetate extract. TLC was employed to select the best mobile phase for this extract and it was then fractionated further using column chromatography, the mobile phase being chloroform: ethyl acetate (3:1). The

chlorophyll fraction was collected and discarded. Four fractions were collected thereafter.



**Figure 5.1** TLC plate of crude ethyl acetate extract in chloroform, ethyl acetate (3:1)

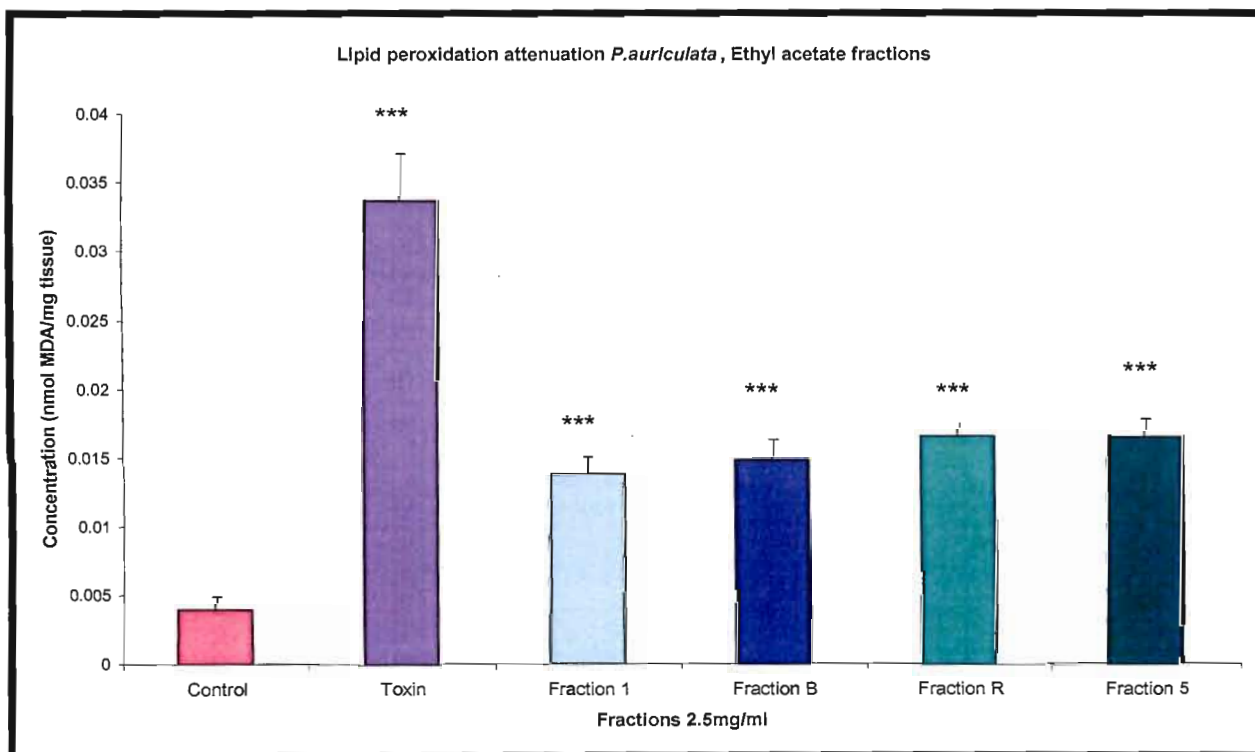
The TBARS assay was employed to measure the antioxidant activity. It was used because it showed the best antioxidant results for the crude extracts. The method given in 4.1.4 was used. Of these four fractions, fraction 1 had the best antioxidant activity (Table 5.1; Figure 5.2).

#### 5.4.1 TBARS assay on fractions of ethyl acetate extract

The TBARS assay was done as a quick way to compare the antioxidant activities of each extract. This explains why only one concentration (2.5 mg/ml) of each fraction was used. The results obtained were effective in choosing the best fraction.

**Table 5.1** Mean of the concentration of MDA tissue for each concentration of extract.

	Concentration nmol MDA/mg tissue n=5	± S.E.M
Control	0.004	0.001
Toxin	0.034	0.003
Fraction 1	0.014	0.001
Fraction B	0.015	0.001
Fraction R	0.017	0.001
Fraction 5	0.017	0.001



**Figure 5.2** Lipid peroxidation graph obtained after exposure of rat brains to fractions of the ethyl acetate extract at 2.5 mg/ml. Each bar represents the mean ± SEM, n = 5; \*\*\*p < 0.001 vs. toxin.

Fraction 1 was subjected to column chromatography using chloroform and ethyl acetate (3:1). Two compounds PS (4.2 mg) and OS (18 mg) were isolated from this fraction. PS is a waxy, white solid, with an R<sub>f</sub> value of 0.72. OS was further purified with a preparative TLC plate to give an orange-red powder. Its R<sub>f</sub> value on TLC was 0.69.



**Figure 5.3** Orange-red OS powder

## 5.5 Characterization of the isolated compounds

### 5.5.1 Instrumentation

A Bruker advance 600 in a 14.09 Tesla magnetic field utilising an ultra shield plus magnet spectrometer was used to record the <sup>13</sup>C, <sup>1</sup>H, DEPT and COSY NMR spectra. The <sup>13</sup>C NMR spectra were recorded at 150.9128712 MHz while the <sup>1</sup>H NMR spectra were recorded at 600.1724007 MHz. Tetramethylsilane was used as the reference point for the chemical shifts. A bandwidth of 1000 MHz at 24 kG was applied for <sup>1</sup>H and <sup>13</sup>C decoupling. Deuterated chloroform (CDCl<sub>3</sub>) was used to dissolve NMR samples. All were reported in parts per million (ppm) relative to the TMS signal ( $\delta = 0$ ).

IR spectra were recorded in KBr on a Nicolet Nexus 470-FT-IR spectrometer over the range 400 - 4000 cm<sup>-1</sup>. The diffuse reflectance method was used.

For the mass spectrometry low resolution APCI and high and low resolution EI were used. The specifications for each of them are as follows:

Low resolution APCI:

Thermo Electron LXQ ion trap mass spectrometer with APCI source set at 300 °C.  
Capillary Voltage = 7,0 V; Corona discharge = 10 uA.

Low resolution EI and high resolution EI:

Thermo Electron DFS magnetic sector mass spectrometer at 70 eV, and 250 °C. Samples were introduced by a heated probe. Perfluorokerosene was used as a reference compound.

### 5.5.2 Compound PS

The IR spectrum of PS showed a broad intense band at 3400  $\text{cm}^{-1}$  (OH). A band at 1050  $\text{cm}^{-1}$  signaled C-O. Peaks signaling alkenes were seen at 1650  $\text{cm}^{-1}$  and between 900 and 950  $\text{cm}^{-1}$ . The  $^{13}\text{C}$  NMR spectrum revealed 29 carbon signals, of which two were located at  $\delta_{\text{C}}$  140.75 ppm and  $\delta_{\text{C}}$  121.73 ppm representing a double bond (spectrum 2). The  $^1\text{H}$  spectrum revealed one signal for a double bond located at  $\delta_{\text{H}}$  5.33 (1H, m) and a signal for the OH at  $\delta_{\text{H}}$  3.50 (1H). The rest of the  $^1\text{H}$  NMR spectrum (spectrum 1) revealed a number of aliphatic proton signals located between  $\delta_{\text{H}}$  1 ppm and  $\delta_{\text{H}}$  2 ppm. Correlation (COSY)  $^1\text{H}$  NMR spectroscopy (spectrum 3) helped further in proving the structure of PS.

Distortionless enhancement by polarization transfer (DEPT)  $^{13}\text{C}$  NMR spectroscopy was employed to distinguish among signals due to  $\text{CH}_3$ ,  $\text{CH}_2$ , CH and quaternary carbons. Spectrum 4 showed 15 signals due to CH and  $\text{CH}_3$  and 12 signals due to  $\text{CH}_2$ .

The  $^1\text{H}$  and  $^{13}\text{C}$  NMR data of PS obtained, corresponded to that described for  $\beta$ -sitosterol (table 5.2) in literature (Nguyen *et al.*, 2004; De Paiva *et al.*, 2005). The DEPT  $^{13}\text{C}$  NMR spectrum had 12 signals due to  $\text{CH}_2$  while  $\beta$ -sitosterol only has 11  $\text{CH}_2$ . It was concluded that impurities gave the 12<sup>th</sup>  $\text{CH}_2$  signal in the DEPT spectra (spectrum 4).

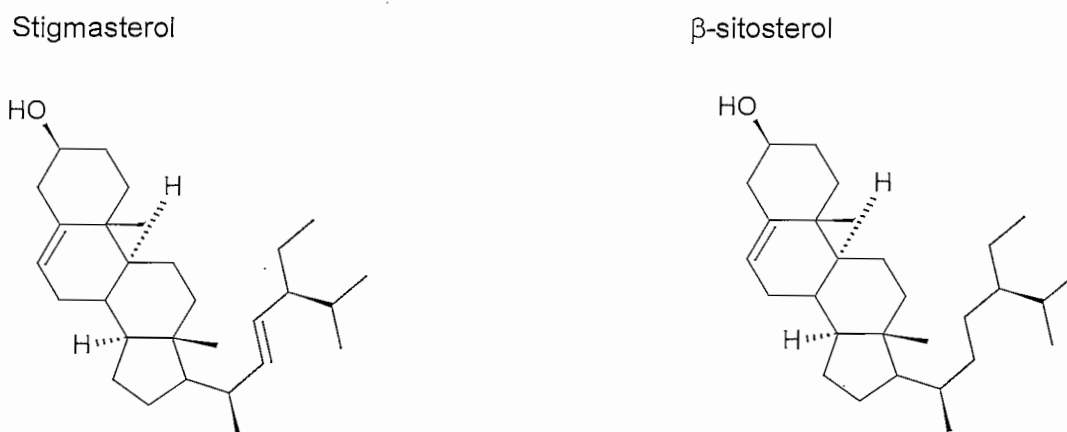
**Table 5.2** Comparison of PS to  $\beta$ -sitosterol

Carbon	$\beta$ -sitosterol		Compound PS	
	$^{13}\text{C}$	$^1\text{H}$	$^{13}\text{C}$	$^1\text{H}$
1	37.27	a.1.06(m)	37.24	a.1.07
		b.1.85(m)		b.1.81
2	29.70	a.1.61	29.69	a.1.64
		b.1.95		b.1.94
3	79.65	3.54 (1H,m)	71.81	b.3.50
4	38.91	a.2.27(1H,m)	37.24	a.2.26
		b.2.36(1H,m)		b.2.28

5	140.30		140.75	
6	122.14	5.38(1H, <i>m</i> )	121.73	5.33
7	31.94	1.98(2H, <i>m</i> )	31.89	1.98
8	31.88	1.52( <i>m</i> )	31.65	1.51
9	50.15	0.93( <i>m</i> )	50.11	0.96
10	36.70		36.50	
11	21.07	a.1.02( <i>m</i> )	21.07	a.1.05
		b.1.56( <i>m</i> )		b.1.56
12	39.76	a.1.18( <i>m</i> )	39.76	a.1.17
		b.2.02( <i>m</i> )		b.2.00
13	42.33		42.31	
14	56.76	1.01( <i>m</i> )	56.75	1.03
15	24.30	a.1.08( <i>m</i> )	24.30	a.1.09
		b.1.12( <i>m</i> )		b.1.13
16	28.26	a.1.83( <i>m</i> )	28.24	a.1.81
		b.1.86( <i>m</i> )		b.1.94
17	56.11	1.12( <i>m</i> )	56.03	1.13
18	11.85	0.68(3H, <i>s</i> )	11.85	0.65
19	19.37	1.00(3H, <i>s</i> )	19.39	0.99
20	36.19	1.36( <i>m</i> )	36.14	1.36
21	18.76	0.92(3H, <i>d</i> , 6.4)	18.77	0.90
22	33.94	a. 1.00( <i>s</i> )	33.93	0.99
		b.1.34( <i>m</i> )		1.32
23	26.10	1.18(2H, <i>m</i> )	26.04	1.17
24	45.81	0.95( <i>m</i> )	45.81	0.96
25	29.16	1.66( <i>m</i> )	29.12	1.65
26	19.02	0.82(3H, <i>d</i> , 6.8)	19.02	0.82
27	19.82	0.84(3H, <i>d</i> , 6.8)	19.82	0.84

28	23.06	1.26( <i>bs</i> )	23.05	1.27
29	11.97	0.84(3H, <i>t</i> , 7.6)	11.97	0.84

A close look at the FT-IR of PS (spectrum 5) compared to the spectrum of stigmasterol (spectrum 6) shows similar spectra. Stigmasterol is a phytosterol with the same basic structure as beta sitosterol but a different side chain (figure 5.4). The EI-MS (spectrum 7) gave data consistent with the  $^1\text{H}$ ,  $^{13}\text{C}$  and the FT-IR. Molecular ion peaks were seen at  $m/z$  (%) 396.39 (100), 381.36 (50), and 414 (10), with the major ion with a mass of 396.39. This ion lacks  $\text{H}_2\text{O}$ ; the reason being the possible fragmentation of the  $\text{H}_2\text{O}$  ion. The molecular formula of PS was established as  $\text{C}_{29}\text{H}_{50}\text{O}$ . The molar mass of  $\beta$ -sitosterol is  $414.71 \text{ g mol}^{-1}$ .



**Figure 5.4** Stigmasterol and  $\beta$ -sitosterol

Compound PS has a basic structure similar to  $\beta$ -sitosterol. It was concluded from the  $^1\text{H}$  NMR,  $^{13}\text{C}$  NMR, DEPT  $^{13}\text{C}$  NMR, COSY NMR, EI-MS and FT-IR spectral information obtained that PS is  $\beta$ -sitosterol. No further assays were performed on PS because the quantity was not enough for any of the assays.  $\beta$ -sitosterol is a known antioxidant as shown in literature (Yasukazu & Etsuo, 2003).

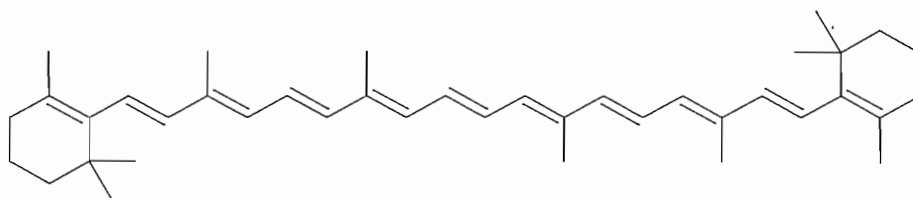
### 5.5.3 Compound OS

Compound OS was obtained as an orange solid that is soluble in chloroform, slightly soluble in methanol and insoluble in water. Its FT-IR spectrum (spectrum 9) showed absorption bands ( $\text{cm}^{-1}$ ) at 2850.79 and 1454.33 (alkanes) and 3026 (alkenes). The infrared spectra of OS did not have an absorption band that signalled the presence of an OH group. The  $^1\text{H}$  NMR spectrum (spectrum 6) revealed a number of aliphatic proton signals located between  $\delta_{\text{H}}$  1 and  $\delta_{\text{H}}$  2 ppm. Due to OS being impure, signals from the impurities possibly clouded the signals for OS.

Signals from known impurities were identified and ruled out.  $^1\text{H}$  NMR of OS does not have a peak between  $\delta_{\text{H}}$  3 ppm and  $\delta_{\text{H}}$  4 ppm suggesting that OS does not have an oxygen carbon bond. Signals at  $\delta_{\text{H}}$  7.24 ppm, and  $\delta_{\text{H}}$  2.15 ppm were for chloroform and acetone respectively. The compound was dissolved in these solvents during the isolation.

The  $^{13}\text{C}$  NMR spectrum (spectrum 9) showed many peaks between  $\delta_{\text{C}}$  120 ppm and  $\delta_{\text{C}}$  140 ppm indicating the presence of many double bonds in the compound.

The  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra of OS gave spectra similar to  $\beta$ -carotene. The molecular formula of  $\beta$ -carotene is  $\text{C}_{40}\text{H}_{56}$ . The spectrum of OS however has many extra peaks suggesting that OS may have many impurities or OS could be a mixture of compounds. Although the data obtained was not conclusive,  $\beta$ -carotene (figure 5.5) was proposed as the structure of OS.



**Figure 5.5**  $\beta$ -carotene

## 5.6 Biological activities of isolated compounds

### 5.6.1 Biological activities of $\beta$ -sitosterol

$\beta$ -sitosterol is the most abundant phytosterol with numerous biological activities. It has been isolated previously from *Plumbago zeylanica* (Nguyen *et al.*, 2004) and *Plumbago auriculata* (De Paiva *et al.*, 2005). Studies by Gomes and his colleagues (2006) showed that a fraction containing a mixture of  $\beta$ -sitosterol and stigmasterol could diminish lethality, cardiotoxicity, neurotoxicity, respiratory changes and Phospholipase  $\text{A}_2$  activity induced by cobra venom. Venom-induced changes in lipid peroxidation and superoxide dismutase activity were also antagonised (Gomes *et al.*, 2006).  $\beta$ -sitosterol is also an effective apoptosis-enriching agent that can therefore be used as a preventive measure for cancer (Nguyen *et al.*, 2004; Awad *et al.*, 2007).

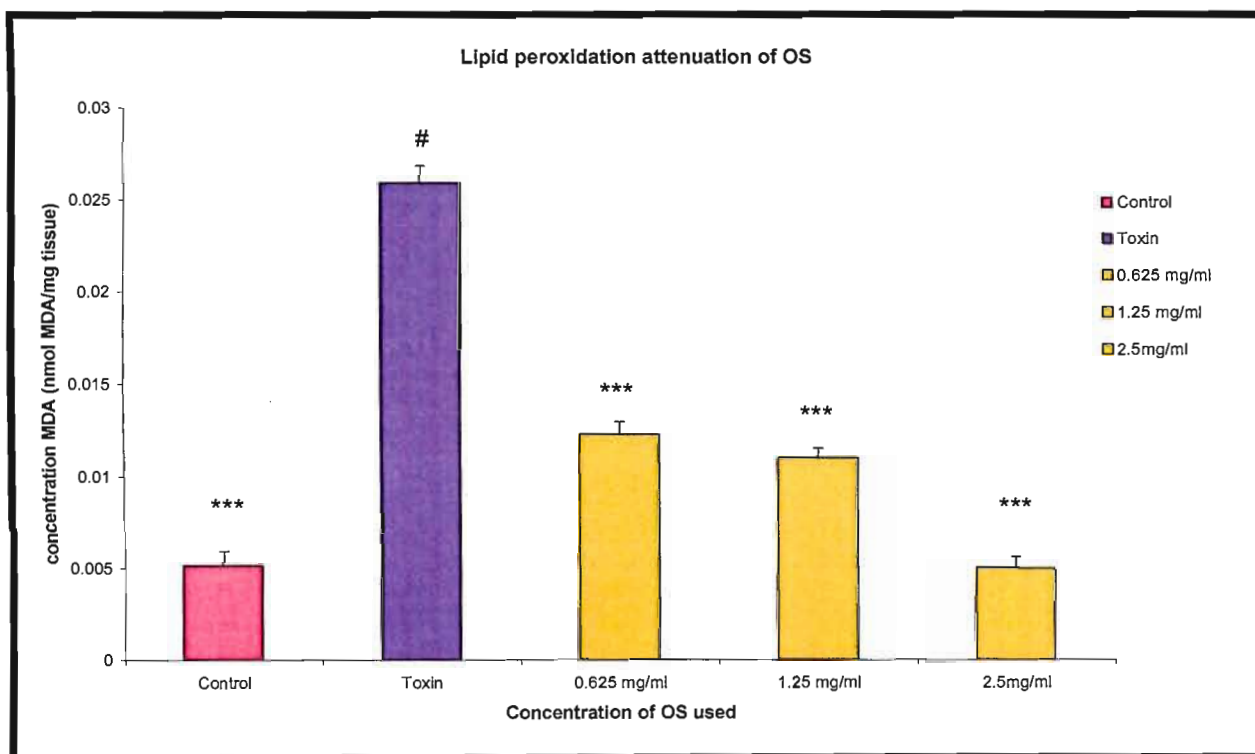
### 5.6.2 Biological activities of $\beta$ -carotene

$\beta$ -carotene is a natural pigment found in most plants. It gives most plants their orange colour. It is a compound found in most vegetables and fruits. The TBARS and MTT assays were performed on  $\beta$ -carotene. The results below show the antioxidant activity (table 5.3, figure 5.6) and toxicity (table 5.4, figure, 5.7) of  $\beta$ -carotene.

## 5.6.2.1 TBARS assay results of OS

**Table 5.3** Mean of the concentration of MDA tissue for each concentration of OS

	Concentration nmol MDA/mg tissue n = 6	± S.E.M
Control	0.005	0.0008
Toxin	0.026	0.0009
0.625mg/ml	0.012	0.0006
1.25mg/ml	0.011	0.0005
2.5mg/ml	0.005	0.0006



**Figure 5.6** Lipid peroxidation graph obtained after exposure of rat brains to the compound, OS at concentrations 0.625 mg/ml, 1.25 mg/ml and 2.5 mg/ml. Each bar represents the mean  $\pm$  S.E.M, n = 6; \*\*\* p < 0.001 vs toxin (#).

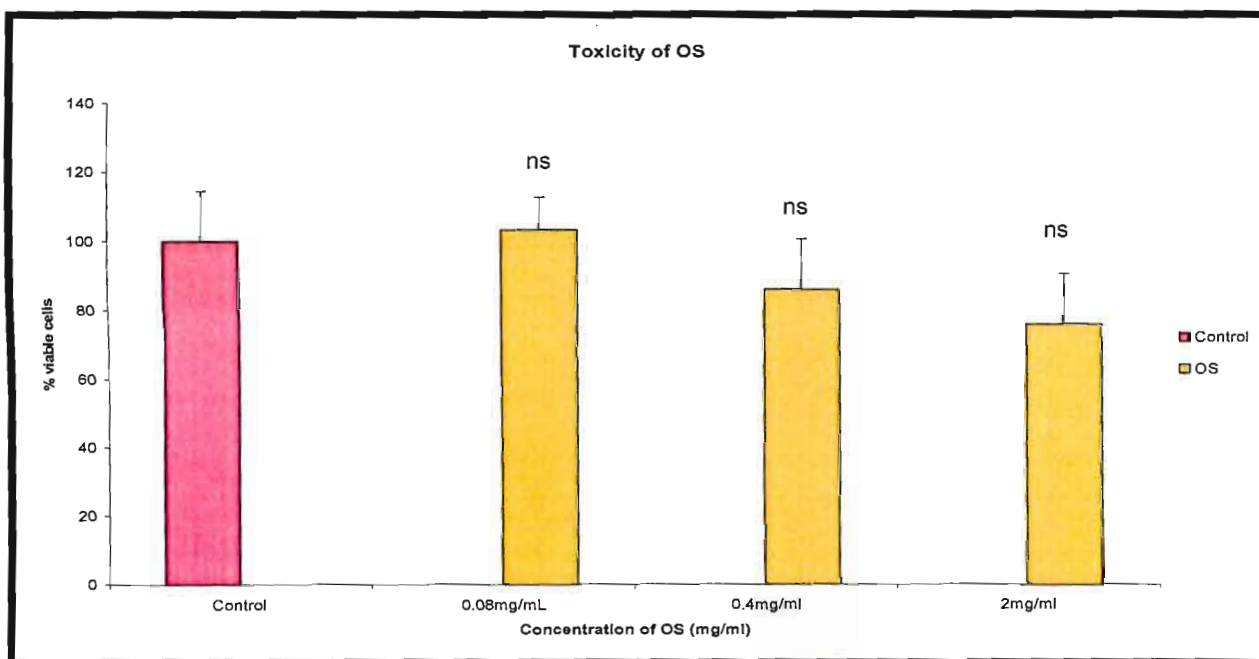
The TBARS assay showed that OS had very high antioxidant activity (table 5.3, figure 5.6). The concentration of MDA/mg of tissue was reduced to 0.05 by 2.5 mg/ml of OS, which is equal to

the MDA /mg observed for the control. This reduction in MDA is compared to the concentration of 0.26 n mol MDA/mg in the brain treated with the toxin

### 5.6.2.2 MTT assay results of OS

**Table 5.4** Percent viable cells after exposure to OS at varying concentrations.

	% viable cells n = 9	± S.E.M
Blank	0	0
Control	100	14.44
OS 0.08 mg/ml	103.28	9.558
0.4 mg/ml	86.06	14.53
2 mg/ml	75.99	14.61



**Figure 5.7** Graph obtained after 24-hour exposure of HeLa cells to 0.08 mg/ml, 0.4 mg/ml and 2 mg/ml concentrations compound OS of *P. auriculata*. Each bar represents ± SEM; n = 9. \*\*\* p < 0.001, ns p > 0.05 vs. control (100 % growth).

## 5.7 Discussion and Conclusion

The aim of this research was to investigate antioxidant properties of *P. auriculata*. To achieve this, bioassay-guided fractionation was done using two assays for antioxidant activity. The ethyl acetate extract, having the highest antioxidant activity was selected for further research. Two compounds were isolated from fraction 1. Compound OS, presumed to be  $\beta$ -carotene, had high antioxidant activity. Unfortunately, PS ( $\beta$ -sitosterol) could not be assayed further because of the small quantities isolated.

A conclusion was made that OS was  $\beta$ -carotene. The  $^{13}\text{C}$  and  $^1\text{H}$  NMR data confirmed this proposal. The orange colour could be because of the conjugated double bonds in this molecule.

The antioxidant activity of  $\beta$ -carotene is not surprising because it is a known natural antioxidant. Carotenoids are abundant in nature. Because of their high antioxidant properties, people who ingest them can reduce the chances of them getting cancer (Naves *et al.*, 1998). Research in 1995 by Kardinaal and colleagues shows that  $\beta$ -carotene can protect against myocardial infarction.  $\beta$ -Carotene is a rapid scavenger of free radicals that are implicated in the initiation of the peroxidation of lipids (Niki *et al.*, 1995; Everett *et al.*, 1996). These free radicals include  $\text{O}_2^\bullet$ ,  $^1\text{O}_2$  and the  $\text{NO}^\bullet$  radical. This explains the attenuation of the peroxidation of lipids in the TBARS assay.

Information obtained from literature showed that  $\beta$ -sitosterol also is a known antioxidant. Thus, bioassay-guided fractionation led to the isolation of two active compounds. FT-IR, MS,  $^{13}\text{C}$ ,  $^1\text{H}$ , DEPT  $^{13}\text{C}$  and COSY NMR were employed in proving that PS was indeed  $\beta$ -sitosterol.

### Conclusion

Parkinson's disease, a disease characterised by a slow and progressive loss of dopaminergic neurons in the substantia nigra *pars compacta* is one of the common neurological disorders affecting the elderly. Loss of neurons is caused by many factors, of which oxidative stress and damage by free radicals are some of the factors. Oxidative stress results in the peroxidation of lipids (Halliwell & Chirico, 1993), necrosis and apoptosis (Franco *et al.*, 2009), which all lead to neurodegeneration.

Data from past experiments show that phagocytosis and the inhibition of superoxide dismutase result in the formation of  $O_2^{\bullet-}$  (Davies & Edwards, 1991). Superoxide dismutase, an antioxidant enzyme, glutathione peroxidase, and the total antioxidant status are significantly lower in Parkinson's disease patients than in normal subjects (Yuan *et al.*, 2000). Given the evidence that oxidative stress leads to neurodegeneration, antioxidants can be used to attenuate the effects of oxidative stress and therefore slow down the destruction of dopaminergic neurons (Chinta & Andersen, 2008).

The aim of this study was to investigate the antioxidant properties and the toxicity of the leaves of *Plumbago auriculata*

The following objectives were met:

Twenty-one plants were selected after getting information from literature about their use traditionally. The FRAP and ORAC assays were used to show the total antioxidant capacities of these plants. The results from these experiments led to the selection of five plants of which *P. auriculata* had the fourth highest activity in the ORAC assay and the seventh highest activity in the FRAP assay. *P. auriculata* was selected for this study as the other four plants were being studied by co-workers.

The soxhlet extraction method was used to extract material from the leaves of the plant. Four solvents: petroleum ether, dichloromethane, ethyl acetate and ethanol in order of increasing polarity were used. From these four extracts, the ethyl acetate fraction had the most antioxidant activity in the NBT and TBARS assays.

Activity guided fractionation was used every step of the separation and isolation. The ethyl acetate fraction was subjected to column chromatography which resulted in two compounds, PS and OS being isolated from fraction 1 of this extract.  $^1H$  NMR,  $^{13}C$  NMR, DEPT  $^{13}C$  NMR and

COSY NMR, MS and FT-IR were employed to characterise the structures of these compounds. PS was found to be  $\beta$ -sitosterol, while OS was proposed to be  $\beta$ -carotene. The amount of  $\beta$ -sitosterol was too little for any further assays to be done on it.  $\beta$ -carotene however had high antioxidant activity as indicated in the TBARS assay.  $\beta$ -carotene also had insignificant toxicity on HeLa cells. Although the proposed structure was not conclusive, information from literature shows that  $\beta$ -carotene has high antioxidant activity, hence the results from the TBARS assay. A number of authors showed that carotenoids are readily oxidised by a variety of oxidants. They however have pro-oxidant activity in the presence of iron because they react in the Fenton reaction (Polyakov *et al.*, 2001):

$\beta$ -carotene is a lipophilic antioxidant (Oshima *et al.*, 1993). Due to its lipophilicity, it is able to suppress oxidation induced by either lipophilic or hydrophilic free radicals (Niki *et al.*, 1995). The compound  $\beta$ -carotene is a scavenger of peroxy free radicals (Kennedy & Liebler, 1992) and other free radicals (Everett *et al.*, 1996), thus it inhibits lipid peroxidation as indicated by the results obtained in the TBARS assay.

$\beta$ -sitosterol has been previously isolated from *P. zeylanica*. It was found to be cytotoxic on Bowes cells and to inhibit growth and stimulate apoptosis of breast cancer cells (Nguyen *et al.*, 2004). De Paiva *et al.* (2005) isolated  $\beta$ -sitosterol from *P. auriculata*. This compound is very common in plants, thus its isolation from *P. auriculata* was not surprising.

The aim of this study was achieved as seen in the results from Chapters 3, 4 and 5. *P. auriculata* had high antioxidant properties in its ethyl acetate fraction. Bioassay-guided fractionation using TBARS, NBT and column chromatography were employed to isolate two pure active compounds from the most active fraction. The two compounds that were isolated are very common in most plants. These two compounds are found in high concentrations in most plants as seen in this research also. Because of their concentrations, these compounds are readily isolated. Plant secondary metabolites are found in very small concentrations in plants and are only isolated from extracts of which the high concentration compounds are eliminated. This was not achieved during this study, but I propose further work on *P. auriculata* to isolate more antioxidant compounds especially from the ethyl acetate and ethanol extracts as they had the best antioxidant activity.

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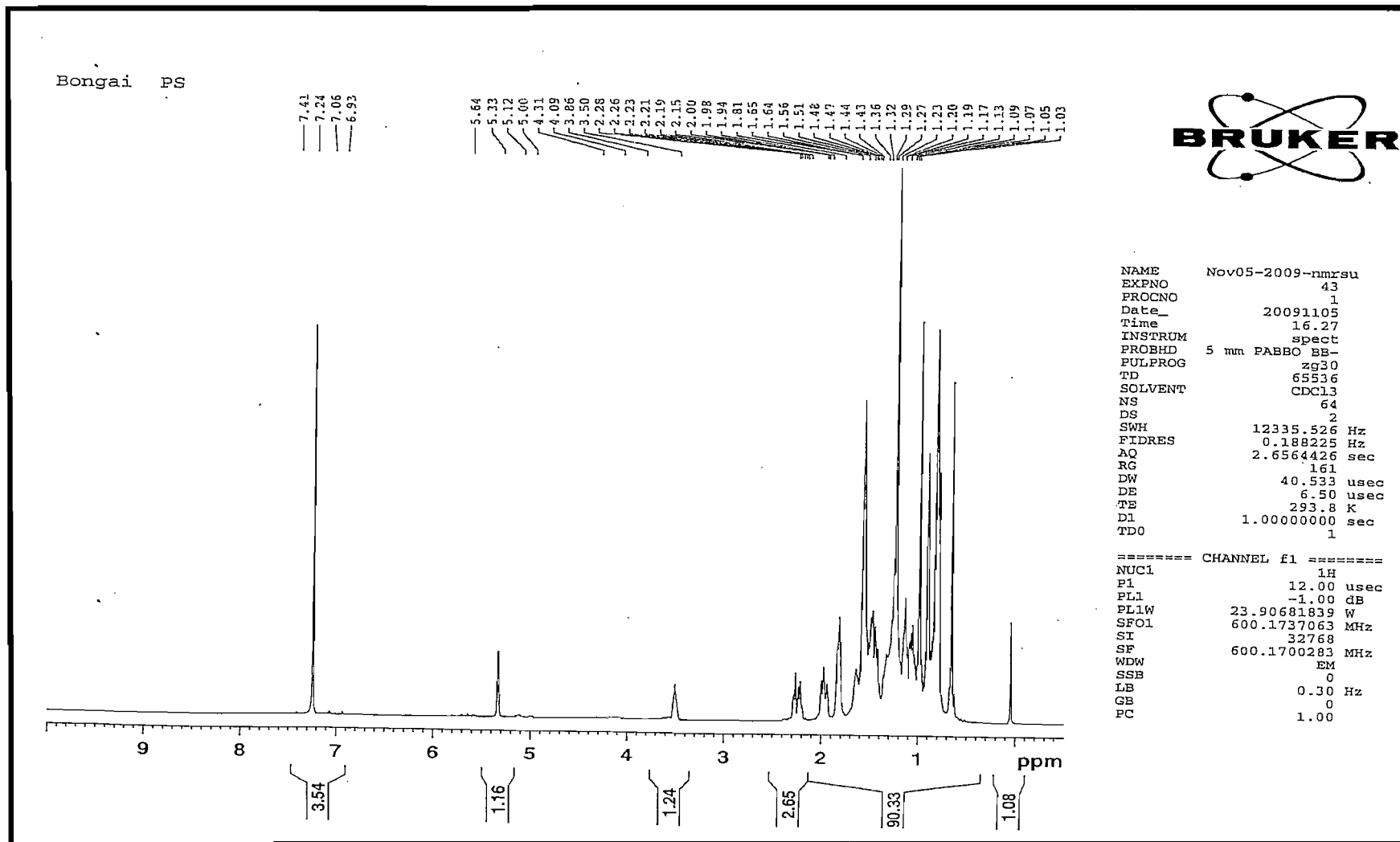
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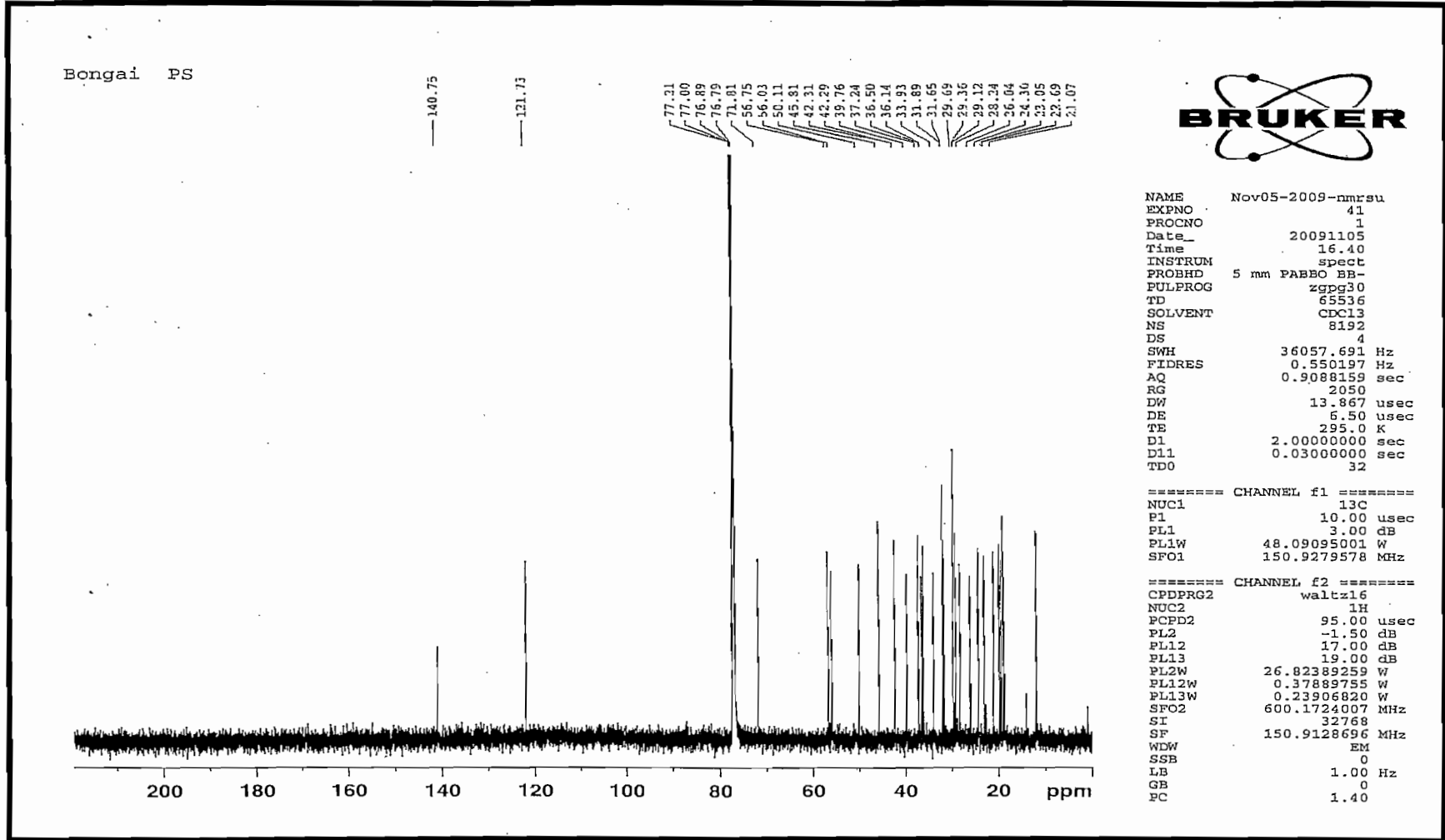
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# SPECTRA

SPECTRUM 1



SPECTRUM 2

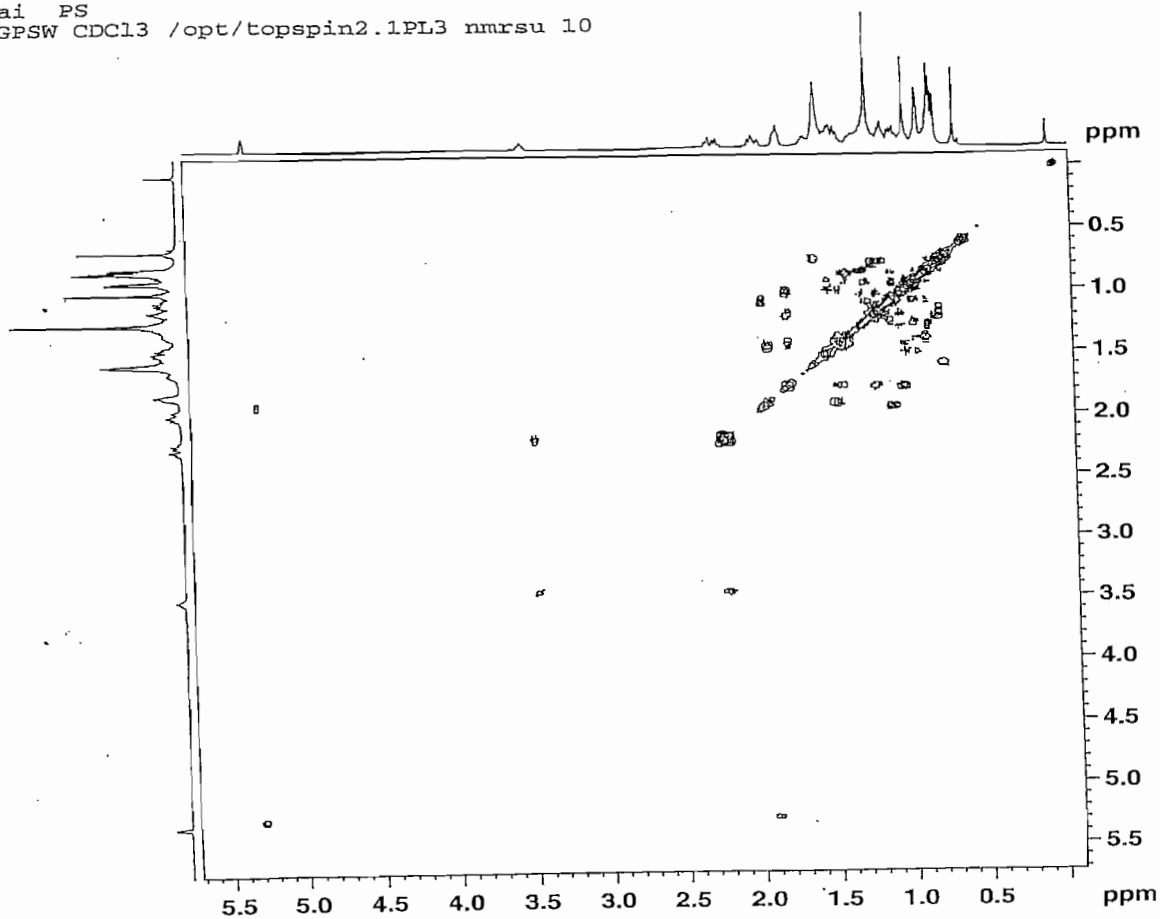


## SPECTRUM 3

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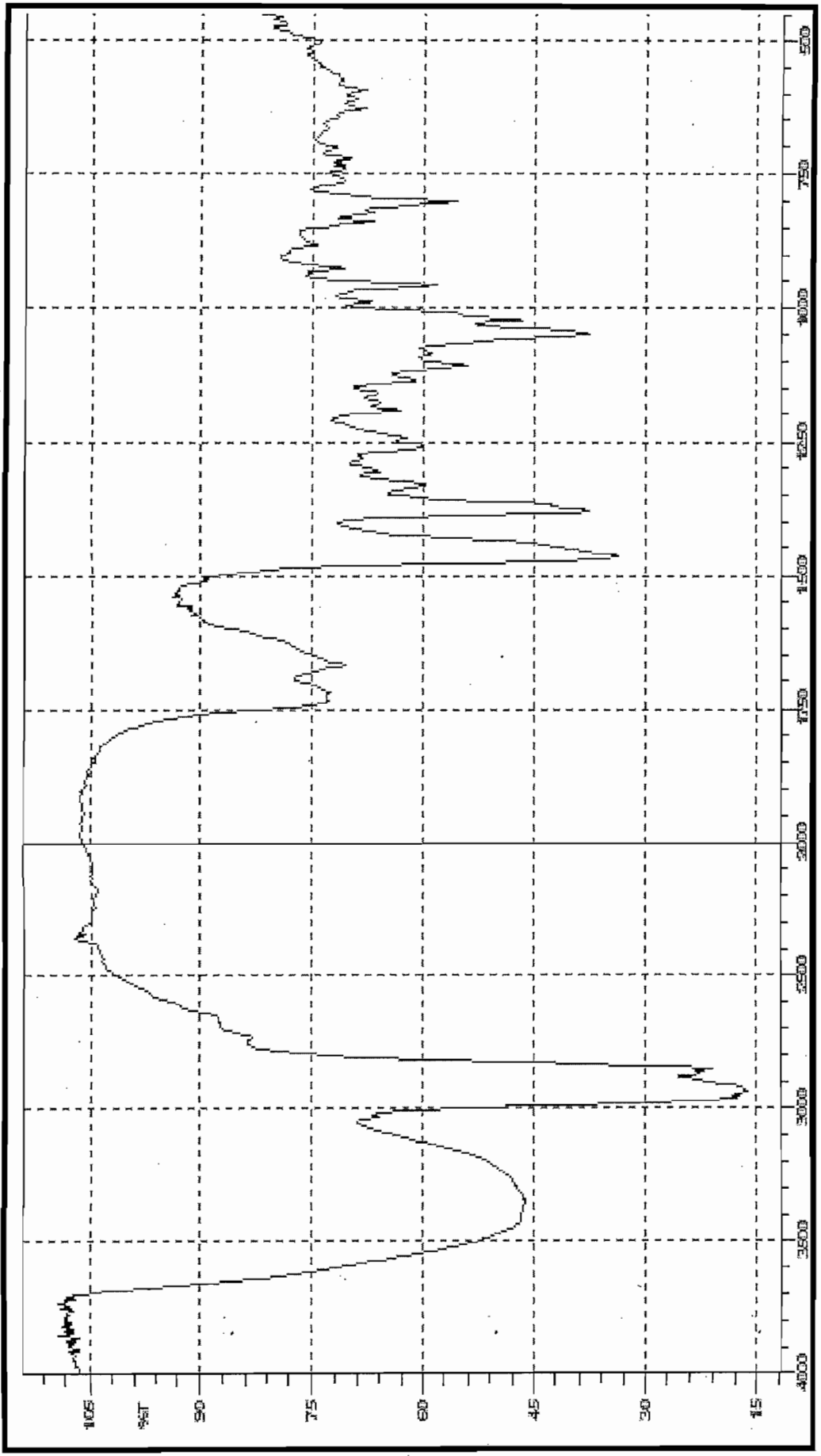


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RG	64
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DE	6.50 usec
TE	293.9 K
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D1	1.32182300 sec
D13	0.00000400 sec
D16	0.00010000 sec
INO	0.00028600 sec
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P0	12.00 usec
P1	12.00 usec
PL1	-1.00 dB
PL1W	23.90681839 W
SFO1	600.1717434 MHz
----- GRADIENT CHANNEL -----	
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GFZ1	10.00 %
P16	1000.00 usec
ND0	1
TD	128
SFO1	600.1717 MHz
FIDRES	27.316433 Hz
SW	5.826 ppm
FMODE	QF
SI	1024
SF	600.1700551 MHz
WDW	SINE
SSB	0
LB	0.00 Hz
GB	0
PC	1.40
SI	1024
MC2	QF
SF	600.1700551 MHz
WDW	SINE
SSB	0
LB	0.00 Hz
GB	0

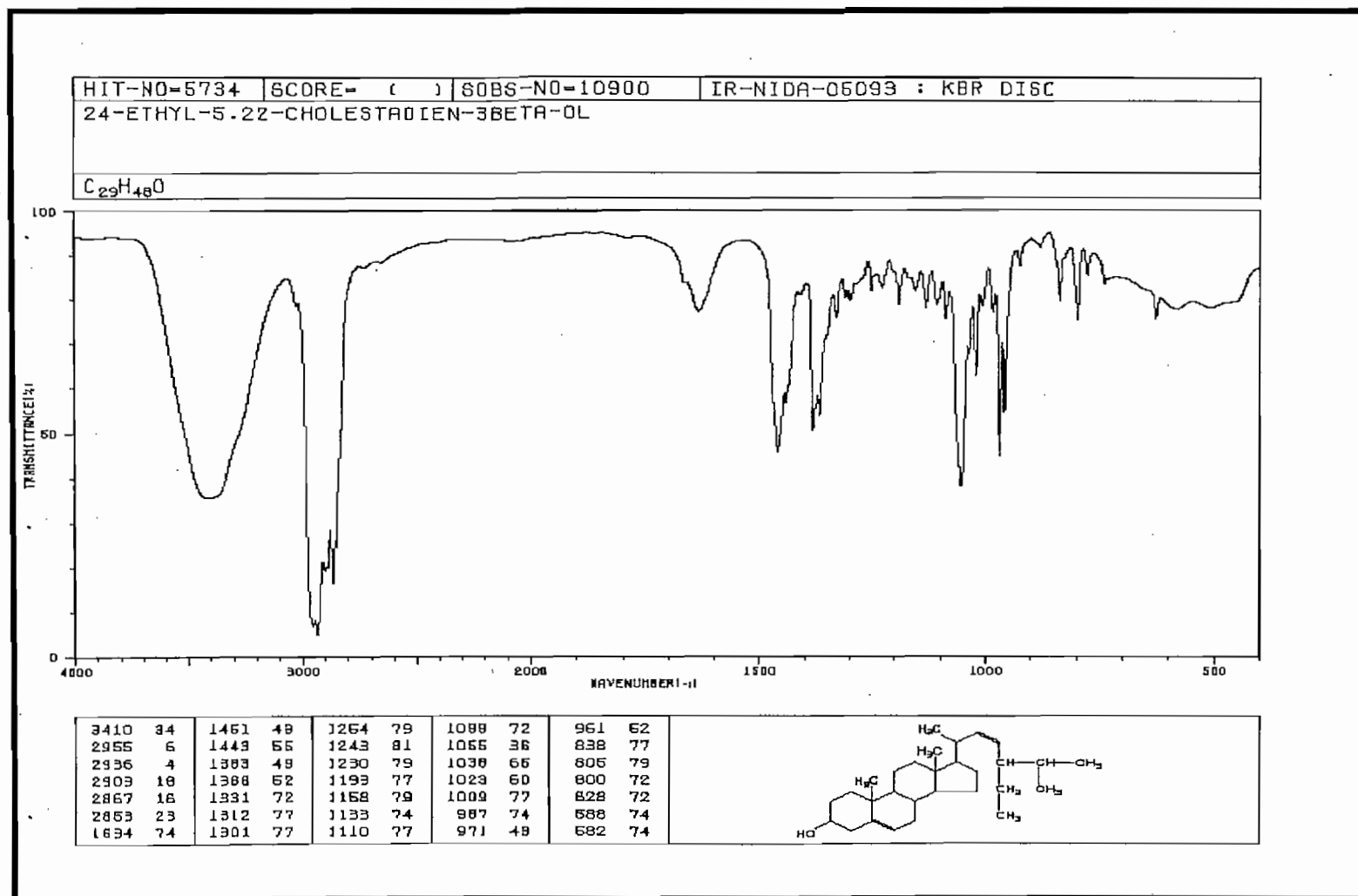




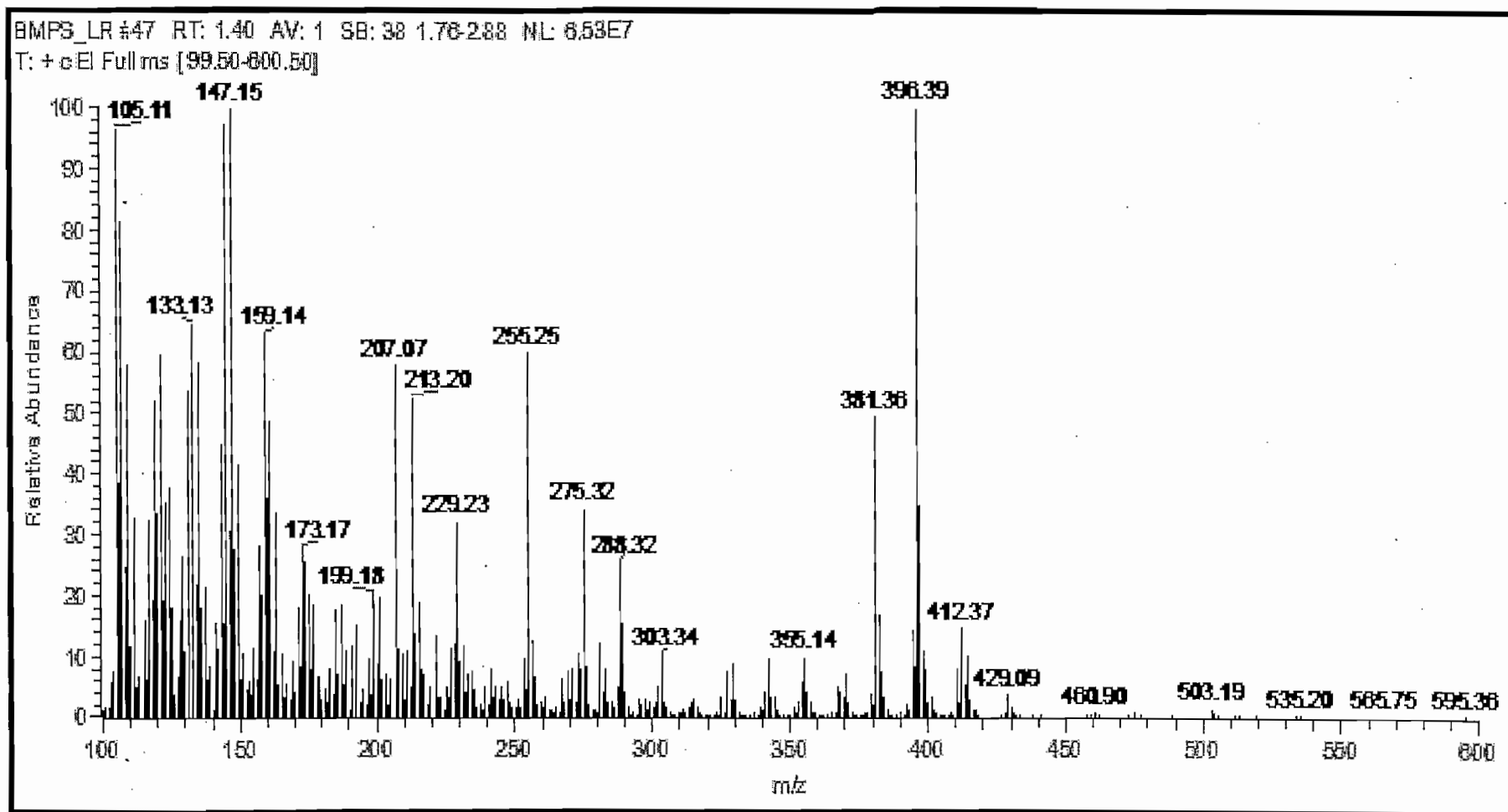
SPECTRUM 5 (FT-IR PS)



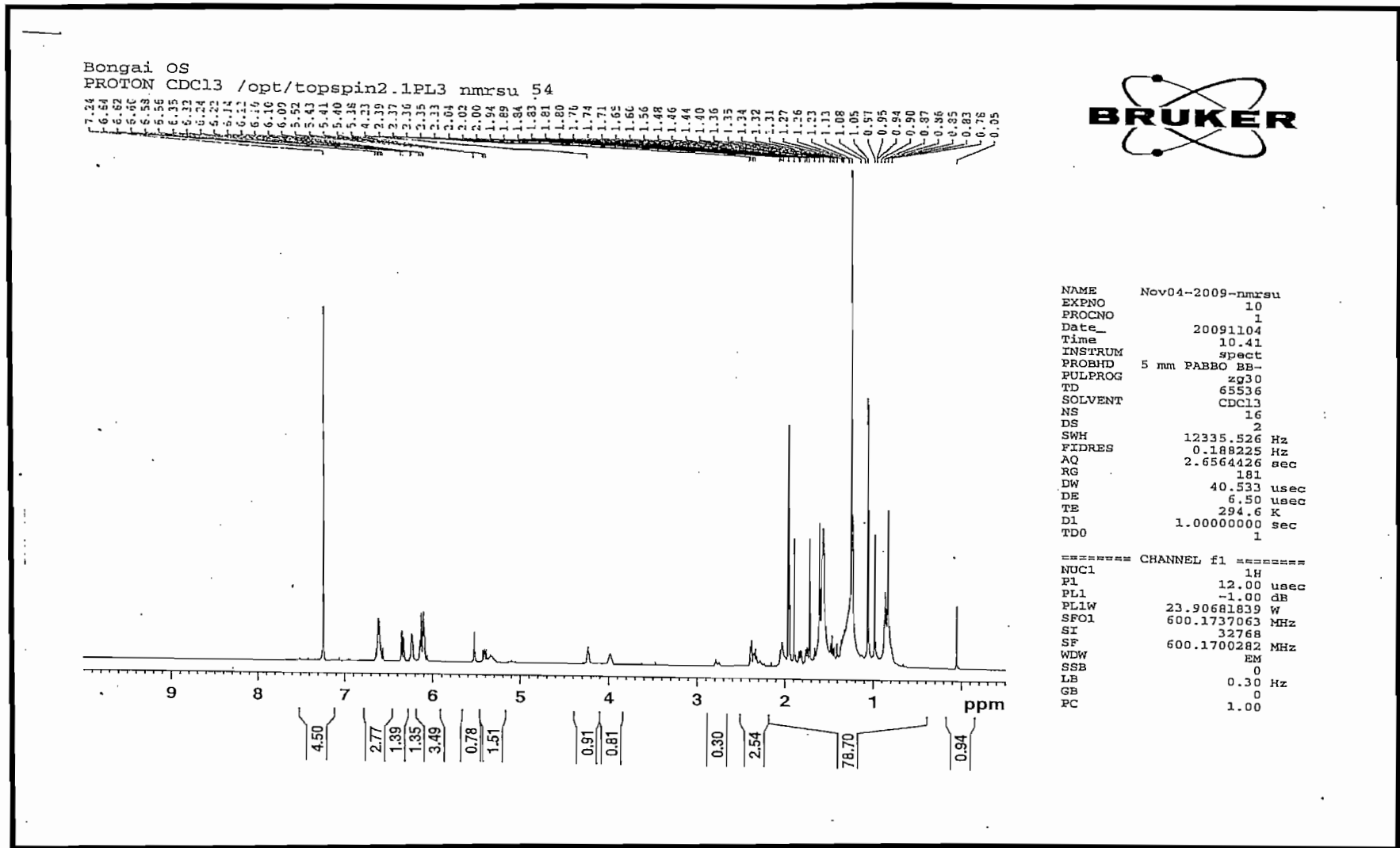
## SPECTRUM 6 (SDBS, 2009)



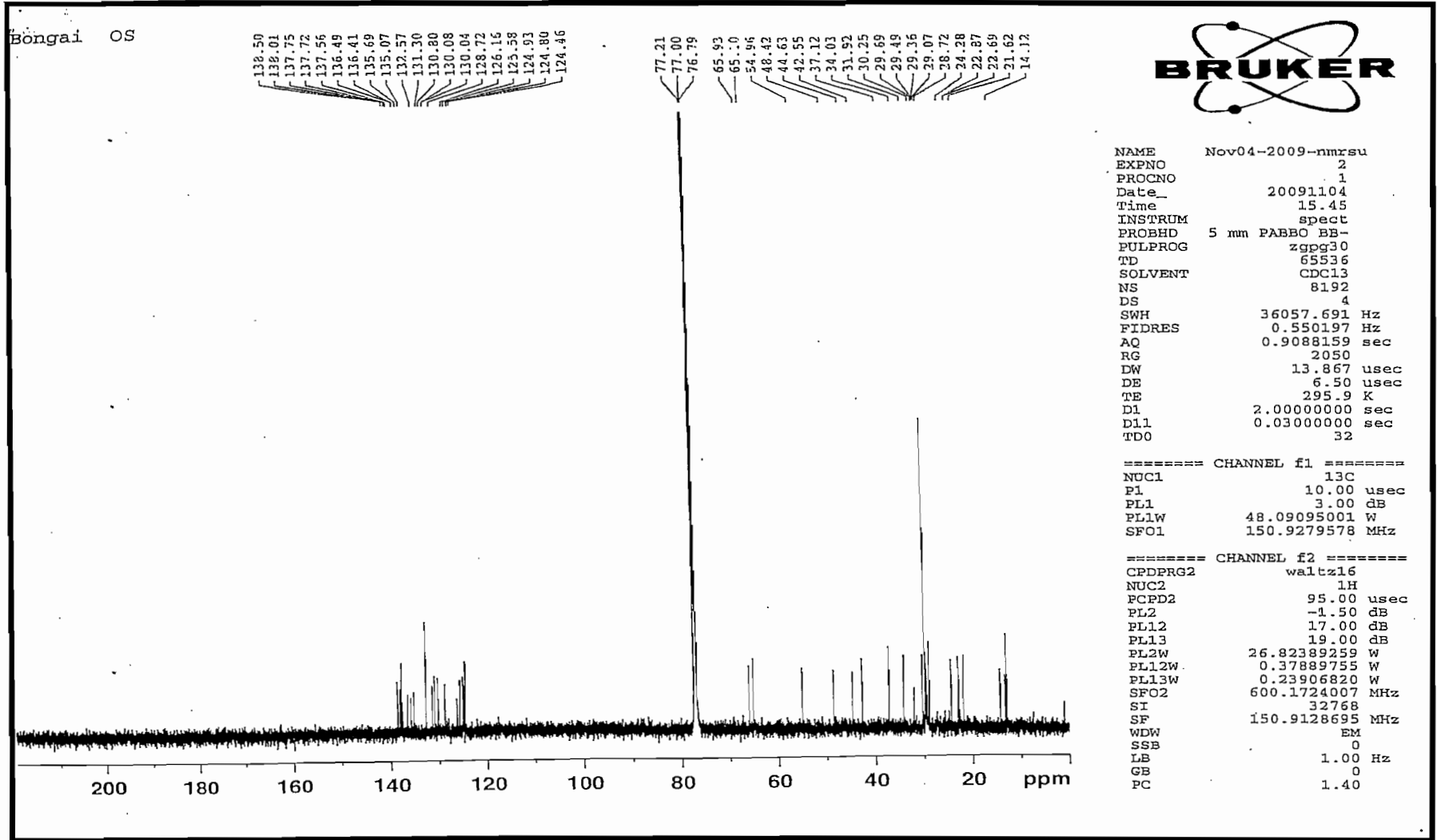
## SPECTRUM 7 MS of PS



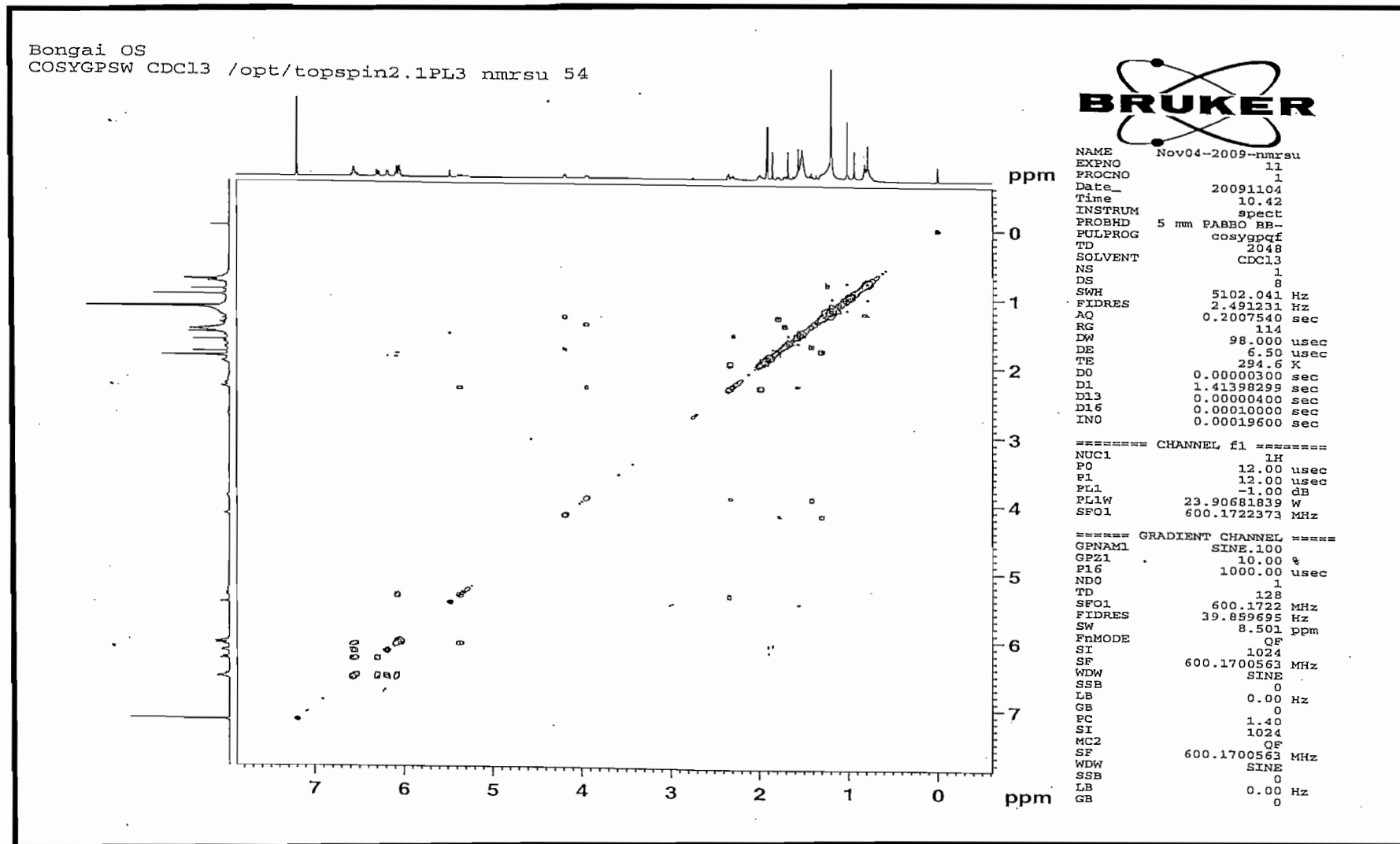
SPECTRUM 8



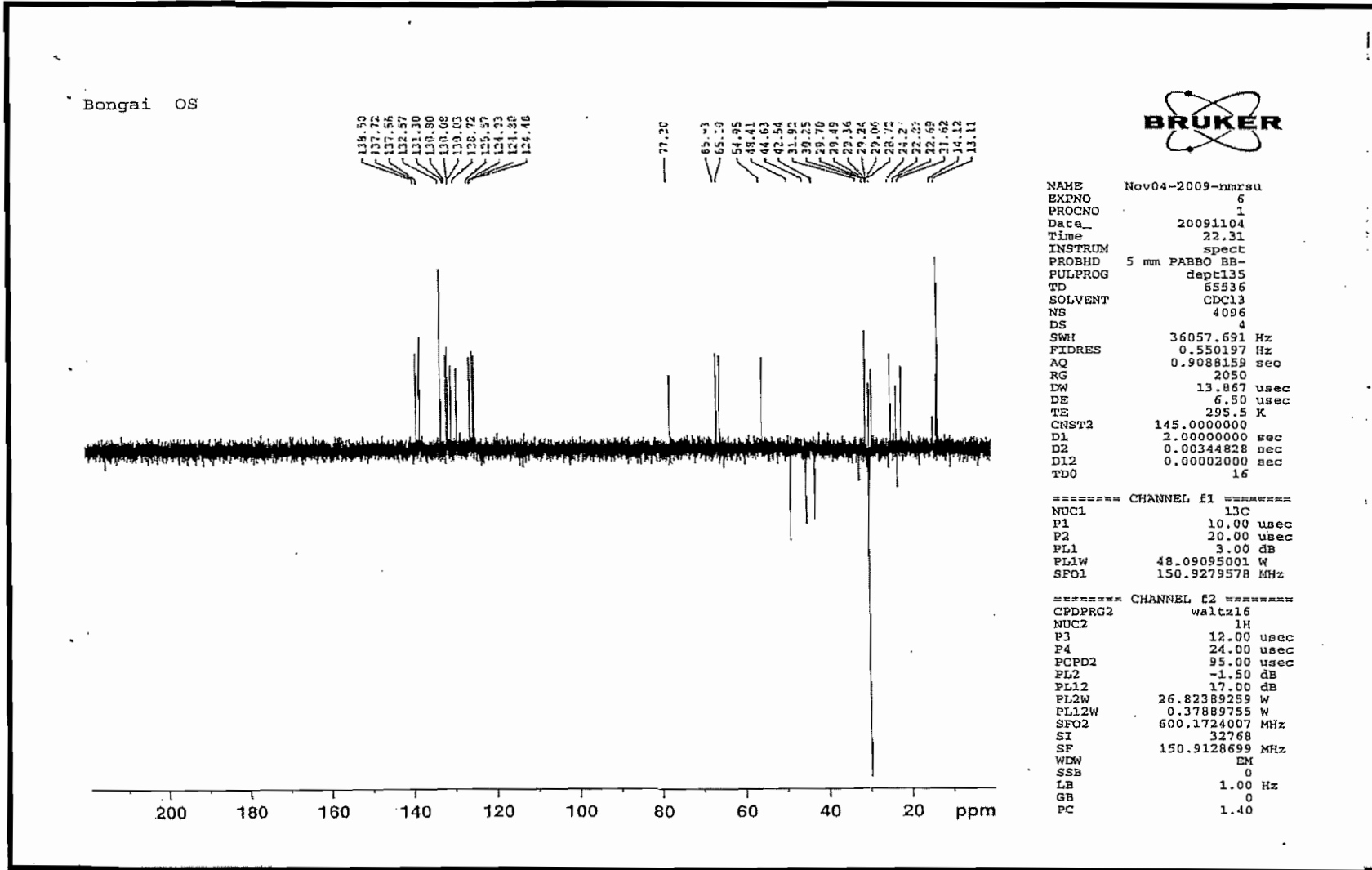
SPECTRUM 9



## SPECTRUM 10



SPECTRUM 11 (DEPT OS)



SPECTRUM 12 (FT-IR OS).

