ANTIOXIDANT PROPERTIES OF 4-HYDROXYQUINOLINES

Susan Elizabeth Neethling

Dissertation submitted in partial fulfilment of the requirements for the degree

MAGISTER SCIENTIAE

in

Pharmaceutical Chemistry

at the North-West University, Potchefstroom Campus

Supervisor: **Dr. S Van Dyk**

Co-Supervisor: **Prof. S.F. Malan**

So many of our dreams at first seem impossible, then they seem improbable, and then, when we summon the will, they soon become inevitable

Christopher Reeve

ACKNOWLEDGEMENTS

On life's highway, one often comes across certain milestones, meeting certain people helping one to achieve such milestones. And without these people, none would be possible. In this regard I wish to express my deepest and sincerest appreciation to the following in helping me realise this dissertation:

My Father God, in heaven, for creating opportunities, encouraging me to take it on, providing me with perseverance to complete this study, and always seeing that everything worked out for the best (Rom 8:24), for I can achieve all things through Christ who strengthens me (Phil. 4:13).

My supervisor, Doctor Sandra van Dyk, for her expert supervision, constant encouragement and unfailing support throughout this study and for her expeditious yet thorough attention in reviewing this dissertation, when time mattered most. Thank you for your constructive criticism, it truly was a privilege working with you, and your invaluable guidance is appreciated extremely.

My co-supervisor Professor Sarel Malan for his supervision, guidance, help and constant support.

Professor Santy Daya, for all his help, advice and support. Thank you very much for the use of your facilities and a great time in Grahamstown.

Professor Francois van der Westhuizen for his supervision, enthusiasm, guidance and patience. It was great pleasure working with you.

Doctors Himant and Deepa Maharaj for your unselfish help and outlasting patience in assisting me. Thank you for your friendship, guidance, encouragement and support. I am truly grateful for everything you have done for me.

Elbie Smith for kindly assisting me with the synthesis and purification of compounds. Your help, friendship and support means a lot to me.

My friends Marnus, Landi, Handre, Sorita, Alla and Gerrit for your everlasting friendship, making my time in Potchefstroom one never to be forgotten. Without your support none of this would be possible. Special thanks to Gerrit, to whom I could call on anytime if I needed anything. Thank you for listening and always encouraging me. Words fail me in describing your immeasurable friendship, help and support.

My friends Sumie, Marle, Bunny and especially Müke and Ash, for your encouragement, love and support. Special thanks to Müke and Ash for understanding and supporting me when things didn't go according to plan, and for always cheering me up when things seemed worst. Your friendship will be cherished forever.

My fellow M. Sc and Ph. D friends, especially Corlea, Cecile and Melanie for the warmth and high spirits provided during the course of my work. Thank you for your understanding and encouragement.

Corlea, for all your invaluable help, constant support and undeviating encouragement. Everything you have done for me is appreciated immensely, and this past few years wouldn't have been the same without you.

Mr. and Mrs. Jean and Marie Coetzee, for always encouraging and motivating me, putting the world into a different light and allowing me to think about the other side of things. Your love and support had an immeasurable worth in sculpturing my existence.

My parents Johann and Francien Neethling, and my sister, Wilna Grobler for your unconditional love and support and for never giving up on me, even when I at times did. The sacrifices you have made in order for me to achieve my goals did not go unnoticed, and it is appreciated from the bottom of my heart.

ABSTRACT

Oxygen, although vital for human survival, is the main source of reactive oxygen species, which can cause damage to essential biomolecules. Production of reactive oxygen species is linked to normal cellular processes; therefore eukaryotes have evolved a specific antioxidant system that curbs this toxic threat, protecting biomolecules against oxidative damage. Imbalance between the level of reactive oxygen species and antioxidants causes a deleterious condition referred to as oxidative stress. Oxidative stress has been implicated in the ageing process as well as the pathogenesis of various neurodegenerative disorders. It is thus crucial to identify compounds with antioxidative activity, which can counteract the oxidative attack and conditions such as Alzheimer's and Parkinson's diseases.

Various hydroxyquinolines have been shown to protect biological systems against induced oxidative damage. Hence, with the aim to clarify the antioxidant properties, a series of 4-hydroxyquinolines were selected as target compounds, synthesized and assayed.

4-Hydroxyquinolines with a nitro-, amino- and dibuthylamino-group in the 6 or 7 positions respectively were synthesized according to the Gould-Jacobs reaction followed by a number of transformation reactions and characterized by means of physical data. The antioxidative properties of the compounds were assayed in terms of: the oxygen radical absorbance capacity, the ability to reduce free chelatable iron, which was proven to play an active role in producing the highly toxic hydroxyl anion, the ability to scavenge superoxide anions and the ability to reduce lipid peroxidation.

Results obtained in this study indicate that the 4-hydroxyquinolines have antioxidative activity as it was shown to scavenge induced superoxide and peroxyl radicals, reduce free chelatable iron and inhibit induced lipid peroxidation. The nitro-4-hydroxyquinolines were the best scavengers of superoxide anions. However, the amino-4-hydroxyquinolines, especially with the amino group present in the 6 position, have the most promising potential for antioxidative activity.

All the compounds tested have the ability to scavenge induced peroxyl radicals. Compounds containing substituents in the 6 position showed more oxygen radical absorbance capacity than the 7-isomers, as well as significantly more iron reducing power. The amino compounds had more activity compared to the other compounds, and furthermore, 6-amino-4-hydroxyquinoline showed more radical absorbance capacity as well as ferric reducing power than the rest of the compounds. All the test compounds significantly curbed the lipid peroxidation induced *in vitro* by 1mM KCN in a dose dependent manner. The compounds substituted in the 6 position have more activity and the amino-4-hydroxyquinolines offered the most protection *in vitro*. In accordance to the *in vitro* studies, 6-amino- and 6-dibuthylamino-4-hydroxyquinolines reduced lipid peroxidation *in vivo*, induced intrastriatally with MPP^+.

The increase in superoxide level induced by 1mM KCN as well as MPP^+, *in vitro* and *in vivo* respectively, was significantly curbed by all the test compounds. *In vitro*, 7-nitro-4-hydroxyquinoline showed to be the best scavenger of superoxide anions, as it was the only compound able to reduce the increased level of superoxide anions in a dose-dependent manner to a level below that of the control. In contrast to the *in vitro* study, the dibuthylamino-4-hydroxyquinolines offer the most protection *in vivo*.

Because intraperitoneal treatment with 4-hydroxyquinolines reduced the level of superoxide anion generation and lipid peroxidation induced intrastriatally with MPP^+, it can be assumed that these compounds crossed the blood brain barrier.

From this study it is possible to conclude that 4-hydroxyquinolines exert various antioxidative properties and may thus be used in the development of antioxidant strategies against neurodegenerative diseases associated with oxidative stress.

UITTREKSEL

Suurstof, hoewel van kardinale belang vir menslike oorlewing, is die hoofbron van vry radikale wat skade aan essensiële molekules kan veroorsaak. Produksie van vry radikale is gekoppel aan normale prosesse in die lewendige sel, dus het eukariotiese selle 'n spesifieke antioksidant stelsel ontwikkel wat die toksiese bedreiging van vry radikale stuit, en sodoende essensiële komponente teen oksidatiewe skade beskerm. Wanbalans tussen die vlak van vry radikale en antioksidante veroorsaak 'n uiters gevaarlike toestand bekend as oksidatiewe stres. Oksidatiewe stres speel 'n kritiese rol in die verouderingsproses asook in verskeie neurodegeneratiewe toestande. Dit is van uiterse belang om verbindings met antioksidatiewe eienskappe te identifiseer, om die aanval op biologiese molekules teen te werk en toestande soos Alzheimer en Parkinson se siektes te voorkom.

Verskeie studies het getoon dat hidroksiekinoliene biologiese sisteme teen geïnduseerde oksidatiewe skade beskerm. Daarom is 'n reeks 4-hidroksikinoliene geselekteer en gesintetiseer ten einde die antioksidanteienskappe daarvan te bepaal.

4-Hidroksikinoliene met 'n nitro-, amino- en dibutielaminogroep in die 6 of 7 posisie, respektiewelik, is gesintetiseer volgens die Gould-Jacobs reaksie, gevolg deur 'n aantal transformasie reaksies en die strukture daarvan is bevestig met behulp van fisiese data. Die antioksidant effekte is bepaal in terme van suurstofradikaalabsorberingskapasiteit, die vermoë om vry cheleerbare yster, wat 'n aktiewe rol speel tydens die produksie van die toksiese hidroksielioon, te reduseer asook die vermoë om superoksiedanione op te ruim en lipiedperoksidase te inhibeer.

Resultate het getoon dat hierdie 4-hidroksikinoliene antioksidant aktiwiteit het deurdat dit superoksied sowel as peroksielradikale opruim, vry cheleerbare yster reduseer en geïnduseerde lipiedperoksidase inhibeer. Die nitro-4-hidroksikinoliene was die beste opruimers van superoksiedanione, maar die amino-verbindings, veral die met die amino-groep in die 6 posisie het die mees belowende potensiaal vir antioksidant aktiwiteit getoon.

Al die toetsverbindings het die vermoë getoon om geïnduseerde peroksielradikale te absorbeer. Kinoliene met die substituent op die 6 posisie toon meer absorberingsvermoë as die 7-isomeer, asook beter ysterreduserende vermoë. Die aminoverbindings het meer aktiwiteit getoon in vergelyking met die ander toetsverbindings, en veral 6-amino-4-hidroksikinolien het meer radikaalabsorberingskapasiteit en ysterreduserings vermoë gehad. Alhoewel al die getoetsde 4-hidroksikinoliene die 1mM kaliumsianied geïnduseerde lipiedperoxidase *in vitro* betekenisvol in 'n konsentrasie–afhanklike manier gestuit het, behalwe in die geval van 7-nitro-4-hidroksikinolien, het die 6-gesubstitueerde hidroksikinoliene meer aktiwiteit getoon en die amino-4-hidroksikinolien het die meeste beskerming getoon. In ooreenstemming met die *in vitro* studie, het die 6-amino en 6-dibutielamino-hidroksiekinoliene *in vivo* die lipiedperoksidase, intrastriataal geïnduseer deur MPP^+, betekenisvol verminder.

Die vlak van superoksiedanione, geïnduseer deur 1mM kaliumsianied *in vitro* en MPP^+ *in vivo* is betekenisvol verlaag deur al die toetsverbindings. 7-Nitro-4-hidroksikinolien was die beste opruimer van superoksiedanione, omdat dit die enigste verbinding was wat die vlak van superoksiedanione in 'n konsentrasie afhanklike manier kon verlaag tot 'n vlak laer as die van die kontrole. Hoewel al die verbindings *in vivo* die geïnduseerde vlak van superoksiedanione kon verlaag, het die dibutielamino-4-hidroksiekinoliene die beste beskerming getoon.

Intraperitoneale toediening van die 4-hidroksikinoliene het intrastriataal geïnduseerde superoksiedanione en lipiedperoksidase verlaag, dus kan dit afgelei word dat hierdie verbindings die bloedbreinskans gekruis het.

Hierdie studie toon dat 4-hidroksiekinoliene oor antioksidant aktiwiteit beskik en dus in die ontwikkeling van antioksidant strategieë teen neurodegeneratiewe siektes geassossieer met oksidatiewe stres, gebruik kan word.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

°C	-	Degrees Celsius
α-TOH	-	α-Tocopherol
A	-	Absorbance
$A^{\bullet}$	-	Endogenous antioxidant radical
AA	-	Ascorbic acid
AAPH	-	2,2'-azo-bis(2-amidinopropane)hydrochloride
ADP	-	Adenosine diphosphate
ANOVA	-	One way analysis of variance
ATP	-	Adenosine triphosphate
AUC	-	area under curve
Bcl-2	-	B-cell leukaemia
BHT	-	Butylated hydroxytoluene
BSA	-	Bovine serum albumin
Ca^{2+}	-	Calcium
CoQ_{10}	-	Co enzyme Q_{10}
CQ	-	7-Chloro-4-hydroxyquinoline
CQCA	-	7-Chloro-4-hydroxyquinoline-3-carboxylic acid
CQCE	-	Ethyl-7-chloro-4-hydryoxyquinoline
Cu^{+}	-	Copper I
$CuSO_4.5H_2O$	-	Aqueous coppersulphate-solution
CuZn-SOD	-	Copper zinc superoxide dismutase
DAT	-	Dopamine transporter carriers
DEEMM	-	Diethyl ethoxymethylenemalonate
DPPC	-	Dipalmitoyl phosphatidylcholine
DSC	-	Differential scanning calorimetry
EC-SOD	-	Extracellular superoxide dismutase
EAA	-	Excitatory amino acid
Fe^{2+}	-	Ferrous (iron II)
Fe^{3+}	-	Ferric (iron III)
Fe^{3+}-$(TPTZ)_2$	-	Ferric tripyridyltriazine

Fe^{2+}-$(TPTZ)_2$	-	Ferrous tripyridtriazine
FQ	-	7-Fluoro-4-hydroxyquinoline
FQCA	-	7-Fluoro-4-hydroxyquinoline-3-carboxylic acid
FQCE	-	Ethyl-7-fluoro-4-hydryoxyquinoline
FRAP	-	Ferric reducing antioxidant power
g	-	Gram(s)
GSH	-	Glutathione (reduced)
GSSG	-	Oxidized glutathione
H_2O_2	-	Hydrogen peroxide
HCl	-	Hydrochloric acid
$HO_2^{\bullet}$	-	Hydroperoxyl radical
HOCl	-	Hypochlorite
i.m.	-	Intramuscular
iNOS	-	Inducible nitric oxide synthase
i.p.	-	Intraperitoneally
IR	-	Infra red
IRPs	-	Iron regulatory proteins
IRP1 and -2	-	Iron regulatory proteins 1 and 2
KCl	-	Potassium chloride
KCN	-	Potassium cyanide
kg	-	Kilogram
ℓ	-	Litre
L-Dopa	-	Levodopa
LH	-	Polyunsaturated fatty acids
$LOO^{\bullet}$	-	Peroxyl radical
LOOH	-	Polyunsaturated fatty acid
M	-	Molar concentration ($mole.l^{-1}$)
mM	-	Millimolar
MAO	-	Monoamine Oxidase
Mn-SOD	-	Manganese superoxide dismutase
MDA	-	Malondialdehyde

Min		Minutes
ml	-	Millilitre
μg	-	microgram
μl	-	Microlitre
mp	-	Melting point
$MPDP^+$	-	1-Methyl-4-phenyl-2,3-dihydropyridinium
MPP^+	-	1-Methyl-4-phenyl pyridinium
MPTP	-	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
$MPDP^+$	-	1-Methyl-4-phenyl-2,3-dihydropyridinium
mRNA	-	Messenger ribonucleic acid
MS	-	Mass spectrometry
NaCl	-	Sodium chloride
Na_2CO_3	-	Disodiumcarbonate solution
NAD	-	Nicotinamide adenine dinucleotide
NADPH	-	Reduced nicotinamide adenine dinucleotide phosphate
NaOH	-	Sodium hydroxide
NBD	-	Nitro-blue diformazan
NBT	-	Nitroblue Tetrazolium
NFκB	-	Nuclear factor κB
NAD	-	Nicotinamide adenine dinucleotide
nm	-	Nanometre
NMDA	-	N-methyl-D-aspartate
nmole	-	Nanomole
NMR	-	Nuclear magnetic resonance
NO	-	Nitric oxide
NOS	-	Nitric oxide synthase
iNOS	-	Inducible Nitric oxide synthase
nNOS	-	Neuronal nitric oxide synthase
$O_2^{\bullet-}$	-	Superoxide anion
$ONOO^-$	-	Peroxynitrite
ORAC	-	Oxygen radical absorbance capacity

PARP	-	Poly(ADP-ribose) polymerase
PBS	-	Phosphate buffered saline
Pd/C	-	Palladium on carbon
PGE_2	-	Prostaglandin E_2
p.o.	-	Orally
ppm	-	Parts per million
PTP	-	Permeability transition pore
PUFA	-	Polyunsaturated fatty acid
$R^{\bullet}$	-	Carbon radial
RFU	-	Relative fluorescence units
RMCD	-	Randomly methylated β-cyclodextrin
$ROO^{\bullet}$	-	Peroxyl radical
S.E.M.	-	Standard Error of Means
SN	-	Substantia nigra
SN/pc	-	Substantia nigra pars compacta
SOD	-	Superoxide dismutase
TBA	-	Thiobarbituric acid
TBA/MDA	-	Thiobarbituric acid-malondialdehyde
TBARS	-	Thiobarbituric acid reactive substances
TCA	-	Trichloroacetic acid
TEP	-	1,1,3,3-Tetraethoxypropane
TNF- α	-	Tumor necrosis factor- α
$TO^{\bullet}$	-	Tocopheroxyl-radical
TOH	-	α-Tocopherol
TPTZ	-	2,4,6-tripiridyl-*s*-triazine
w/v	-	Weight/volume

CHAPTER 1. INTRODUCTION

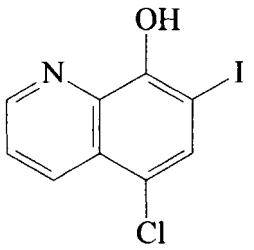
The vast majority of eukaryotic organisms require atmospheric oxygen in order to survive as oxygen is the terminal electron acceptor in the respiratory chain during the production of adenosine triphosphate (ATP), the energy stores used to drive chemical reactions within the cell (McCord, 2000; Seeley *et al.,* 2000; Melov, 2002). An inevitable consequence of respiration is the production of highly reactive oxygen species, which may cause oxidative damage to essential biological structures such as DNA, proteins and lipids and consequentially disrupt cellular function (Girotti *et al.*, 2002; Melov, 2002). Since free radical formation is linked to several normal cellular processes including cell metabolism and mitochondrial respiration, eukaryotes have evolved a specific antioxidant defence system which curb the toxic threat from reactive oxygen species, keeping it integrated with the pathways of healthy metabolism, thus protecting themselves against the detrimental effects induced by reactive oxygen species (Ashok and Ali, 1999, Kidd, 2000).

However, when the balance between the production of oxidants and the protective antioxidants is disturbed, oxidative stress is introduced. This can be extremely toxic to cells as it leads to rapid cell death (Giasson *et al.,* 2002; Jones *et al.*, 2002; Granot and Kohen, 2004). Oxidative stress is implicated in ageing as well as various neurodegenerative disorders (Schwemmer *et al.*, 2000; Naidu *et al.*, 2003).

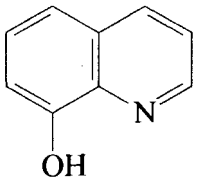
Parkinson's disease is one of the most debilitating diseases in the United States, and is highly age-dependent, probably due to accumulative oxidative damage and the steady decrease of antioxidant activity seen during the ageing process (Kidd, 2000). The hallmark of Parkinson's disease has been shown to be the dramatic selective degeneration of dopaminergic neurons in the substantia nigra, and it is to this profound reduction in dopamine content in the striatum to which most of the disabling abnormalities are attributed (Alexi *et al*, 2000; Kidd, 2000; Przedborski and Vila, 2001). However, iron-dependent oxidative stress, impaired mitochondrial function and alteration in the antioxidant defence system within the brain have also been shown to be major pathogenic factors of Parkinson's disease (Alexi *et al.*, 2000; Kidd, 2000; Giasson *et al.,* 2002).

Treatment is currently focused on replenishing the brain with dopamine, which initially alleviates clinical symptoms. However, these benefits rarely persist and in addition, this treatment does not alter the progressive degeneration of the dopaminergic neurons, nor does it address the closely associated oxidative stress. Taking this information in consideration, it is thus imperative to identify compounds with antioxidative activity, counteracting the attack of reactive oxygen species, preventing diseases associated with such attack.

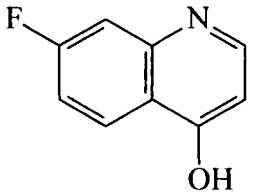
Several researchers have shown that compounds containing a quinoline moiety, such as clioquinol **(1)**, 8-hydroxyquinolines **(2)** and fluoro-4-hydroxyquinolines **(3)**, may act as free radical scavengers (Zheng *et al*, 2005), hence inhibit the highly deleterious reaction of self-perpetuating lipid peroxidation, and may thus have the potential to protect biological systems against induced oxidative damage (Liu *et al.*, 2002).



(1)



(2)



(3)

In this regard a series of 4-hydroxyquinolines, with a nitro-, amino- or dibuthylamino-group in the 6- or 7 positions was selected for this study in order to determine the antioxidative effects *in vitro* as well as *in vivo.*

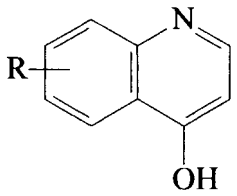
1.1 Research Objectives

The aim of this study was to investigate the possible free radical scavenging effects of the proposed 4-hydroxyquinolines.

The investigation comprised of a chemical component which included the synthesis, purification, and structural identification of a series of 4-hydroxyquinolines proposed for possible antioxidative effects. The biological component included the determination of the ability of the compounds to reduce induced oxidative damage, by screening for

oxygen radical absorbance capacity (ORAC) and ferric reducing/antioxidant power (FRAP), the ability to scavenge induced superoxide anions (NBT) as well as the ability to inhibit induced lipid peroxidation (TBARS).

Compounds **(4)-(9)** were proposed for this study. These compounds differ in substitution at positions 6 and 7. Functional groups at the different positions included nitro-, amino- and dibuthylamino substitution.



(4) R = 6-NO_2
(5) R = 7-NO_2
(6) R = 6-NH_2
(7) R = 7-NH_2
(8) R = 6-$N(C_4H_9)_2$
(9) R = 7-$N(C_4H_9)_2$

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

- Synthesis and purification of the proposed 4-hydroxyquinolines.
- Structural confirmation of the prepared 4-hydroxyquinolines by standard analytical techniques.
- *In vitro* determination of the ability to scavenge peroxyl radicals, induced by 2,2'-azo-bis(2-amidinopropane)hydrochloride, using the ORAC assay.
- Measuring the ability of the compounds to reduce iron as direct measurement of antioxidant activity using the FRAP assay, *in vitro*.
- *In vitro* determination of the ability of compounds to reduce superoxide anions induced by cyanide, according to the reduction of nitroblue tetrazolium to nitroblue diformazan (NBT assay).

- Determining whether these compounds cross the blood brain barrier to scavenge MPP^+-induced superoxide anions *in vivo*, using the nitroblue-tetrazolium assay.

- Determining the ability of the compounds to inhibit lipid peroxidation induced *in vitro* with potassium cyanide, in terms of reducing the levels of malondialdehyde equivalents, by means of the thiobarbituric acid reactive substances assay.

- Determining whether these compounds would reduce MPP^+-induced lipid peroxidation *in vivo* via the thiobarbituric acid reactive substances assay.

CHAPTER 2. LITERATURE REVIEW

2.1 Reactive Oxygen Species, Reactive Nitrogen Species and Free Radicals

Oxygen, present in the atmosphere as a stable triplet biradical ($^3O_2$) in the ground state is a vital component for the survival of the human being (McCord, 2000, Gülçin *et al.*, 2002). Although vital for human survival, molecular oxygen is also the main source of deleterious free radicals and reactive oxygen species in the biological system (Inal *et al.*, 2001; Akyol *et al.*, 2002).

The vast majority of eukaryotic organisms require atmospheric oxygen in order to survive (Melov, 2002). Oxygen is the terminal electron acceptor in the respiratory chain of the mitochondria during the production of adenosine triphosphate (ATP), energy stores that is used to drive chemical reactions within the cell (McCord, 2000; Seeley *et al.*, 2000; Melov, 2002). An unavoidable by-product during this respiration is the production of reactive oxygen species, primarily within the matrix of the mitochondria (Melov, 2002).

Free radicals, defined as any molecular species capable of independent existence, contain at least one unpaired electron in an atomic orbital and it is this unpaired electron that results in certain common properties shared by most radicals (Karlson, 1997; Girotti *et al.*, 2002; Young and Woodside, 2001). Many radicals are highly reactive and can either donate an electron to or extract an electron from other molecules, thus behaving as oxidants or reductants (Young and Woodside, 2001; Girotti *et al.*, 2002). As a result of this high reactivity, most radicals are usually unstable and have very short half lives in biological systems; however, certain species may survive for much longer (Girotti *et al.*, 2002; Young and Woodside, 2001). Oxygen-centred free radicals are referred to as a reactive oxygen species, and have a high degree of electrophilicity, giving them the ability to oxidize other molecules, making it the most important free radicals in many disease states (Driver *et al.*, 2000; Young and Woodside, 2001).

A free radical is formed when a covalent bond between two entities is broken and one electron remains with each newly formed entity (Karlson, 1997).

Biological structures such as nucleic acids, proteins and lipids can be damaged and cellular functions may be disrupted (Girotti *et al.*, 2002). A highly reactive free radical would take an electron from another molecule, leaving the latter as an electron-deficient free radical (Lee, 2004). This newly formed radical then acts to return to its ground state by stealing electrons with antiparallel spins from surrounding cellular structures, and may in this way start a chain reaction involving oxidation processes leading to a cascade and finally resulting in the disruption of a living cell (Goldfarb, 1999; Girotti *et al.*, 2002; Lee, 2004).

2.1.1 Production of Reactive Oxygen Species

Free radical formation is linked to several normal cellular processes including cell metabolism, mitochondrial respiration, prostaglandin synthesis and phagocytosis, which may increase in the maturing brain (Alexi *et al.*, 2000; Allen and Tresini, 2000; Driver *et al.*, 2000; Tahara *et al.*, 2001; Giasson *et al.*, 2002; Kim *et al.*, 2002; Parikh *et al.*, 2003).

Oxidants can be classified in various ways; according to their reactivity towards biological targets, their chemical nature or according to their belonging to a radical or non-radical subgroup. The radical group includes species such as superoxide ($O_2^{\bullet-}$), hydroxyl ($OH^{\bullet}$), and nitric oxide radicals (Granot and Kohen, 2004). The non-radical oxidant group includes metabolites like hydrogen peroxide, hypochloric acid and aldehydes (Granot and Kohen, 2004). These species can cause damage on their own or may serve as a source for more reactive and damaging species (Granot and Kohen, 2004).

2.1.2 Sources of Reactive Oxygen Species

While the molecular divalent oxygen is the main source of reactive oxygen species, radical formation in the body occurs by several mechanisms, involving both endogenous and environmental factors (Fig. 2.1) (Inal *et al.*, 2001; Akyol *et al.*, 2002).

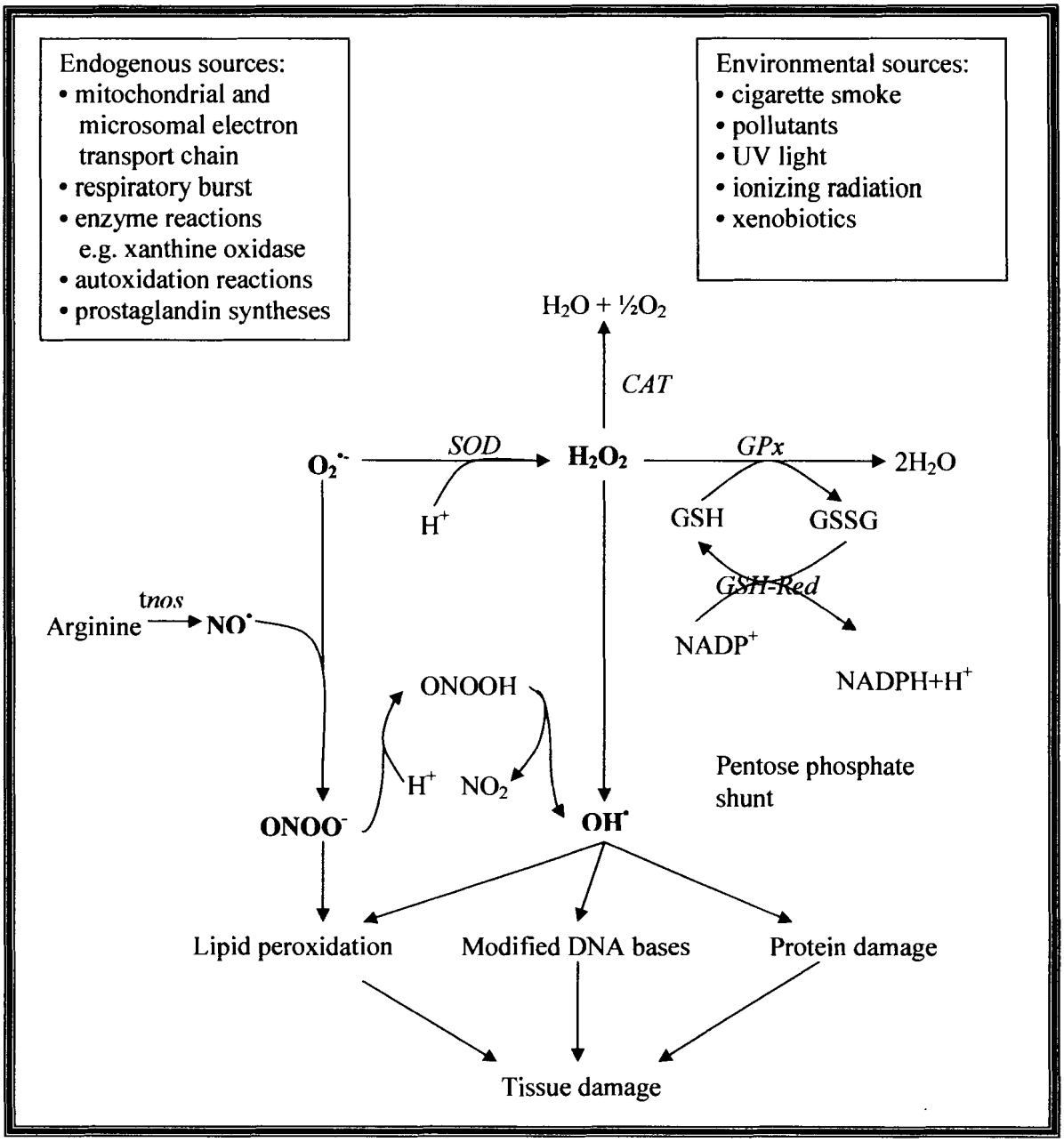


Figure 2.1 Schematic representation of the relationships among reactive oxygen species formation, enzymatic antioxidant systems and the consequences of free radical damage (Young and Woodside, 2001; Akyol et al., 2002).

2.1.2.1 Endogenous sources

Significant levels of reactive oxygen species are produced within cells (Gaboriau *et al.*, 2002). In normal aerobic respiration, endogenous sources may include enzymes which can indirectly produce reactive oxygen species, stimulated polymorphonuclear leukocytes and macrophages, and peroxisomes (Gülçin *et al.*, 2002; Granot and Kohen, 2004).

(a) Autoxidation

Several molecules, including adrenaline, thiol compounds and glucose, can autooxidize in the presence of oxygen, producing superoxide anions. These reactions are greatly accelerated by the presence of transition metals such as iron or copper (Young and Woodside, 2001).

Dopamine has a strong tendency to spontaneously break down into oxidant metabolites due to "autooxidation". Most reactive among these auto-metabolites are 6-hydroxydopamine, quinone and dopamine aminochrome (Youdim *et* al., 1989; Kidd, 2000).

(b) Enzymatic oxidation

Multiple enzymes using molecular oxygen as a substrate can produce oxidants within cells (Finkel, 2003). Their generation leads off with the production of superoxide, by NADPH-oxidase of activated leukocytes, occurring at inflammatory sites, and/or by xanthine oxidase activated in ischemia/reperfusion. Over-dismutation either spontaneous or catalyzed by superoxide dismutase, leads to the generation of hydrogen peroxide (Janisch *et al.*, 2002).

Other enzymes which may play a role in the generation of reactive oxygen species are caspases and nitric oxide synthase (NOS) (Shou *et al.*, 2000; Alexi *et a.*, 2000; Granot and Kohen, 2004). Enzymes in the prostaglandin synthesis pathway, cyclooxygenases and lipooxygenase, are also involved in the formation of reactive oxygen species and can lead to lipid peroxidation (Akyol *et al.*, 2002).

(c) Respiratory burst

Neutrofils serve as major contributors of reactive oxygen species. Following activation, these cells undergo a respiratory burst resulting in the release of an efflux of oxidants as well as proteinase, cationic proteins and other compounds which may act synergistically to cause oxidative damage in tissues (Granot and Kohen, 2004). In the nervous system, activated

microglia also undergo respiratory burst activity and release superoxide anions in the environment of neurons (Agbas *et al.*, 2002).

(d) Subcellular organelles

Organelles such as mitochondria, microsomes, peroxisomes and nuclei have been shown to generate superoxide radicals (Halliwell, 1995).

Within the cell, the semi-independent organelles, mitochondria produce reactive species during normal aerobic respiration (Alexi *et al.*, 2000; Gaboriau *et al.*, 2002). Over 90% of the oxygen consumed by mammals is utilized by mitochondria, thus generating 90% or more of these species that make up the endogenous oxidative burden, making the mitochondria the primary site for the production of reactive oxygen species (Michaelis, 1998; Kidd, 2000; Agbas *et al.*, 2002; Melov, 2002; Somayajulu *et al.*, 2005). Of this 1-3% is diverted to form superoxide ($O_2^{\bullet-}$) (Harman, 1998).

Mitochondria have their own DNA and manage the oxidative phosphorylation process in which ATP is generated (Fig. 2.2) (Kidd, 2000; Somayajulu *et al.*, 2005).

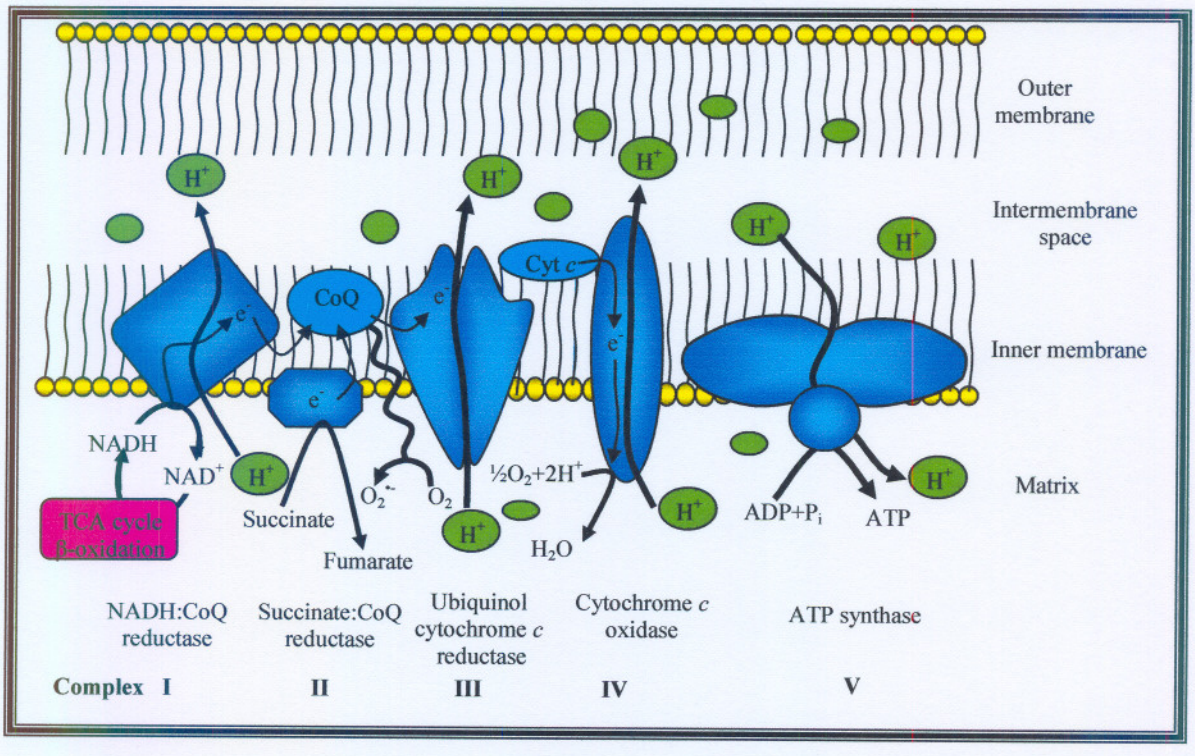


Figure 2.2 The mitochondrial electron transport complexes. (Kidd; 2000; Seeley *et al.*, 2000)

ATP, a ubiquitous store of energy is needed for transport across membranes for all synthetic processes and for the mechanical work involved in motor activities of the cell, thus driving all life processes. Energetically compromised mitochondria may thus have detrimental effects on the survival of the cell (Kidd, 2000; Maharaj, 2003; Somayajulu *et al.*, 2005).

The oxidative phosphorylation complexes are aggregates of enzymes, functionally linked and distributed in groups throughout the inner membranes of the mitochondria (Fig. 2.2) (Kidd, 2000). The complexes I, II, III, IV and V occur in spatial sequences that optimize electron transfer efficiency while minimizing the possibilities for single-electron "leakage" to oxygen that would generate reactive oxygen species (Kidd, 2000). The system is finely balanced: damage to any one complex both reduces ATP yield and worsens the inevitable leakage of oxidative species from the system (Kidd, 2000).

Disruption of the mitochondrial respiratory chain results in over-production of reactive oxygen species. These mitochondrial derived oxygen species are responsible for oxidative stress and the activation of apoptotic mediators, causing progressive and specific neuronal degeneration (Inal *et al.*, 2001; Delibas *et al.*, 2002; Gaboriau *et al.*, 2002; Somayajulu *et al.*, 2005). It is likely that the life span of an individual is determined by the rate of damage to the mitochondria, particularly to mitochondrial DNA, which is associated with progressively higher rates of $O_2^{\bullet-}$ or hydrogen peroxide production and decreased formation of ATP with age (Harman, 1998). Mitochondrial dysfunction has also been implicated in the neurodegeneration of Alzheimer's and Parkinson's diseases (Maharaj, 2003).

(e) Transition metal ions

Iron is carried by transferrin and is stored in the protein ferritin. However, a pool of non-protein-bound iron moving between transferrin, cell cytoplasm, mitochondria and ferritin provides iron as a catalyst for the Fenton reaction generating the highly toxic hydroxyl radical from hydrogen peroxide (Halliwell and Gutteridge, 1984; Prior and Cao, 1999; Ou *et al.*, 2001; Young and Woodside, 2001; Zheng *et al.*, 2005).

The pivotal role for iron in neurodegeneration has been strengthened by the identification of iron regulatory proteins 1 and 2 (IRP1 and IRP2) in various regions of rodent brain including the striatum and substantia nigra, as well as increased levels of iron in the substantia nigra of subjects suffering from Parkinson's disease (Alexi *et al.*, 2000; Shachar *et al.*, 2004).

The regulation of iron metabolism in mammalian cells is controlled by the interaction of IRPs with iron responsive elements and nitric oxide (Shachar *et al.*, 2004). In mammalian cells, IRP1 and 2 sense cytosolic iron levels and regulate expression of genes controlling iron metabolism and transport (Levine, 2002; Shachar *et al.*, 2004).

Although the iron binding proteins effectively chelate iron and prevent any appreciable redox effects under normal physiological conditions, this protection can break down in disease states (Young and Woodside, 2001). Neither Fe^{2+} nor Fe^{3+} is able to directly cause oxidative damage to essential biological molecules, but Fe^{2+} participate in the Fenton reaction creating the highly toxic hydroxyl radical and is therefore regarded as a source of reactive oxygen species (Prior and Cao, 1999; Young and Woodside, 2001).

Free iron is also thought to be pro-inflammatory, responsible for the activation of NFκB and increased release of cytotoxic cytokines and TNF- α, due to its cellular abundance, profound redox state, and decompartmentation from ferritin (Shachar *et al.*, 2004).

Another essential trace element, copper, a divalent cation is an important component in the brain as it is found to be a functional component of several important intracellular and extracellular proteins and enzymes, such as cytochrome oxidase, superoxide dismutase, ceruloplasmin and monoamine oxidase (Santamaria, *et al.*, 2003).

At neurochemical level copper is accumulated in synaptic vesicles and might be released from nerve terminals in a calcium-dependent manner, influencing neuronal transmitter systems. Low levels of copper may induce different neuropathological conditions (neurodegeneration, mental retardation, seizures, etc.) due to neurotoxic effects (Santamaria, *et al.*, 2003). The role of copper is analogous to that described for iron (Young and Woodside, 2001).

(f) Inflammation

Inflammatory processes all involve the release of reactive oxygen species, originating from respiratory burst in activated neutrophils (Janisch *et al.*, 2002; Granot and Kohen, 2004). Activated macrophages and polymorphonuclear cells also contribute to tissue damage in several inflammatory diseases by releasing highly reactive oxygen species (Malfroy *et al.*, 1997).

During chronic inflammation associated with different pathologies, nitric oxide (NO) production increases and the NO reacts with $O_2^{\bullet-}$ in the presence of iron yielding peroxynitrite and the hydroxyl radical, which can eventually cause severe damage to the DNA (Virgili *et al.*, 1998; Lee, 2004). Hydroxyl radicals are the major mediators of oxidative damage in this setting (Granot and Kohen, 2004). Also, during inflammation, reactive oxygen species may act as signalling molecules, contributing to cell injury and degenerative processes (Grimm, 2004).

2.1.2.2 Exogenous sources

Although free radical production occurs as a consequence of endogenous reactions and play an important role in normal cellular function, exogenous environmental factors can also promote radical formation (Young and Woodside, 2001). Exogenous sources of free radicals include air, tobacco smoke, ionizing radiation, certain pollutants, organic solvents, natural deleterious gases (e.g. ozone and high concentrations of oxygen or hyperbaric oxygen) and pesticides (Fig. 2.1) (Ghiselli *et al.*, 2000; Polidori *et al.*, 2001; Gülçin *et al.*, 2002; Granot and Kohen, 2004; Wan *et al.*, 2005).

(a) UV light

Ultraviolet light induces the formation of singlet oxygen and other reactive oxygen species in the skin (Young and Woodside, 2001).

(b) Pollutants

Atmospheric pollutants such as ozone and nitrogen dioxide cause radical formation and antioxidant depletion in bronchoalveolar lining fluid, and this may exacerbate respiratory disease (Young and Woodside, 2001).

(c) Cigarette smoke

Cigarette smoke contains over 4 000 chemical species, including high concentrations of oxidants, along with other toxins that may injure the respiratory tract (Young and Woodside, 2001; Carnevali *et al.*, 2003). This makes cigarette smoke a potent source of oxidative stress, and oxidation to structural and functional molecules, like DNA. Apoptosis has been shown to play a major role in the different toxic effects of cigarette smoke (Carnevali *et al.*, 2003).

(d) Xenobiotics

Various xenobiotics, including paraquat, paracetamol, bleomycin and anthracyclines, cause tissue damage as consequence of free radical generation, (Young and Woodside, 2001).

(e) Drugs

Chronic treatment with neuroleptics, such as haloperidol, increases the production of cytotoxic free radicals and suppresses the activity of certain detoxifying enzymes, such as superoxide dismutase, leaving cells unprotected especially if basal enzyme activity is low or the free radical-scavenging mechanisms are less effective, therefore increasing oxidative stress. Associated neuronal loss in the striatum has been reported in animals treated chronically with neuroleptics (Naidu *et al.*, 2003).

Haloperidol causes a sequence of cellular alteration that leads to cell death and oxidative stress due to increased reactive oxygen species generation from the mitochondria. Alterations in the activity of antioxidative enzymes play an integral part of this cascade (Akyol *et al.*, 2002; Parikh *et al.*, 2003; Naidu *et al.*, 2003).

Chlorpromazine metabolites have also been suggested to generate hydrogen peroxide by autoxidation (Parikh *et al.*, 2003).

(f) Ionizing Radiation

Ionizing radiation generates ions and leads to the formation of free radicals and aqueous electrons, which reacts with oxygen to produce superoxide radicals, which in turn may react with each other to produce hydrogen peroxide and singlet oxygen (Wan *et al.*, 2005).

2.1.3 Types of free radicals

2.1.3.1 Superoxide anion

Mitochondria is known to be the major source of superoxide anion ($O_2^{\bullet-}$) generation within the cell (Michaelis, 1998; McCord, 2000).

Superoxide ($O_2^{\bullet-}$) is produced by the addition of a single electron to oxygen, and several mechanisms exist by which $O_2^{\bullet-}$ can be produced *in vivo*. During the process of ATP production, the electron transport chain in the inner mitochondrial membrane performs the reduction of oxygen to water (Fig. 2.1 and 2.2), in addition free radical intermediates are

generated, which are tightly bound to the components of the transport chain. However, there is a constant leak of a few electrons into the mitochondrial matrix, and this result in the formation of superoxide anions (Young and Woodside, 2001).

Because increases in intracellular calcium (Ca^{2+}) accumulation are handled by energy-dependent transport of Ca^{2+} into mitochondria, it is likely that excessive Ca^{2+} accumulation in the cytoplasm of neurons may lead to enhanced mitochondrial electron transport and thus the formation of $O_2^{\bullet-}$ (Michaelis, 1998).

Another way of $O_2^{\bullet-}$ formation is via lipid metabolism (Michaelis, 1998). For example, entry of Ca^{2+} into neurons activates the enzyme phospolipase A_2, leading to the formation of arachidonic acid and, consequently, the metabolism of arachidonic acid, by cyclooxygenases and lipooxygenase leads to the generation of $O_2^{\bullet-}$ (Michaelis, 1998; Akyol *et al.*, 2002).

The activity of several other enzymes, such as cytochrome P_{450} oxidase in the liver and enzymes involved in the synthesis of adrenal hormones, also results in the leakage of a few electrons into the surrounding cytoplasm and hence superoxide generation (Young and Woodside, 2001). Macrophages and other phagocytic white blood cells generate superoxide, using a membrane-associated NADPH oxidase that directly reduces molecular oxygen (Heinecke, 2002):

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

The toxicity of $O_2^{\bullet-}$ is evident in its ability to inhibit enzymes, attenuating vital metabolic pathways, as well as its ability to damage biological macromolecules. Superoxide is also known to be a mediator of inflammation (McCord, 2000).

2.1.3.2 Hydrogen peroxide

Any biological system generating superoxide will also produce hydrogen peroxide (H_2O_2) as a result of a spontaneous dismutation of the superoxide anion (Heinecke, 2002; Young and Woodside, 2001):

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

In addition, several enzymatic reactions, including those catalyzed by glycolate oxidase and D-amino acid oxidase, might produce H_2O_2 directly (Young and Woodside, 2001).

H_2O_2 is not a free radical itself, but is usually included under the general heading of reactive oxygen species (Young and Woodside, 2001; Wan *et al.*, 2005), as a pro-oxidant molecule, because it is the substrate for the Fenton reaction that generates the highly reactive hydroxyl radical (Bush, 2002).

H_2O_2 may also contribute to the generation of hypoclorite (HOCl) via myeloperoxidase on Cl^- ions in activated leukocytes (Janisch *et al.*, 2002).

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

H_2O_2 is freely permeable across all cell membranes (Halliwell, 1995) and due to this ability, H_2O_2 formed in one location might diffuse a considerable distance before reacting with reduced metal ions (Fe^{2+}, Cu^+) to decompose, yielding the highly reactive hydroxyl radical (Bush, 2002; Young and Woodside, 2001), which is likely to mediate most of the toxic effects ascribed to H_2O_2. Therefore, H_2O_2 acts as a conduit to transmit free radical induced damage across the cell compartments and between cells (Young and Woodside, 2001).

H_2O_2 mediates oxidative stress and subsequent neuronal death, which partly mimic the dopamine response. However, this effect is entirely due to the oxidative properties of the peroxide and independent of the dopamine D1 receptor (Wersinger *et al.*, 2004). H_2O_2-mediated neurotoxicity does not require protein kinase A and only part of the H_2O_2 effects are mediated by NOS (Wersinger *et al.*, 2004). Due to the fact that H_2O_2 is a weak oxidizing agent, it may damage proteins and enzymes containing reactive thiol groups (Young and Woodside, 2001). The toxicity of H_2O_2 is observed in the induction of base oxidation and single strand breaks (Collins *et al.*, 1997) and by the induction of apoptotic stimuli that depend on the mitochondrial respiratory chain (Somayajulu *et al.*, 2005).

2.1.3.3 Hydroxyl radical

The hydroxyl radical ($OH^\bullet$) is the most reactive of all the reactive oxygen species (Ashok and Ali, 1999; Janisch *et al.*, 2002), and the strongest oxidant in the body and cells do not have an enzymatic defence system against this radical (Delibas *et al.*, 2002).

The most important mechanism of generating the $OH^\bullet$ radical *in vivo* is the transition metal catalyzed decomposition of superoxide and hydrogen peroxide. Hydrogen peroxide can react with iron II (Fe^{2+}) or copper I (Cu^+) to generate the hydroxyl radical, a reaction first described by Fenton in 1894 (Young and Woodside, 2001):

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

Superoxide and hydrogen peroxide can react directly to produce the $OH^{\bullet}$ radical, but the rate constant for this reaction in aqueous solution is virtually zero. However, if transition metal ions are present, a reaction proceeding at a rapid rate is established. The net result of the reaction sequence illustrated below is known as the Haber-Weiss reaction (Young and Woodside, 2001):

$Fe^{3+} + O_2^{\cdot} \rightarrow Fe^{2+} + O_2$

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

Net result

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

Hydroxyl radicals may also be formed due to background exposure to radiation, the reaction of $NO^{\bullet}$ with $O_2^{\bullet-}$ and the reaction of hypochloric acid with $O_2^{\bullet-}$ (Halliwell, 1995).

The hydroxyl radical is probably the final mediator of most free radical induced tissue damage. $OH^{\bullet}$ reacts, with extremely high rate constants, with almost every type of molecule found in living cells including sugars, amino acids, lipids, especially polyunsaturated fatty acids (PUFAs), nucleotides and enzymes. It is thought that the reactive $OH^{\bullet}$ initiates a process of lipid peroxidation that results in cell membrane fluidity and finally cell death by a further cascade of events (Young and Woodside, 2001, Janisch *et al.*, 2002; Shachar *et al.*, 2004).

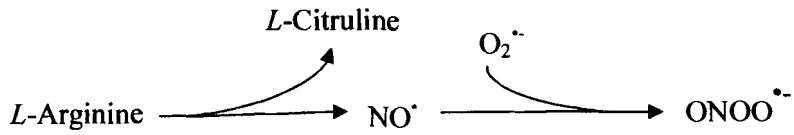
$OH^{\bullet}$ reacts rapidly with deoxyribose and DNA bases (Ashok and Ali, 1999). These modified bases are eliminated by DNA repair enzymes (Ashok and Ali, 1999), inducing radical chain reactions with a multitude of organic molecules (Janisch *et al.*, 2002).

$OH^{\bullet}$ can be scavenged by hydrogen abstraction, addition and electron transfer (Janisch *et al.*, 2002).

2.1.3.4 Nitric Oxide

Nitric oxide (NO) is a molecule that is known to be both a reactive oxygen species and a universal neurotransmitter in the central - and the peripheral nervous system. Nitric oxide is a free radical with a short half life and acts independently; it may also cause neuronal damage in cooperation with other reactive oxygen species (Akyol *et al.*, 2002; Bellé *et al.*, 2004).

Nitric oxide is produced in mammalian cells constitutively or can be induced by various cell activators through the oxidation of $_L$-arginine by the NOS enzymes (Virgili *et al.*, 1998; Akyol *et al.*, 2002).



The toxicity of nitric oxide is believed to involve the formation of superoxide anions by neurons and the reaction of NO with $O_2^{\bullet-}$ to form a powerful oxidizing intermediate, peroxynitrite ($ONNO^-$) and possibly hydroxyl radicals (Michaelis, 1998; Virgili *et al.*, 1998; Akyol *et al.*, 2002; Heinecke, 2002).

This makes the highly reactive nitric oxide a potent pro-oxidant molecule, as the hydroxyl radical formed from the peroxynitrate radical (Fig.2.1) is highly cytotoxic and can result in profound cellular injury and cell death (Virgili *et al.*, 1998; Akyol *et al.*, 2002).

2.1.3.5 Peroxynitrite

Arising from $O_2^{\bullet-}$ and NO, both occurring in activated leukocytes (Janisch *et al.*, 2002), peroxynitrite ($ONOO^-$) is a harmful compound to cellular structures (Akyol *et al.*, 2002).

Peroxynitrite is a strong oxidizing agent that can lead to efficient oxidation of proteins, lipid peroxidation chain reactions, DNA fragmentation and enhanced apoptosis in many systems (Michaelis, 1998; Schwemmer *et al.*, 2000; Janisch *et al.*, 2002). Peroxynitrite and its products have been linked to several interactions that may contribute to cellular injury, including inactivation of sodium channels, and interactions with different metals with redox potential such as iron and copper (Akyol *et al.*, 2002).

Peroxynitrite also causes nitration of proteins – especially tyrosine residues, to form nitrotyrosine (Przedborski and Vila, 2001; Akyol *et al.*, 2002). Nitrotyrosine can be harmful as it can inactivate enzymes and receptors that depend on tyrosine residues for their activity and prevent phosphorylation of tyrosine residues important for signal transduction (Przedborski and Vila, 2001; Heinecke, 2002).

This molecule can cause DNA strand breaks and activation of poly ADP ribosyl synthetase (PARS) activity, leading to the phenomenon of "PARS suicide", a form of cell death that has the characteristics of apoptosis (Michaelis, 1998).

$ONOO^-$ is also an indicator for inflammation (Michaelis, 1998; Janisch *et al.*, 2002).

2.2 Antioxidants

All the cells in the body that generate life energy are chronically exposed to oxidants from both endogenous and exogenous sources, making the resultant oxidative burden an obligatory, unavoidable by-product of aerobic respiration (Kidd, 2000; Zaidi and Banu, 2004).

Because reactive oxygen species have the capacity to react in an indiscriminate manner leading to damage to almost any cellular component, eukaryotes have evolved a specific antioxidant defence system which curb the toxic threat from reactive oxygen species, keeping it integrated with the myriad pathways of healthy metabolism (Kidd, 2000; Young and Woodside, 2001), thus protecting themselves against the detrimental effects induced by oxidants (Ashok and Ali, 1999; Prior and Cao, 1999; Inal *et al.*, 2001; Tahara *et al.*, 2001; Gülçin *et al.*, 2002; Melov, 2002; Zaidi and Banu, 2004).

Such defence systems include enzymes like superoxide dismutase (SOD) and a host of other proteins and peptides, like vitamin E and glutathione (Ashok and Ali, 1999; Granot and Kohen, 2004), with the function to reduce the cumulative load of reactive oxygen species within the cell, or intracellular space (Melov, 2002).

2.2.1 Definition of antioxidants

An antioxidant can be defined as a substance, which when present at low concentrations compared with those of an oxidizable substrate (essential biological structures) significantly delays or prevents the pro-oxidant initiated oxidation of that substrate (Halliwell, 1995; Harman, 1998; Prior and Cao, 1999; Aruoma, 2003; Young and Woodside, 2001).

Antioxidative molecules exhibit scavenging and chelating properties, removing reactive oxygen species and catalytic metal ions involved in the Fenton reaction and in this way interfering with the oxidation process caused by such deleterious species (Gaboriau *et al.*, 2002; Gülçin *et al.*, 2002; Aruoma, 2003; Lee, 2004; Somayajulu *et al.*, 2005). Therefore, reactive oxygen species produced as by-products of the mitochondrial electron transport chain are quenched by antioxidants and converted to non-toxic compounds by free radical scavenging enzymes.

Antioxidant molecules have loosely attached electrons, and can function as electron donors without becoming electron-deficient free radicals themselves (Lee, 2004). Thereby it reduces a pro-oxidant with the formed products having no or low toxicity (Prior and Cao, 1999). In this regard, acidic compounds (including phenols) usable in foods, cosmetics and pharmaceutical preparations, which can readily donate an electron or a hydrogen atom to a peroxyl radical to terminate a lipid peroxidation chain reaction, regenerate a phenolic compound or effectively chelate a pro-oxidant transition metal may also be classified as antioxidants (Gülçin *et al.*, 2002; Aruoma, 2003). Phenolic compounds function as free radical terminators (Gaboriau *et al.*, 2004).

Antioxidants also act by inhibiting the formation of reactive oxygen species (Gaboriau *et al.*, 2002; Aruoma, 2003).

The physiological role of antioxidants, according to definition, is to prevent damage to cellular components arising as a consequence of chemical reactions involving free radicals (Young and Woodside, 2001). To prevent progressive neuronal loss based on antioxidant activity, the antioxidant must be able to cross the blood brain barrier and occur at the respective brain region for neuroprotection (Aruoma, 2003). Antioxidants that accumulate in brain and neuronal tissue are potential candidates for prevention or treatment of disorders involving oxidative damage (Arivazhagan *et al.*, 2002).

2.2.2 Types of Antioxidants

Antioxidants can be divided into three main groups: chain breaking antioxidants, enzymatic antioxidants and transition metal binding proteins (Fig. 2.3) (Young and Woodside, 2001).

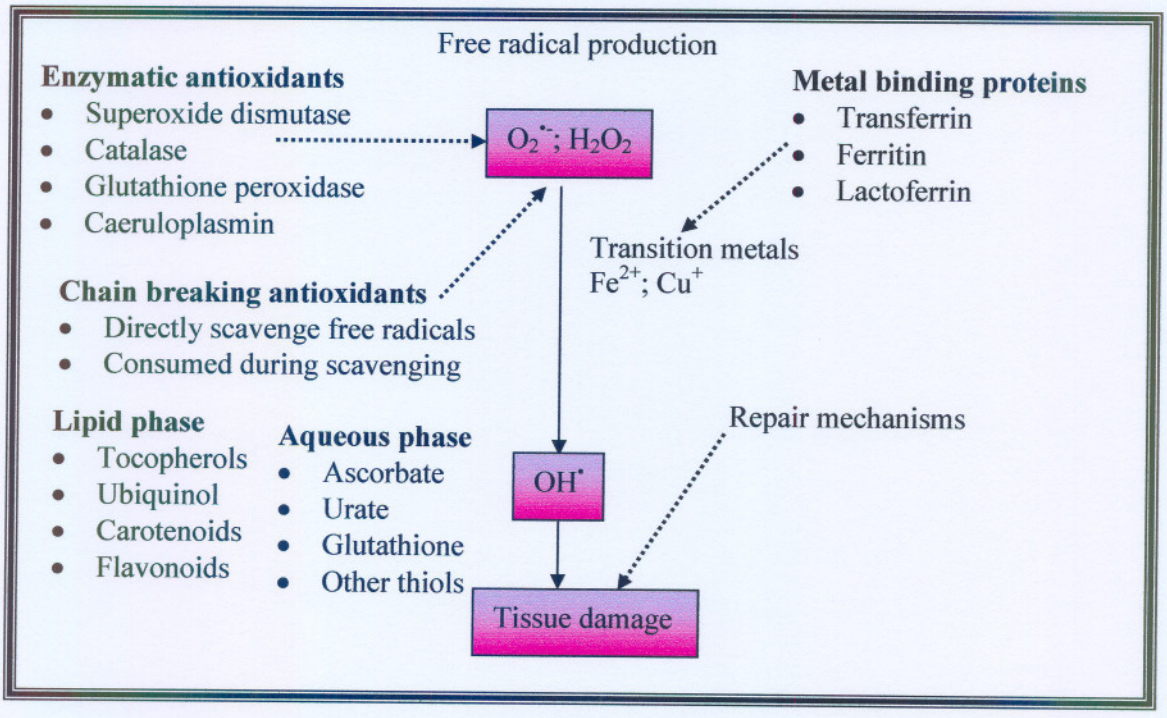


Figure 2.3 Antioxidant defences against free radical attack (Young and Woodside, 2001).

2.2.2.1 Chain breaking antioxidants

The human body contains a variety of radical-scavenging antioxidants, including glutathione, uric acid, α-tocopherol (vitamin E), ascorbic acid (vitamin C), β-carotene and flavonoids (Prior and Cao, 1999; Aruoma, 2003), which constitute an important aspect of the antioxidative defence system (Prior and Cao, 1999).

Whenever a free radical interacts with another molecule, secondary radicals may be generated that can then react with other targets to produce yet more radical species. The classic example of such a chain reaction is lipid peroxidation, and the reaction will continue to propagate until two radicals combine to form a stable product or the radicals are neutralized by a chain breaking antioxidant (Young and Woodside, 2001). Chain breaking antioxidants shorten the propagation phase of lipid peroxidation (Harman, 1998).

Chain breaking antioxidants are powerful electron donors and react preferentially with free radicals before important target molecules are damaged. In doing so, the antioxidant is oxidized and must be regenerated or replaced. By definition, the antioxidant radical is relatively unreactive and unable to attack further molecules (Harman, 1998; Young and Woodside, 2001).

Chain breaking antioxidants can also receive an electron from a radical with the formation of stable products (Harman, 1998; Young and Woodside, 2001). In general, the charge associated with the presence of an unpaired electron becomes dissociated over the scavenger and the resulting product will not readily accept an electron from or donate an electron to another molecule, preventing the further propagation of the chain reaction (Young and Woodside, 2001).

Chain breaking antioxidants can conveniently be divided into aqueous phase and lipid phase antioxidants (Fig 2.3) (Young and Woodside, 2001).

(a) Lipid phase chain breaking antioxidants

These antioxidants scavenge radicals in membranes and lipoprotein particles and are crucial in preventing lipid peroxidation. The most important lipid phase antioxidant is probably vitamin E (Young and Woodside, 2001).

(i) Vitamin E

Vitamin E is the most widely used antioxidant in clinical trials (Heinecke, 2002), and occurs in nature in eight different forms (tocopherols), which differ greatly in their degree of biological activity (Young and Woodside, 2001).

α-Tocopherol is the most potent antioxidant of the tocopherols and is the most abundant in humans (Laranjinha *et al.*, 1995; Young and Woodside, 2001). As an antioxidant, α-tocopherol react more rapidly with peroxyl radicals, than does polyunsaturated fatty acids, forming a relatively stable tocopheroxyl radical, with an extra electron being dispersed across the chromanol ring (Laranjinha *et al.*, 1995; Aruoma, 2003; Young and Woodside, 2001), hence acting to break the chain reaction of lipid peroxidation by trapping the peroxyl radicals. α-Tocopherol will not prevent the initial formation of carbon centred radicals in a lipid rich environment, but does minimise the formation of secondary radicals (Young and Woodside, 2001).

(ii) Carotenoids

The carotenoids are a group of lipid soluble antioxidants based on an isoprenoid carbon skeleton and the most important of these is β-carotene, although at least 20 other may be present in membranes and lipoproteins (Young and Woodside, 2001).

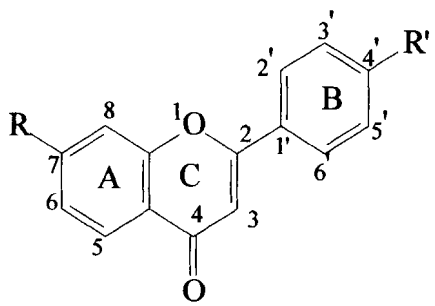
These are efficient scavengers of singlet oxygen, but can also trap peroxyl radicals at low oxygen pressure with efficiency at least as great as that of α-tocopherol. Carotenoids may play a role in preventing lipid peroxidation *in vivo* and plays an important role as precursors of vitamin A (retinol), which also has antioxidant properties (Young and Woodside, 2001).

Carotenoids react with peroxyl radicals (e.g. benzylperoxyl radicals) only slightly more reactive than lipid peroxyl radicals neither by electron transfer nor by hydrogen atom abstraction, but by adduct formation (Aruoma, 2003). Therefore, the ability of carotenoids to scavenge singlet oxygen suggests different reaction mechanisms (Arriaga-Alba *et al.*, 2000; Aruoma, 2003).

(iii) Flavonoids

People who drink wine occasionally seem to be less prone to develop senile dementia and Alzheimer's disease. Flavonoids are ubiquitously distributed in foods of plant origin such as vegetables, fruit, tea as well as in olive oil and red wine (Ho and Saville, 2001; Gaboriau *et al.*, 2002; Roig *et al.*, 2002; Aruoma, 2003; Naidu *et al*, 2003; Kang *et al.*, 2004; Young and Woodside, 2001).

Flavonoids have various biological and pharmacological activities. Researchers hypothesize that the beneficial health effects, as well as the neuroprotective effects are attributed to the anti-inflammatory, antioxidant activity and free radical scavenging properties of flavonoids (Gaboriau *et al.*, 2002; Heim *et al.*, 2002; Kang *et al.*, 2004).



(10)

Flavonoids (**10**) are a broad class of naturally occurring low molecular weight polyphenolic diphenylpropanes, characterized by a flavan nucleus (Gaboriau *et al.*, 2002; Heim *et al.*, 2002) bearing one or more hydroxyl groups and are therefore potentially able to quench free radicals by forming resonance-stabilized phenoxyl radicals (Packer *et al.*, 1999).

Structurally flavonoids have variations in the C ring that characterizes the different types namely, flavonols, flavones, isoflavones, flavonones, flavanol and anthocyanins (Cai *et al.*, 1997; Aruoma, 2003; Kang *et al.*, 2004; Kuti, 2004).

Flavonoids has several mechanisms by which it can act as antioxidants. Quercetin, have been reported to efficiently quench reactive nitrogen species, such as NO (Virgili *et al.*, 1998; Packer *et al.*, 1999). Flavonoids may also scavenge free radicals, with the formation of stable oxidized products (Virgili *et al.*, 1998; Roig *et al.*, 2002; Naidu *et al.*, 2003), act as radioprotectors (Cai *et al.*, 1997), alter glutathione metabolism, quench reactive oxygen species, like the superoxide- and hydroxyl radicals (Packer *et al.*, 1999) or inhibit Ca^{2+} influx (Gaboriau *et al.*, 2002) and activate detoxifying/defence proteins (Roig *et al.*, 2002). Another suggested mechanism of the antioxidant action of flavonoids involves the suppression of free radical generating enzymes (Naidu *et al.*, 2003).

Some authors proposed that the ability of flavonoids to provide health benefits may be because of the ability of their metabolites to interact with cell-signalling cascades, to influence the cell at a transcriptional level and to down-regulate pathways leading to cell death (Roig *et al.*, 2002; Kang *et al.*, 2004).

Another postulated mechanism is transition metal chelation, preventing the conversion of H_2O_2 to $OH^{\cdot}$ (Roig *et al.*, 2002; Naidu *et al.*, 2003). Phenolic molecules may behave as metal chelators owing to their catechol structure (Packer *et al.*, 1999; Gülçin *et al.*, 2002), which is also known to give excellent radical scavenging properties (Ishige *et al.*, 2001).

The liver is the key organ in the metabolism of flavonoids and is extensively exposed to the production of oxidant intermediates (Roig *et al.*, 2002).

(iv) Co enzyme Q_{10}

Co enzyme Q_{10} (ubiquinone; CoQ_{10}) is present in all human tissues; it is transported in the circulation by lipoproteins and cholesterol, and synthesized by the mevalonate pathway (Jiménez-Jiménez *et al.*, 2000).

Located in the inner mitochondrial membrane, CoQ_{10} is an important hydrophobic component of the mitochondrial respiratory chain and a potent antioxidant acting as a free radical scavenger (Jiménez-Jiménez *et al.*, 2000; Kidd, 2000; Papucci *et al.*, 2003; Somayajulu *et al.*, 2005).

CoQ_{10} is well known for its role as election carrier in the lipid phase of the mitochondrial membrane (Jiménez-Jiménez *et al.*, 2000). CoQ_{10} is the electron acceptor for mitochondrial complexes I and II (Jiménez-Jiménez *et al.*, 2000; Kidd, 2000), and transports electrons between complexes I (NADH-ubiquinone oxidoreductase), II (succinate-ubiquinone oxidoreductase) and III (ubiquinone-cytochrome *c* oxidoreductase) (Papucci, *et* al., 2003; Somayajulu *et al.*, 2005).

Ubiquinol (reduced coenzyme Q_{10}) is an effective lipid soluble chain breaking antioxidant (Young and Woodside, 2001). It scavenges lipid peroxyl radicals with higher efficiency than either α-tocopherol or the carotenoids, and can also regenerate membrane bound α-tocopherol from the tocopheryl radical (Aruoma, 2003; Young and Woodside, 2001). Ubiquinol may thus be important in preventing the propagation of lipid peroxidation (Young and Woodside, 2001).

CoQ_{10} concentrations are significantly reduced in mitochondria taken from the brain and platelets of patients suffering from Parkinson's disease, and lower complex I activity is strongly correlated with reduced mitochondrial content of CoQ_{10} (Kidd, 2000). Studies showed that the oral administration of CoQ_{10} is beneficial to patients suffering from Parkinson's disease at an early stage to slow the functional decline. It increases the CoQ_{10} concentration in plasma, and probably the ubiquinol in many organs to protect them form oxidative stress (Sohmiya *et al.*, 2004). In MPP^+ lesioned animals, treatment with this metabolic supplement, CoQ_{10} attenuated the nigral degeneration and the decrease of striatal dopamine concentration (Alexi *et al.*, 2000).

(b) Aqueous phase chain breaking antioxidants

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

(i) Vitamin C

It is widely believed that ascorbic acid can reduce the peroxyl radical of tocopherol back to α-tocopherol (Aruoma, 2003). Ascorbic acid (vitamin C) is often claimed to be the most important antioxidant *in vivo* (Aruoma, 2003).

In humans, ascorbate acts as an essential cofactor for several enzymes catalyzing hydroxylation reactions. It provides electrons for enzymes that require prosthetic metal ions in a reduced form to achieve full enzymatic activity (Young and Woodside, 2001).

A major function of ascorbate is that it acts as a key chain breaking antioxidant in the aqueous phase. Ascorbate has been shown to scavenge superoxide, hydrogen peroxide, the hydroxyl radical, hypochlorous acid, aqueous peroxyl radicals as well as singlet oxygen (Young and Woodside, 2001).

(ii) Uric acid

Uric acid has powerful antioxidant activity (Ghiselli *et al.*, 2000). It may be particularly important in providing protection against certain oxidizing agents, such as ozone (Young and Woodside, 2001).

Uric acid scavenges radicals, like peroxynitrite and attenuates apoptosis (Jones *et al.*, 2003; Young and Woodside, 2001). Part of the antioxidant effect of uric acid may be attributable to the formation of stable non-reactive complexes with iron (Young and Woodside, 2001).

(iii) Albumin bound bilirubin

Albumin bound bilirubin is also an efficient radical scavenger. It may have a crucial role in protecting the neonate from oxidative damage, because deficiency of other chain breaking antioxidants is common in the newborn (Young and Woodside, 2001).

Albumin is the predominant plasma protein and makes the major contribution to plasma sulphydryl groups. Albumin contains 17 disulphide bridges and has a single remaining cysteine residue, and it is this residue that is responsible for the capacity of albumin to react

with and neutralize peroxyl radicals. This property is important in view of the role albumin plays in transporting free fatty acids in the blood (Young and Woodside, 2001).

In addition, albumin has the capacity to bind copper ions and will inhibit copper dependent lipid peroxidation and hydroxyl radical formation. It is also a powerful scavenger of the phagocytic product hypochlorous acid, and provides the main plasma defence against this oxidant (Young and Woodside, 2001).

Because albumin itself is damaged when it acts as an antioxidant, it has been regarded as a sacrificial molecule that prevents damage occurring to more vital species. The high plasma concentration of albumin and a relatively short half life mean that any damage suffered is unlikely to be of biological importance (Young and Woodside, 2001).

(iv) Thiol groups

Another major chain breaking antioxidant in plasma is the protein bound thiol groups. The sulphydryl groups present on plasma proteins function as antioxidants by donating an electron to neutralize a free radical, with the resultant formation of a protein thiyl radical (Young and Woodside, 2001).

However, protein thiyl radicals can themselves act as a potential source of reactive oxidants by abstracting an electron from polyunsaturated fatty acids to initiate the process of lipid peroxidation (Young and Woodside, 2001). The antioxidant effects of albumin and other proteins have been shown to decrease at high concentrations, probably because thiyl radicals can cause oxidative damage to other molecules (Young and Woodside, 2001).

(v) Glutathione

Glutathione (GSH) is a potent molecular antioxidant and a conjugation cofactor for the liver P_{450} system (Samiec *et al.*, 1998; Kidd, 2000).

Glutathione functions directly as an antioxidant by scavenging a variety of reactive oxygen species, eliminating toxic peroxides and aldehydes (Samiec *et al.*, 1998; Jones *et al.*, 2003; Young and Woodside, 2001), and indirectly in maintaining vitamin C and E in their reduced and functional forms (Samiec *et al.*, 1998). Glutathione also acts as an essential factor for the glutathione peroxidase enzyme (Samiec *et al.*, 1998; Kidd, 2000; Young and Woodside, 2001), reducing cellular oxidative stress (Jones *et al.*, 2003), and has also been shown to attenuate apoptosis (Jones *et al.*, 2003).

The activity of glutathione as an antioxidant can be expressed in two ways: as a function of its concentration and as a function of the redox state of the reduced glutathione (GSH) to the oxidized glutathione (GSSG) (the GSH/GSSG pool). Enzymes that utilize glutathione as a substrate may be dependent solely upon its concentration whereas redox-sensitive processes may be dependent upon the redox state of GSH (Samiec *et al.*, 1998).

Figure 2.4 shows that neurodegenerative diseases also points to glutathione depletion (Kidd, 2000). The features of this model most relevant to Parkinson's disease are glutathione degradation in the substantia nigra (SN), and its overlap with the presence of oxidative stressors (Kidd, 2000).

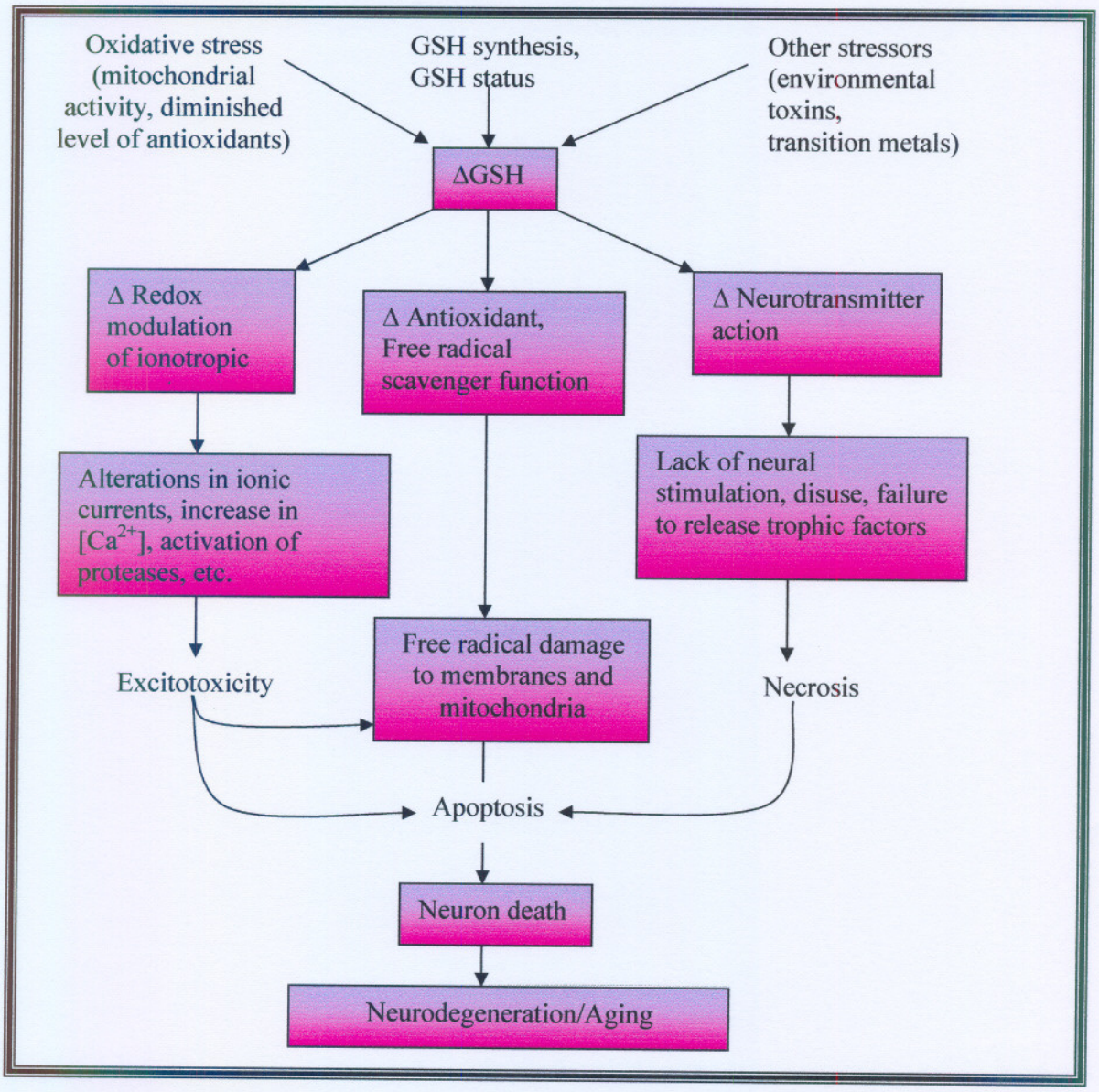


Figure 2.4 Proposed GSH-depletion model for neurodegenerative disorders (Kidd, 2000).

Glutathione depletion can arise in various ways, including genetic propensity, poor diet, pharmaceutical treatment (as with acetaminophen), or decreased release from tissues, either as a consequence of decreased intracellular concentrations or due to decreased glutathione transporter functions associated with ageing (Samiec *et al.*, 1998; Kidd, 2000). Oxidation of glutathione in plasma could also reflect generalized oxidative stress or a decline in antioxidant systems in tissues (Samiec *et al.*, 1998).

Reduced glutathione is a major source of thiol groups in the intracellular compartment but is of little importance in the extracellular space (Young and Woodside, 2001). Oxidation of the thiol-disulfide redox state has dramatic effects on cellular functions because transcriptional regulation of gene expression is sensitive to the redox state and this may affect the cell proliferation rate and apoptosis (Samiec *et al.*, 1998).

Oxidized glutathione (GSSG) appears to be released from most cells as a consequence of oxidative stress so that an oxidation of the cellular pool could shift the balance of glutathione and GSSG efflux and change the extracellular redox state (Samiec *et al.*, 1998).

Vitamin C deficiency results in decreased plasma glutathione and vitamin E supplementation increases plasma glutathione (Samiec *et al.*, 1998). Both vitamin C and E concentrations in plasma decrease with age, suggesting that glutathione may also decrease in plasma with age (Samiec *et al.*, 1998). Such decrease can contribute to development and/or progression of age-related toxicities (Samiec *et al.*, 1998) and evidence is growing that glutathione depletion contributes to neurodegenerative diseases (Kidd, 2000).

2.2.2.2 Antioxidant Enzymes

The first line of the antioxidant system includes enzymes, such as superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) (Delibas *et al.*, 2002; Kim *et al.*, 2002; Roig *et al.*, 2002). Antioxidant enzymes catalyze the breakdown of free radical species, usually in the intracellular environment (Young and Woodside, 2001).

Under normal circumstances, these antioxidative enzymes can protect against oxidative injury (Parikh *et al.*, 2003). Antioxidant enzymes can inhibit free radical production by chelating the transition metal catalysts, breaking chain reactions and reducing concentrations of reactive oxygen species (Aruoma, 2003).

(a) Superoxide dismutase

Superoxide dismutase (SOD) protects against oxygen free radicals by catalyzing the removal of superoxide radicals ($O_2^{\bullet-}$), which damages membrane and biological structures (McCord, 2000; Arivazhagan *et al.*, 2002).

Superoxide dismutase destroys $O_2^{\bullet-}$ by accelerating its conversion to hydrogen peroxide, which in turn is decomposed to water and oxygen by catalase and glutathione peroxidase, thereby preventing the formation of hydroxyl radicals (Inal *et al.*, 2001; Parikh *et al.*, 2003; Roig *et al.*, 2002; Aruoma, 2003; Young and Woodside, 2001) :

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

Three forms of superoxide dismutase are found in mammalian tissues, each with a specific subcellular location and different tissue distribution (Young and Woodside, 2001). Copper-zinc superoxide dismutase (CuZn-SOD) is found in the cytoplasm and organelles of virtually all mammalian cells (Akyol *et al.*, 2002; Aruoma, 2003), and contains catalytically active copper and zinc atoms (Young and Woodside, 2001). Manganese superoxide dismutase (Mn-SOD) is found in the mitochondria of almost all cells and contains a single manganese atom (Young and Woodside, 2001). The amino acid sequence of Mn-SOD is entirely dissimilar to that of CuZn-SOD and it is not inhibited by cyanide, allowing Mn-SOD activity to be distinguished from that of CuZn-SOD in mixtures of the two enzymes (Young and Woodside, 2001). Extracellular superoxide dismutase (EC-SOD), described by Marklund in 1982, is a secretory copper and zinc containing superoxide dismutase distinct form the CuZn-SOD described above. EC-SOD is synthesized by only a few cell types, including fibroblasts and endothelial cells, and is expressed in the cell surface where it is bound to heparin sulphates (Young and Woodside, 2001). EC-SOD is the major superoxide dismutase detectable in extracellular fluids and is released into the circulation from the surface of vascular endothelium following the injection of heparin (Young and Woodside, 2001).

(b) Catalase

Catalase was the first antioxidant enzyme to be characterized and is responsible for the detoxification of significant amounts of hydrogen peroxide by catalyzing the two stage conversion of H_2O_2 to water and oxygen (Inal *et al.*, 2001; Arivazhagan *et al.*, 2002; Aruoma, 2003; Young and Woodside, 2001):

Catalase-Fe^{3+} + H_2O_2 → compound I

Compound I + H_2O_2 → catalase-Fe^{3+} + $2H_2O$ + O_2

H_2O_2 + H_2O_2 → $2H_2O$ + O_2

Catalase is largely located within cells in peroxisomes (Aruoma, 2003; Young and Woodside, 2001) which also contain most of the enzymes capable of generating hydrogen peroxide (Young and Woodside, 2001).

Catalase is inhibited by $O_2^{\bullet-}$ in two distinct ways. One of these is a rapid inhibition, which can be prevented and reversed by superoxide dismutase (Kono and Fridovich, 1982). The second is a slow inhibition, which can be prevented but not reversed by superoxide dismutase, but can be both prevented and reversed by ethanol (Kono and Fridovich, 1982).

The rate constant for the reaction described above is extremely high (~10^7M/sec), implying that it is virtually impossible to saturate the enzyme *in vitro* (Young and Woodside, 2001). Catalase requires NADPH for its regeneration from the inactive form (Arivazhagan *et al.*, 2002).

Kono and Fridovich, 1982 proved synergistic interactions between catalase and superoxide dismutase (Kono and Fridovich, 1982).

(c) Glutathione peroxidase and Glutathione reductase

Glutathione peroxidase may be the most important H_2O_2-removing enzyme in the brain and in human cells (Arivazhagan *et al.*, 2002; Aruoma, 2003), protecting cell membranes from lipid peroxidation (McCord, 2000; Arivazhagan *et al.*, 2002).

Glutathione peroxidase removes H_2O_2 and peroxides originating from polyunsaturated fatty acids, by catalyzing the reduction of H_2O_2 and hydroperoxides to water (Fig. 2.1) (Inal *et al.*, 2001; Akyol *et al.*, 2002). Glutathione peroxidase uses the hydroperoxides to oxidize glutathione to GSSG (Aruoma, 2003; Shila *et al.*, 2005; Young and Woodside, 2001):

ROOH + 2GSH → GSSG + H_2O + ROH

Other peroxides, including lipid hydroperoxides, can also act as substrates for these enzymes, which might therefore play a role in repairing damage resulting from lipid peroxidation (Young and Woodside, 2001).

Glutathione peroxidase uses NADPH as the reducing species for hydrogen peroxide (McCord, 2000):

$$NADPH + H^+ + H_2O_2 \rightarrow 2H_2O + NADP^+$$

These enzymes require selenium (as selenocysteine) at the active site for their action (Inal *et al.*, 2001; Aruoma, 2003; Young and Woodside, 2001).

The activity of the enzyme is also dependent on the constant availability of glutathione (Young and Woodside, 2001). The ratio of GSH to GSSG is kept very high as a result of the activity of the enzyme glutathione reductase (Young and Woodside, 2001), a flavine nucleotide dependent enzyme, which regenerates glutathione from the oxidised GSSG, with NADPH as a source of reducing power (Aruoma, 2003; Young and Woodside, 2001):

$$GSSG + NADPH + H^+ \rightarrow 2GSH + NADP^+$$

Within the cells, the highest concentrations are found in liver, although glutathione peroxidase is widely distributed in almost all tissues. The predominant subcellular distribution is in the cytosol and mitochondria, suggesting that glutathione peroxidase is the main scavenger of hydrogen peroxide in these subcellular compartments. Glutathione reductase has a similar tissue distribution to glutathione peroxidase (Young and Woodside, 2001).

2.2.2.3 Transition metal binding proteins

Transition metal binding proteins (ferritin, transferrin, lactoferrin, and caeruloplasmin) (section 2.1.2.1*e*) act as a crucial component of the antioxidant defense system by sequestering iron and copper to prevent the interaction of these metals with hydrogen peroxide and superoxide producing the highly reactive hydroxyl radicals (Young and Woodside, 2001).

2.3 Oxidative Stress

A condition, termed oxidative stress, occurs when the balance between the level of oxidants to reductants (antioxidative defences) in the living organism is disrupted, in which the former predominates (Jones *et al.*, 2002; Granot and Kohen, 2004; Junqueira *et al.*, 2004; Young and Woodside, 2005; Wan *et al.*, 2005).

This deleterious condition occurs when the production of reactive oxygen species is accelerated or when the mechanisms involved in maintaining the normal reductive cellular milieu, are impaired. The excess of reactive oxygen species overwhelms the capacity of the antioxidant defence mechanisms, causing insufficient removal of such oxidants, ultimately leading to damage of all intracellular macromolecules (Ghiselli *et al.*, 2000; Polidori *et al.*, 2001; Giasson *et al.*, 2002; Kim *et al.*, 2002; Wan *et al.*, 2005).

The source of oxidative stress need not be a toxin. The steady state between pro-oxidants and antioxidants may also be disturbed in case of insufficiency of antioxidants or mineral enzyme cofactors, which may occur endogenously or as a consequence of a diminished dietary intake, impairment of antioxidant enzyme synthesis, or the overall decline of antioxidant defence capacity associated with ageing (Kidd, 2000; Polidori *et al.*, 2001).

Free chelatable iron, more than any other transitional metal, plays a pivotal role in the processes of oxidative stress, inflammatory processes and cell death in many non-neuronal and neuronal diseases, because of its role in the Fenton reaction yielding the highly toxic hydroxyl radical (Shachar *et al.*, 2004).

A state of oxidative stress can be extremely toxic to cells and can lead to rapid cell death (Giasson *et al.*, 2002). A number of neurological diseases are associated with locally enhanced oxidative stress, affecting functionality of neuronal cells (Schwemmer *et al.*, 2000; Naidu *et al.*, 2003). Oxidative/nitrative stress plays a role in the formation of intracellular pathological lesions, cellular dysfunction and the demise of cells in Alzheimer's and Parkinson's diseases (Giasson *et al.*, 2002). Oxidative stress has been proposed as one of the major causes of nigral degeneration (Carrasco and Werner, 2002).

However, late onset neurodegenerative diseases typically progress over a period of many years or decades, indicating that aberrant oxidative conditions occurring in these disorders may be characterized by short periods of oxidative/reductive imbalance, which may lead to

the progressive accumulation of damaged biomolecules and/or cell death. Over time, the summation of oxidative and nitrative modification leads to the formation of lesions, which contributes to impairment of cellular homeostasis by disrupting cellular morphology and creating obstructions to intracellular movement (Giasson *et al.*, 2002).

2.3.1 Consequences of Oxidative Stress

Reactive oxygen species and free radicals participate in a wide variety of deleterious oxidative reactions (Allen and Tresini, 2000) causing irreversible damage to practically all macromolecules in the living cell as well as disrupting the redox signalling mechanisms (Cai *et al.*, 1997; Inal *et al.*, 2001; Tahara *et al.*, 2001; Jones *et al.*, 2002). This damage manifests as the peroxidation of membrane polyunsaturated fatty acid chains, modification of DNA and carbonylation and loss of sulphydryl groups in proteins, among other changes (Arriaga-Alba *et al.*, 2000; Inal *et al.*, 2001; Heim *et al.*, 2002; Parikh *et al.*, 2003; Bellé *et al.*, 2004). This type of damage leads to cell dysfunction (Somayajulu *et al.*, 2005). Cell injury can be amplified by the reactive by-products of oxidative damage and may eventually lead to cell death (Bellé et al., 2004).

Oxidative damage contributes to the development of chronic diseases (Zaidi and Banu, 2004). Reactive oxygen species plays a critical role in neurodegeneration, including Parkinson's and Alzheimer's diseases as well as schizophrenia (Zaidi and Banu, 2004). It is also known that reactive oxygen species contributes to cellular ageing, mutagenesis, carcinogenesis and coronary heart disease (Arriaga-Alba *et al.*, 2000; Heim *et al.*, 2002).

Cellular responses stimulated by reactive oxygen species can be divided into five broad categories, including (i) modulation of cytokine, growth factor, or hormone action and secretion; (ii) ion transport; (iii) transcription; (iv) neuromodulation and (v) apoptosis (Allen and Tresini, 2000).

2.3.2 Molecular targets of Oxidative Stress

During the course of their lifetime, biological structures are continuously exposed to harmful oxidative stresses (Giasson *et al.*, 2002; Girotti *et al.*, 2002). Free radical reactions have been observed to influence molecular and biochemical processes and to directly cause some of the changes observed in cells during differentiation, ageing, and transformation (Allen and Tresini, 2000). Proteins, DNA and unsaturated lipids are the primary candidates for oxidation and oxidative attack (Granot and Kohen, 2004).

2.3.2.1 Nucleic acids

DNA can undergo a series of reactions with reactive oxygen species leading to DNA stand breaks (double or single), base modifications, which may result in genetic mutations and cell death, damage to DNA repair enzymes (Granot and Kohen, 2004) and nucleic acids and protein cross-linking (Giasson *et al.*, 2002).

8-Hydroxy-2'-deoxyguanosine has been the most frequently used biomarker of nucleic acid damage, because it can easily be measured biochemically (Giasson *et al.*, 2002), and the degree of spontaneous chromosomal breakage has been shown to be directly related to cellular oxygen tension (Finkel, 2003). DNA damage is often measured as single-strand breaks, double-strand breaks or chromosomal aberrations (Aruoma, 2003).

2.3.2.2 Proteins

Amino-acids, aromatic amino acids, histidine, methionine, and cysteine are particularly sensitive to oxidative stress (Giasson *et al.*, 2002).

Reactive oxygen species can react directly with the protein or with molecules such as sugars and lipids generating products that then react with the protein (Levine and Stadtman, 2001). Oxidation of proteins in the presence of monosaccharides, such as glucose, can result in the formation of irreversible advanced glycation end-products. These products are formed because primary amines, like lysine residues, can undergo a slow and reversible non-enzymatic reaction with monosaccharides. However, in the presence of transition metals, the glycated proteins undergo rearrangement into irreversible products. Further oxidative reactions can result in the formation of lysine and arginine cross-links (Giasson *et al.*, 2002).

Within the protein, either the peptide bond or the sidechain may be targeted. The protein may be cleaved to give lower molecular weight products, or it may be cross-linked giving higher-molecular weight products (Levine and Stadtman, 2001; Levine, 2002). Many of the reactions mediated by free radicals are usually in a site-specific manner and frequently influenced by redox cycling metal cations, especially iron or copper (Levine, 2002).

Protein oxidative damage can result in the modification in the structure, enzymatic activity and signalling pathways (Ashok and Ali, 1999).

2.3.2.3 Fatty acids

The lipid component of biological membranes is especially vulnerable to oxidative damage, due to its abundance in fatty acids, especially polyunsaturated, and may undergo a self perpetuating chain peroxidation process termed lipid peroxidation (Giasson *et al.*, 2002; Granot and Kohen, 2004).

(a) Lipid peroxidation

Reactive oxygen species can easily propagate the initial attack on lipid rich membranes of the brain to cause lipid peroxidation (Zaidi and Banu, 2004); leading to the accumulation of lipid peroxides (Gülçin *et al.*, 2002).

Lipid peroxidation is an autocatalytic process of which cell death is a common consequence (Inal *et al.*, 2001; Heim *et al.*, 2002). This process may cause peroxidative tissue damage to polyunsaturated fatty acids in inflammation, cancer, toxicity of xenobiotics and ageing (Inal *et al.*, 2001). Lipid peroxidation might also be a cause of alterations in antioxidant enzyme activity (Delibas *et al.*, 2002). Marked depletion of glutathione content in the brain, a guarding factor against oxidative stress, may also cause enhanced lipid peroxidation. The glutathione depletion may be a result of decreased activities of glutathione-S-transferase, superoxide dismutase and catalase (Zaidi and Banu, 2004). Lipid peroxidation is a well-established index of cellular peroxidative membrane injury associated with increased oxidative stress (Parikh *et al.*, 2003).

Under conditions of oxidative stress, the defence system cannot prevent the escape of reactive oxygen species especially from the mitochondria, and their effects on other intracellular compartments (Akyol *et al.*, 2002). Polyunsaturated fatty acids located in cellular membranes of the central nervous system can readily react with free radicals and undergo peroxidation, altering membrane function, such as transport mechanisms (Inal *et al.*, 2001; Akyol *et al.*, 2002).

The lipid peroxidation process is characterized by 3 major stages mainly (1) initiation, (2) propagation and (3) termination, as illustrated in fig. 2.5 (Harman, 1998; Granot and Kohen, 2004):

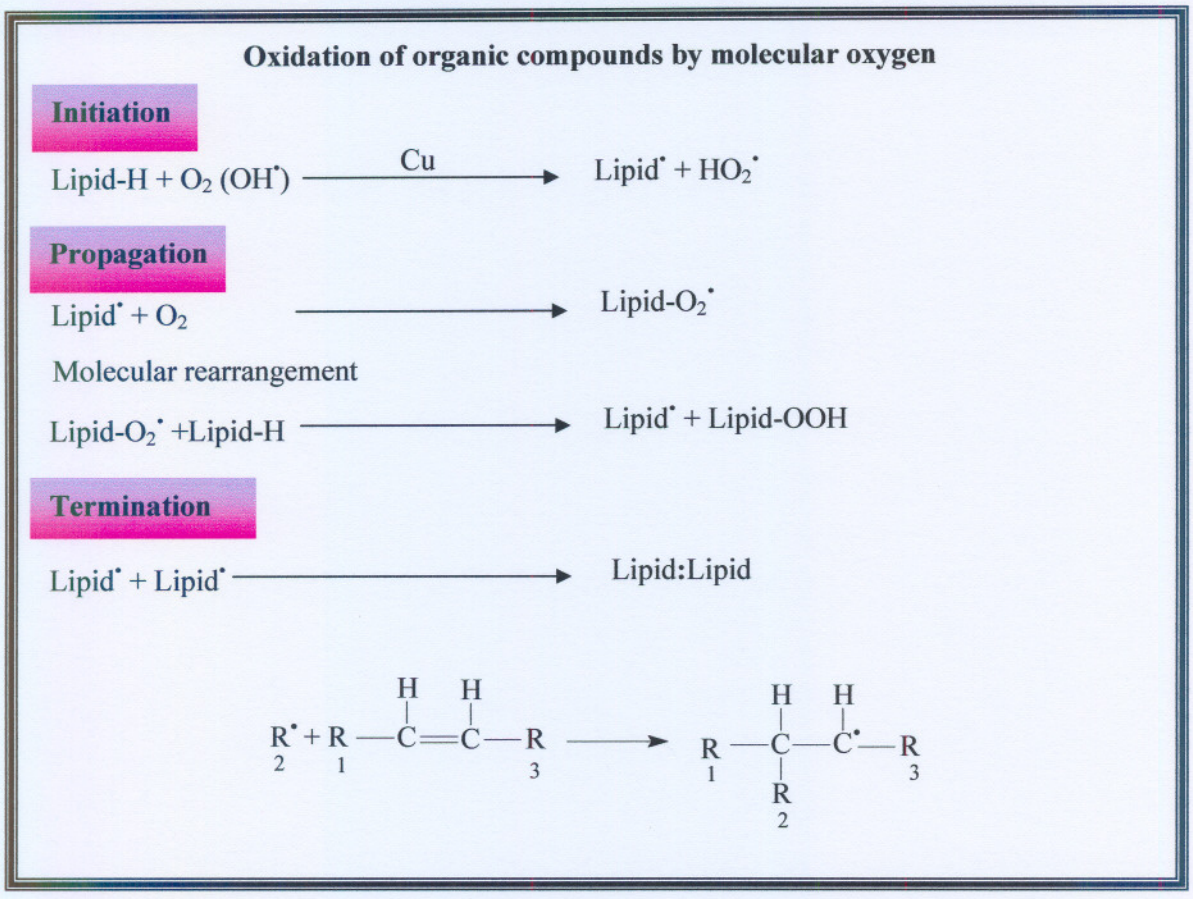


Figure 2.5 Free radical reactions: reaction of O_2 with organic compounds (Halliwell and Gutteridge, 1984; Harman, 1998).

(i) The initiation stage

Initiation includes an oxidant or any species with sufficient reactivity, abstracting a hydrogen atom from a polyunsaturated lipid, leaving behind an unpaired electron on the carbon atom, i.e. leaving the lipid in a radical form (Halliwell and Gutteridge, 1984; Granot and Kohen, 2004).

(ii) The propagation stage

Reactive oxygen species propagates the initial attack on lipid rich membranes of the brain causing lipid peroxidation (Zaidi and Banu, 2004). The carbon radical tends to stabilize by molecular rearrangement to produce a conjugated diene, which rapidly reacts with oxygen generating a peroxyl radical, which itself can abstract hydrogen from a neighbouring lipid resulting in the production of lipid hydroperoxide and a new lipid radical. In this way peroxyl radicals stimulate the self-perpetuating chain reaction of lipid peroxidation (Halliwell and Gutteridge, 1984; Granot and Kohen, 2004).

(iii) The termination stage

Finally (termination) the lipid hydroperoxide or peroxyl radical can undergo further oxidation, via its interaction with reduced metals (Fe^{2+}), yielding a variety of products (Granot and Kohen, 2004).

The amount of a compound converted to products per unit time by a free radical reaction depends on the rate of initiation and the number of times the propagation phase is repeated before termination, in other words the chain-length (Harman, 1998). Since lipid peroxidation is a self-propagating chain reaction the initial oxidation of only a few lipid molecules can result in significant tissue damage and disease, especially so in brain tissues rich in polyunsaturated fatty acids (Zaidi and Banu, 2004).

The products of lipid peroxidation are complex (Giasson *et al.*, 2002). The two products most widely studied is short chain aldehydes (i) *Trans*-4-hydroxy-2-hexenal (4-HNE), one of the many unsaturated aldehydes formed, because of its reported toxicity and the availability of antibodies to its protein adducts that can be used *in situ*; and (ii) Malondialdehyde (MDA) which is universally used as an index of lipid peroxidation (Giasson *et al.*, 2002). Lipid peroxidation also give rise to a variety of other products including alkanes and alkenes, conjugated dienes and a variety of hydroxides and hydroperoxides, lipofucine and volatile hydrocarbons such as ethane and pentane, depending on the lipid substrate (Granot and Kohen, 2004; Young and Woodside, 2005). The peroxidation products and their secondary oxidation products, such as MDA and 4-HNE are highly reactive; and can amplify cell injury causing further damage to biological structures (Gülçin et al., 2002; Bellé et al., 2004). In addition, these peroxidation products appear to contribute to the etiology of a number of chronic diseases including neurodegenerative disorders (Bellé *et al.*, 2004).

It has been concluded that peroxidative damage increases during the ageing process (Inal *et al.*, 2001; Tahara *et al.*, 2001; Delibas *et al.*, 2002). Either an age-dependent increase in the rate of protein and lipid peroxidation or a decrease in active oxygen scavengers can explain the age-related increase in amounts of lipid peroxidation products and oxidized protein (Okatani *et al.*, 2002).

2.3.3 Oxidative stress and the brain

Throughout life, the ageing brain is continuously exposed to oxidative stress and a number of diseases of the brain have been hypothesized to involve free radical induced oxidative damage, either as a cause or consequence of the disease process (Arivazhagan *et al.*, 2002).

Since the brain consumes a disproportionately high share (20-25%) of the oxygen taken into the human body, more reactive oxygen species per gram tissue are generated in the brain than in any other organ (Arivazhagan *et al.*, 2002; Lee, 2004), creating an exceptionally high endogenous oxidative burden (Kidd, 2000). Therefore, of all the organs in the body the central nervous system is thought to be particularly susceptible to the toxic effects of oxidative damage (Arivazhagan *et al.*, 2002; Bellé *et al.*, 2004; Zaidi and Banu, 2004). In addition, other factors that contribute to this vulnerability include its high content of polyunsaturated fatty acids and the abundance of transition metals such as iron and copper (Driver *et al.*, 2000; Akyol *et al.*, 2002; Okatani *et al.*, 2002).

Since mitochondria are the primary site for the production of reactive oxygen species and neuronal cells depend on mitochondrial oxidative phosphorylation for their energy need and having an exceptionally high rate of oxidative metabolic activity (e.g catecholamine degradation), neurons are at a particular risk for oxidative stress (Michaelis, 1998; Akyol *et al.*, 2002; Somayajulu *et al.*, 2005). Containing myelin sheaths, the neurons are particularly enriched with polyunsaturated fatty acids, which may be oxidized to form peroxides (Michaelis, 1998; Driver *et al.*, 2000; Kidd, 2000; Akyol *et al.*, 2002; Arivazhagan *et al.*, 2002; Okatani *et al.*, 2002; Zaidi and Banu, 2004).

Also rendering the brain particularly vulnerable is its relatively low content of protective enzymatic and non-enzymatic cytosolic antioxidants compared to other tissues (Michaelis, 1998; Driver *et al.*, 2000; Akyol *et al.*, 2002; Arivazhagan *et al.*, 2002; Okatani *et al.*, 2002; Bellé *et al.*, 2004; Zaidi and Banu, 2004). Activities of the antioxidant enzymes catalase and peroxidase are abnormally low in the brain. Although superoxide dismutase are active, acquiring $O_2^{\cdot-}$ as it leaks out of the mitochondrial complexes and converting it to H_2O_2, the virtual absence of catalase and peroxidase (normally detoxifying peroxide products), shunts the burden for detoxifying H_2O_2 onto the glutathione peroxidase, which uses glutathione as an essential cofactor, and when it is adaptively induced the brain's glutathione reserves are likely rendered more prone to depletion from oxidative attack (Kidd, 2000).

2.3.3.1 Striatum

The striatum is one of the brain regions most sensitive to the effects of hypoxia/ischemia and to free radical damage, not only because it lies deeper and is subject to greater acidification, but also because it is rich in dopaminergic neuronal endings and dopamine (Barc *et al.*, 2004). Striatal extracellular dopamine is significantly increased in the ischemic or hypoxic brain injury model, and the extracellular level of dopamine is related to the severity of the ischemic insult (Barc *et al.*, 2004). It has been shown that regions like the cortex, hypothalamus, hippocampus and the striatum are more susceptible to oxidative damage when compared to the cerebellum (Arivazhagan *et al.*, 2002).

2.3.3.2 Substantia Nigra

The substantia nigra pars compacta (SN/pc) innate especially vulnerable to oxidative challenge (Kidd, 2000; Carrasco and Werner, 2002). In its most healthy state the substantia nigra is more vulnerable to oxidative attack than probably any other brain region and with the increase of age antioxidant defences tend to become less competent (Kidd, 2000).

The substantia nigra's unique biochemistry renders it more vulnerable to oxidative stress than the brain as a whole (Kidd, 2000). The substantia nigra's unique biochemical features are:

(1) A high content of dopamine, consequent to the high density of dopaminergic neurons (Kidd, 2000). Dopamine has a strong tendency to spontaneously break down into oxidant metabolites by autooxidation; which can be accelerated by free (ionized) iron or by other redox-active elements such as copper, zinc or manganese (Kidd, 2000). The most reactive among these auto-metabolites are 6-hydroxydopamine, quinone and dopamine aminochrome.

(2) An extremely high content of iron, higher even than in the liver (Kidd, 2000; Shachar *et al.*, 2004). Iron, when present in such high cellular concentrations, can escape buffer control by iron-binding proteins, and, via Fenton reaction, catalytically convert H_2O_2 to highly reactive hydroxyl radicals, damaging all classes of biomolecules (Kidd, 2000; Giasson *et al.*, 2002).

(3) High activities of the two monoamine oxidase enzymes (MAO–A and –B), which increases with ageing, functioning to degrade dopamine to products that include

hydrogen peroxide, making hydrogen peroxide particularly prevalent in the substantia nigra (Alexi *et al.*, 2000; Kidd, 2000).

(4) High content of melanin (dopamine-melanin), a complex macromolecule formed from the autooxidation of dopamine (Kidd, 2000), normally functioning as a scavenger of free radicals. However, when infiltrated with high levels of ionized iron, it can drive the Fenton reaction and exacerbate the conversion of endogenous H_2O_2 to the potent hydroxyl radical. Melanin within the substantia nigra could act as a support matrix upon which ionized iron would catalyze the generation of oxidants from available H_2O_2 or from neuromelanin itself (Kidd, 2000).

(5) Low glutathione content relative to other brain areas (Kidd, 2000). Depletion of nigrostriatal glutathione enhance sensitivity to oxidants and to complex I impairment (Kidd, 2000). Glutathione depletion seemingly occurs early in neurodisease pathogenesis, and may thus be a central factor in the process. Studies confirmed that the substantia nigra is abnormally depleted of glutathione in patients suffering from Parkinson's disease (Kidd, 2000).

(6) The substantia nigra also carries the mitochondrial complex I defect, which lowers energy production and intensifies endogenous production of reactive oxygen species (Kidd, 2000).

Carrying so many pro-oxidant biochemical factors, the substantia nigra pars compacta could well be considered an "oxidative accident waiting to happen" (Kidd, 2000).

2.3.4 Compounds used to induce oxidative stress

Animal models of nigral degeneration take advantage of the well defined anatomical and biochemical nature of these neurons (Alexi *et al.*, 2000).

2.3.4.1 Rotenone

Rotenone is a classical, high affinity non-competitive inhibitor for ubiquinone at complex I (Reil *et al.*, 1997) and is typically used to define the specific activity of the complex (Giasson *et al.*, 2002; Greenamyre, *et al.*, 2003).

Because it is extremely lipophillic, it crosses biological membranes easily; independent of transporters and it gets into the brain very rapidly. As such, it is well-suited for inducing a

systemic inhibition of complex I in experimental animals. Chronic, uniform, systemic inhibition of complex I causes – over a period of days to weeks- selective degeneration of the dopaminergic neurons in the substantia nigra. Degeneration begins in the nerve terminals and progress retrogradely to the cell bodies. The distribution of pathology accurately matches what is seen in typical parkinsonism, with nigral neurons being most susceptible to degeneration. In the rotenone model of parkinsonism, the expression of α-synuclein is increased and it eventually becomes insoluble, forming cytoplasmic inclusions. Many of the dying neurons contain these cytoplasmic inclusions which, like true Lewy bodies, contain α-synuclein and ubiquitin (Greenamyre, *et al.,* 2003). This aggregation may be due to direct oxidative modifications of α-synuclein or dopamine quinone, and may form adducts with α-synuclein (Greenamyre, *et al.,* 2003).

Rotenone-infused animals also develop symptoms of Parkinson's disease, including bradykinesia and rigidity. Severely affected rats also develop the flexed posture and motor 'freezing' typical of advanced parkinsonism (Greenamyre, *et al.,* 2003).

Studies have shown that rotenone causes a delayed depletion of glutathione, which is accompanied by oxidative damage to proteins and DNA. It is believed that there is a site of electron leakage upstream of the rotenone binding site in complex I, and these electrons combine with molecular oxygen to form reactive oxygen species, which attack macromolecules (Greenamyre, *et al.,* 2003).

Greenamyre *et al.*, 2003, showed that microglia plays an active role in dopaminergic degeneration in the rotenone model, as dramatic activation of microglia is evident in the nigrostriatal tract and, to a lesser extent in the olfactory bulb, but very little activation in non-dopaminergic areas. Microglial activation also begins before there is anatomical evidence of degeneration (Greenamyre *et al.,* 2003).

2.3.4.2 6-Hydroxydopamine

6-Hydroxydopamine, a hydroxylated analogue of dopamine, is a well known neurotoxin that causes nigral degeneration by redox-cycling, reactive oxygen species production and consequent oxidative stress (Carrasco and Werner, 2002).

6-Hydroxydopamine is pro-inflammatory via its ability to increase the release of cytotoxic cytokines IL-1, IL-6 and TNF-α similar to what has been reported in the parkinsonian

striatum, a process thought to be initiated via generation of the hydroxyl radical, by the Fenton reaction. 6-Hydroxydopamine has also been shown to increase the iron content of the substantia nigra pars compacta in rats and monkeys (Shachar *et al.*, 2004).

6-Hydroxydopamine is generally administered unilaterally to the medial forebrain bundle, the striatum, or sometimes directly to the substantia nigra resulting in its selective uptake by dopaminergic (and other nearby catecholaminergic) neurons where it causes oxidative stress and ultimately cell degeneration. The extent of nigral cell degeneration is dose dependent and a nearly full selective ablation of tyrosine hydroxylase activity can be achieved with 6-hydroxydopamine. Cell death occurs in a biphasic pattern with much of the cell loss occurring during the initial acute phase beginning 12 hours after injection and continuing up to approximately 7-10 days post injection. Maximal cell death occurs at 4-6 days post lesioning. The initial phase is followed by a prolonged phase of less cell death to about 30 days, although this timing is dose dependent. Nigral cell degeneration can occur up to 8 weeks or more post lesion with high doses of 6-hydroxydopamine (Alexi *et al.*, 2000).

2.3.4.3 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) has been identified to be a "protoxin" for the mitochondrion causing selective degeneration of the dopaminergic neurons in the substantia nigra pars compacta as well as degeneration of dopaminergic nerve terminals in the putamen (Alexi *et al.*, 2000; Kidd, 2000; Przedborski and Vila, 2001).

A single acute insult to the substantia nigra pars compacta by MPTP can set in motion a self-sustained cascade of events with long-lasting deleterious effects. As illustrated in fig. 2.6, the metabolism of MPTP is a complex, multistep process (Przedborski and Vila, 2001).

MPTP is not toxic itself, but is highly lipophilic and crosses the blood-brain barrier easily. Inside the brain, MPTP is metabolized to 1-methyl-4-phenyl-2,3-dihydropyridinium ($MPDP^+$) by the enzyme monoamine oxidase B (MAO-B) within non-dopaminergic cells, and then (probably by spontaneous oxidation) to the active toxic metabolite 1-methyl-4-phenylpyridinium (MPP^+) (Kidd, 2000; Przedborski and Vila, 2001; Greenamyre, *et al.*, 2003; Khaldy *et al.*, 2003; Pan *et al.*, 2003). MPP^+ is then released, by an unknown mechanism, into the extracellular space (Przedborski and Vila, 2001).

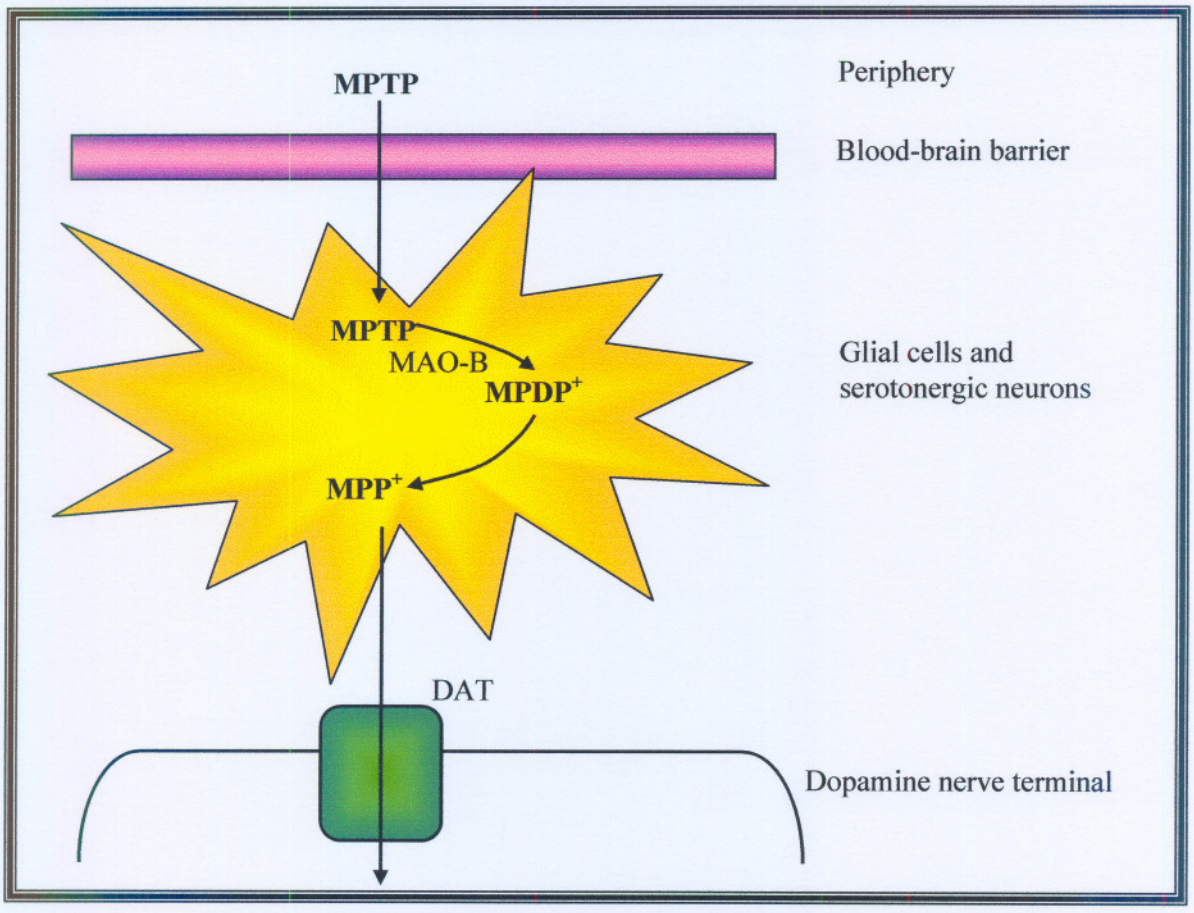


Figure 2.6 Schematic representation of MPTP metabolism. (Przedborski and Vila, 2001)

Since MPP^+ is a polar molecule, it cannot freely enter cells and depends on the plasma membrane dopamine transporter carriers (DAT), to be selectively taken up by active transport into the presynaptic dopaminergic nerve terminals (Fig. 2.6) (Przedborski and Vila, 2001; Greenamyre, *et al.,* 2003; Pan *et al.*, 2003).

Within the dopaminergic neurons MPP^+ concentrates in millimolar concentrations in the mitochondria by an energy dependent process (Kidd, 2000), where it inhibits respiration at the level of complex I of the electron transport chain (Fig. 2.7) (Przedborski and Vila, 2001; Carrasco and Werner, 2002; Giasson *et al.,* 2002; Greenamyre, *et al.,* 2003; Khaldy *et al.,* 2003). The binding of MPP^+ to complex I, interrupts the flow of electrons, resulting in extreme impairment of mitochondrial energy production accompanied by amplification of reactive oxygen species production (Kidd, 2000; Przedborski and Vila, 2001; Pan *et al.*, 2003). Other enzymes, like α-ketoglutarate dehydrogenase, located near complex I, which is also involved in the mitochondrial electron transport chain is also inhibited and contributes to the impaired energy production (Kidd, 2000; Pan *et al.*, 2003).

MPP^+ not only blocks the mitochondrial ATP production, but causes an increase of both superoxide and hydrogen peroxide, depleting the cell from glutathione, which accounts for the decrease of complex I activity (Khaldy *et al.*, 2003). Superoxide is known to play a pivotal role in the MPTP neurotoxic process as MPTP stimulate the formation of hydroxyl radicals *in vivo* (Przedborski and Vila, 2001). The increased production of reactive oxygen species, is believed to mediate the toxic effects of MPP^+, and it is likely that energy failure and oxidative/nitrative stress resulting from mitochondrial dysfunction may act synergistically (Przedborski and Vila, 2001; Carrasco and Werner, 2002; Giasson *et al.*, 2002).

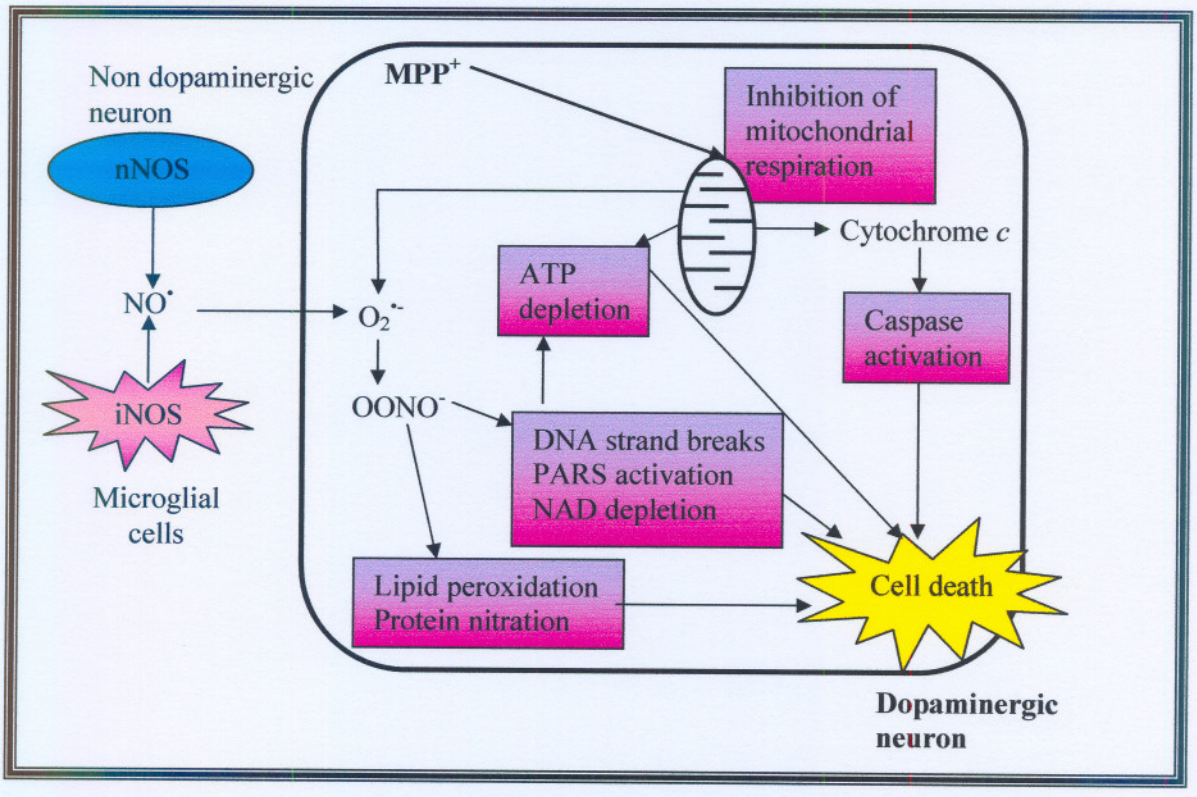


Figure 2.7 Mechanisms of MPTP neurotoxicity (Przedborski and Vila, 2001).

In addition, MPP^+ increases the iron content of the substantia nigra pars compacta in rats and monkeys, which facilitates the production of the hydroxyl radical. The mechanism not yet known, but may depend on the ability of MPP^+ to release iron from ferritin, down regulate transferrin receptors and up regulate the divalent metal transporter I (Shachar *et al.*, 2004).

MPP^+ also induces a massive and rapid outflow of dopamine from intracellular pools, which raises the possibility that intracellular dopamine autooxidation may represent a significant

source of reactive oxygen species (Przedborski and Vila, 2001; Giasson *et al.*, 2002; Khaldy *et al.*, 2003).

Treatment with this inhibitor thus results in oxidative stress as a consequence of disregulation of mitochondrial iron, glutathione, displacement of dopamine vesicular storage sites and inhibition of complex I (Giasson *et al.*, 2002; Shachar *et al.*, 2004).

The expression of inducible NOS (iNOS) and neuronal NOS (nNOS), also contributes to MPTP toxicity. nNOS appears to be involved in nigral and striatal degeneration, while iNOS is only involved in nigral injury (Giasson *et al.*, 2002). NO, which is produced by nNOS and iNOS outside dopaminergic neurons, is membrane-permeable and can diffuse into neighbouring neurons. If the neighbouring cells has elevated levels of superoxide anions, there is an increased probability of $O_2^{\bullet-}$ reacting with NO to produce peroxynitrite, which can damage lipids, proteins, and cause DNA single strand breakage (Fig 2.7). (Przedborski and Vila, 2001). It has been shown that inhibition of NOS attenuates MPTP-induced striatal dopaminergic loss in mice, in a dose-dependent manner (Przedborski and Vila, 2001).

Oxidants cause DNA damage, producing 8-hydroxyguanine and 8-hydroxydeoxyguanosine, two modifications whose levels seem to be increased in the midbrain of post-mortem parkinsonian brains. DNA single strand breakage is an obligatory trigger of the activation of PARP, a phenomenon believed to be a major factor in the MPTP-induced cascade of deleterious events. Activation of PARP results in the cleavage of NAD^+ into ADP-ribose and nicotinamide, resulting in covalently attaching ADP-ribose to diverse proteins, including nuclear proteins, histones, and PARP itself, which then extends the initial ADP-ribose groups into a nucleic acid group-like polymer, poly(ADP-ribose) (Przedborski and Vila, 2001). In this way, activation of PARP can rapidly deplete intracellular stores of NAD^+, impairing glycolysis and mitochondrial electron transport chain activities, consequently worsening the deterioration of ATP production (Przedborski and Vila, 2001).

On the other hand, MPP^+ also induces the release of cytochrome *c* from the mitochondria to the cytosol where it initiates a cascade of caspases activation (Przedborski and Vila, 2001) and create the loss of mitochondrial membrane potential, (Alexi *et al.*, 2000; Kidd, 2000; Carrasco and Werner, 2002; Khaldy *et al.*, 2003; Pan *et al.*, 2003).

Furthermore, the loss of dopaminergic neurons in the MPTP mouse model (and in Parkinson's disease) is associated with a glial response composed mainly of activated

microglial cells and, to a lesser extent, of reactive astrocytes, which is indicative of an active, ongoing process of cell death (Przedborski and Vila, 2001). PGE_2 content has also been shown to be elevated in the substantia nigra pars compacta from Parkinsonian patients (Przedborski and Vila, 2001).

Although the main mediators of MPTP-toxicity, at least shortly after the toxin administration, are energy crisis and oxidative stress, more recently it has become apparent that MPP^+-induced mitochondrial dysfunction is also associated with the activation of specific factors of the apoptotic machinery, and activation of genetic programs leading to cell death. A sub-acute regimen of MPTP administration in mice induces activation of the apoptotic molecular pathways (Przedborski and Vila, 2001). A single injection of MPTP causes nigral degeneration which is mostly completed after 1-2 weeks. A small degree of apoptosis is evident, although this may depend on the dosing regimen (Alexi *et al.*, 2000).

MPTP is regarded to be the best experimental model of Parkinson's disease to date, especially in the context of studies designed to explore molecular mechanisms involved in the degeneration of dopaminergic neurons in the substantia nigra pars compacta (Przedborski and Vila, 2001), despite its shortcoming that Lewy bodies are not formed which may be due to the acute nature of its neurotoxicity (Maharaj *et al.*, 2004). In human and non-human primates, MPTP produces an irreversible and severe parkinsonian syndrome that replicates almost all of the features of Parkinson's disease including tremor, rigidity, slowness of movement, postural instability and even gait freezing. The responses as well as the complications to traditional anti-parkinsonian therapies are virtually identical to those seen in Parkinson's disease. Although in Parkinson's disease it is believed that the neurodegeneration process evolves over several years, the most-active phase of neuronal death is presumably completed in a short period of time following MPTP administration, producing a clinical condition consistent with 'end-stage Parkinson's' in a few days. Post mortem brain samples from patients suffering from Parkinson's disease show a selective defect in the same mitochondrial electron transport chain complex that is affected by MPTP (Przedborski and Vila, 2001).

α-Synuclein needs to be post-translationally modified (either by oxidative stress, in sporadic Parkinson's disease, or by a point mutation, in the familial forms) in order to acquire pathological properties, involving its aggregation and the formation of intracellular inclusions. After MPTP intoxication, it is post-translationally modified by oxidative stress,

resulting in structural modifications that may increase its tendency to aggregate. Tyrosine nitration disrupts its biophysical properties, making α-synuclein a specific target for MPTP-induced oxidative attack (Przedborski and Vila, 2001). It was shown that α-synuclein is up-regulated and accumulated into the cytosol of dopaminergic neurons after a sub-acute MPTP intoxication, and it is thus possible that upon the translocation of cytochrome *c* from the mitochondria to the cytosol of dopaminergic cells, accumulation and subsequent aggregation of α-synuclein can occur (Przedborski and Vila, 2001).

2.3.4.4 Quinolinic acid

Quinolinic acid, 2,3-pyridine dicarboxylic acid, is a well-known endogenous tryptophan metabolite at the kynurenine pathway, produced by macrophages microglia (Santamaria *et al.*, 2003; Bellé *et al.*, 2004). Present in both human and rat brain, it has been implicated in the pathogenesis of a variety of human neurological diseases (Bellé *et al.*, 2004).

Quinolic acid is a potent neurotoxin exhibiting agonist properties at the N-methyl-D-aspartate (NMDA) receptor in the brain, producing a specific pattern of neuronal degeneration (Santamaria *et al.*, 2003; Bellé *et al.*, 2004). Its character as excitotoxin explains most of its toxic effects in the central nervous system (Santamaria *et al.*, 2003).

Studies have shown that a fraction of quinolinic acid toxicity may be independent of NMDA receptor activation and more likely depend on iron through the formation of quinolinic-iron complexes enhancing the Fenton's reaction (Santamaria *et al.*, 2003). Quinolic acid is not readily metabolized in the synaptic cleft and stimulates the NMDA receptor for prolonged periods (Bellé *et al.*, 2004). This sustained stimulation results in opening of calcium channels causing Ca^{2+}-dependent enhancement of free radical production leading to molecular damage (including lipid peroxidation) and often to cell death (Bellé *et al.*, 2004).

2.3.4.5 Cyanide

The brain is the primary target organ for cyanide toxicity (Shou *et al.*, 2000; Maharaj *et al.*, 2003) and the neurotoxicity of cyanide is well recognized (Jones *et al.*, 2003).

In a number of acute cyanide intoxication cases, a Parkinson-like condition associated with degeneration of the substantia nigra, pursues (Jones *et al.*, 2003). This condition can be attributed to production of cellular anoxia in the brain producing tonic and clonic seizures as well as convulsions (Maharaj *et al.*, 2003).

Cyanide is generated endogenously and is normally present in the blood and nervous systems at low levels (Jones *et al.*, 2003). Additionally, individuals can be exposed to cyanide from tobacco smoke, diet and other environmental sources (Jones *et al.*, 2003). Exposure to cyanide under appropriate conditions may be an initiating event in dopaminergic neurodegeneration (Jones *et al.*, 2003).

This mitochondrial toxin causes severe depletion of cellular energy reserves by means of inhibiting the mitochondrial respiratory chain, complex IV (cytochrome *c* oxidase), the terminal electron acceptor enzyme in the electron transport chain. This prevents the utilization of oxygen and causes the disruption of the homeostatic ATP-dependent Na^{2+}/K^{+} and Ca^{+} pumps (Southgate and Daya, 1999; Shou *et al.*, 2000; Jones *et al.*, 2003; Maharaj *et al.*, 2003).

Cyanide produces dopaminergic toxicity, characterized by a loss of dopaminergic neurons in the basal ganglia which is accompanied by impaired motor function (Maharaj *et al.*, 2004). It has also been shown that cyanide potentiates the cytotoxicity of dopamine by increasing dopamine-induced formation of oxidants and -apoptosis (Jones *et al.*, 2003).

After cyanide treatment, different modes of cell death are involved in different brain areas. Cell death in cortical region mainly occurs via apoptosis, whereas necrosis predominates in substantia nigra after the same dose of cyanide treatment (Shou *et al.*, 2000). The mode of cell death also depends on the duration of exposure and concentration. Higher concentrations (>500μM) produce necrosis and in the concentration range of 300 – 400μM, apoptosis occurs (Jones *et al.*, 2003).

It is well-known that cyanide induces neurotoxicity due to oxidative damage resulting in extensive lipid peroxidation of neuronal membranes (Maharaj *et al.*, 2003). Immediately after cyanide treatment, generation of reactive oxygen species is observed and continue to be elevated for up to 3 hours (Shou *et al.*, 2000). Cyanide promotes the release of cytochrome *c* from mitochondria into the cytoplasm which in turn activates the caspase cascade, which may be the initiating event in cyanide-induced neuronal apoptosis (Jones *et al.*, 2003).

Reactive oxygen species generated after cyanide treatment is due partly to NMDA receptor activation and influx of Ca^{2+}. Cyanide acts on the NMDA-receptor either directly through an interaction with the redox regulatory site of the receptor or indirectly by inducing glutamate release from the cytosolic pool (Shou *et al.*, 2000). Increased cytosolic Ca^{2+}, due to both

influx of extracellular Ca^{2+} via NMDA receptor and mobilization of intracellular Ca^{2+} stores, plays an important role in cyanide toxicity, leading to lipid peroxidation and subsequent neuronal damage (Shou *et al.*, 2000; Maharaj *et al.*, 2003). The number of antioxidant enzymes inhibited by cyanide also contributes to the hypothesis that oxidative stress plays an important role in cyanide induced neurotoxicity (Maharaj *et al.*, 2003).

Cyanide causes a rapid and severe depletion of cellular ATP and cell death that is dependent on cellular energy impairment but not necessarily lipid peroxidation (Maharaj *et al.*, 2003). The final transport of electrons across the inner mitochondrial membrane is inhibited with cyanide by inhibition of cytochrome a_1a_3 which reduces the number and rate of electron production by mitochondrial metabolism (Maharaj, 2003).

2.4 Mechanisms of Neurodegeneration: The Lethal Triplet

Three main mechanisms of neuronal cell death, both necrotic and apoptotic in nature, which may act separately or cooperatively to cause neurodegeneration has been described as the lethal triplet comprising of metabolic comprise, excitotoxicity and oxidative stress (Alexi *et al.*, 2000).

In general, necrosis is a form of cell death by 'murder' that involves an injury from which the cell can't recover, causing the cell to lyse. Necrosis is characterized by causing further tissue damage, affecting neighbouring cells and attracting pro-inflammatory cells to the lesion (Alexi *et al.*, 2000; Somayajulu *et al.*, 2005). On the contrary, apoptosis is a form of programmed cell death ('suicide'), whereby the cell activates a self-destruct mechanism causing it to shrink and ultimately be phagocytosed by microglia (Alexi *et al.*, 2000; Somayajulu *et al.*, 2005). Apoptosis is a physiological process designed to maintain normal tissue development and homeostasis, protecting against growth of cells carrying cancerous mutations (Somayajulu *et al.*, 2005).

Metabolic comprise of neurons is caused by stroke, asphyxiation, and certain respiratory (mainly mitochondrial) poisons, like cyanide, MPTP and rotenone, characterized by the inhibition of the mitochondrial electron transport chain and tricarboxylic acid cycle and consequential depletion of cellular energy; ultimately resulting in neurodegeneration preferentially in the basal ganglia. Cell death appears to be both apoptotic and necrotic (Alexi *et al.*, 2000).

Mitochondrial dysfunction results in loss in intracellular Ca^{2+} buffering capacity and in increased production of reactive oxygen species, hence leading to oxidative stress. ATP depletion causes failure of ATP-dependent ion pumps resulting in depolarization of neurons, a loss of ionic integrity and accumulation of intracellular Ca^{2+}, inducing mitochondrial strain, free radical generation and a host of downstream neurotoxic processes (Alexi *et al.*, 2000).

Excitotoxicity is due to the activation of the NMDA receptors, by excitatory amino acids, which leads to an influx and toxic overloading of Ca^{2+}, which causes indiscriminate activation of Ca^{2+}-dependent signals such as phospholipase and protease, as well as oxidative stress. The cytotoxicity of Ca^{2+} may involve not only Ca^{2+} overloading but also disordering of intracellular Ca^{2+} dynamics and mitochondrial ATP synthesis. NMDA has been shown to increase not only cytosolic levels of Ca^{2+} but also the mitochondrial Ca^{2+} concentration (Alexi *et al.*, 2000).

The lethal triplet of metabolic compromise, excitotoxicity and oxidative stress may also act cooperatively in causing neuronal cell death. Metabolic impairment, cause depolarization of neurons and the loss of ionic integrity due to bioenergetic impairment and releases the voltage block on the NMDA receptor, activating it and causing secondary excitotoxicity (Alexi *et al.*, 2000).

The striatum, a glutamaceptive region containing glutamate receptors, is predisposed to excitotoxic mechanisms (Alexi *et al.*, 2000). Metabolic compromise has been shown to involve an excitotoxic component and may also cause oxidative stress by inducing the production of free radicals (Alexi *et al.*, 2000). Reversely, oxidative stress may also cause metabolic impairment and initiate excitotoxic pathways. Oxidative stress can cause lipid peroxidation yielding the byproduct 4-hydroxynonenal, which impairs glucose transport, consequently leading to energetic failure, inhibits Na^{+}/K^{+}-ATPase activity, necessary for maintaining neuronal polarization and therefore the voltage-dependent Mg^{+} block of the NMDA receptor channel. Also, NO producing peroxynitrate causes mitochondrial depolarization, impairing the transport of electrons along the mitochondrial matrix during ATP syntheses resulting in depletion ATP (Alexi *et al.*, 2000). Excitotoxicity also leads to oxidative stress (Alexi *et al.*, 2000).

These events cause a series of intracellular responses which either promote the recovery of the cell or cause it to die and biological insults that cause neuronal cell death generally do so via one or more mechanisms of the lethal triplet. (Alexi *et al.*, 2000).

2.5 Ageing and Age-Related Neurodegenerative diseases

2.5.1 Ageing

Ageing is an inevitable biological process characterized by a general decline in various physiological functions and irreversible injuries associated with accumulation of gradual progressive oxidative changes to cells and tissues (Harman, 1998; Ashok and Ali, 1999; Inal *et al.*, 2001; Tahara *et al.*, 2001 Arivazhagan *et al.*, 2002; Junqueira *et al.*, 2004).

These alterations compromise an organism's ability to meet both internal and external challenges and lead to progressive increases in the chance of disease and death (Harman, 1998; Ashok and Ali, 1999; Arivazhagan *et al.*, 2002). These changes may be attributed to disease, environment, immune dysfunction and genetic defects as well as the inborn ageing process (Harman, 1998; Ashok and Ali, 1999).

The involvement of free radicals in ageing and age-related neurodegenerative diseases, e.g. Parkinson's and Alzheimer's diseases, has been postulated by Harman in 1992 (Arivazhagan *et al.*, 2002). As a consequence of normal ageing, age-related accumulation of oxidative damage is worsened by the consequent decline in the organism's antioxidant defence system, resulting in oxidative stress (Inal *et al.*, 2001; Arivazhagan *et al.*, 2002; Jones *et al.*, 2002; Okatani *et al.*, 2002; Junqueira *et al.*, 2004; Young and Woodside, 2005). Net increases in free radicals within the intracellular environment accelerate cell damage and lead to the pathophysiological changes associated with ageing (Okatani *et al.*, 2002; Finkel, 2003). In addition, cells of older organisms carry an increased burden of oxidatively damaged macromolecules (Jones *et al.*, 2002; Levine, 2002). Oxidative stress thus may be responsible for the progressive deterioration of biological systems with time (Harman, 1998).

Ageing still remains the most important risk factor for neurodegeneration, suggesting that during senescence the brain may become more vulnerable to these insults which can be compounded over long periods of time (Giasson *et al.*, 2002).

2.5.2 Schizophrenia and Dementia

There is much evidence that oxidative cell injury may play an important role in the pathogenesis of schizophrenia, which may be exacerbated by the treatment with antipsychotics with pro-oxidant properties (Akyol *et al.*, 2002; Parikh *et al.*, 2003). Clinical studies have indicated changes in the activities of the antioxidant enzymes due to the

neuroleptic treatment (Akyol *et al.*, 2002). Lipid peroxidation, as a marker for free radical mediated damage, is significantly increased in dementia patients (Delibas *et al.*, 2002).

2.5.3 Alzheimer's Disease

Alzheimer's disease is the major cause of dementia in the United States (Harman, 1998), with a typical age of onset as early as 60 years for sporadic cases and a prevalence that can reach as high as 30% by the age of 80 (Giasson *et al.*, 2002). Increasing numbers of individuals are developing this devastating disease because of the demographic shift of the population to older ages (Melov, 2002a). Patients suffering from Alzheimer's disease can be categorized into 2 groups: (1) late onset, after about age 60, 90-95% of patients, largely sporadic and (2) early onset, before the age 60; 5-10% of Alzheimer's patients, mostly, perhaps all, familial (Harman, 1998).

Alzheimer's disease is a systemic disorder with its major manifestations in the brain (Harman, 1998), and is associated with a progressive deterioration in cognitive, language, executive and behavioural functions, impairments associated with neuronal loss, synaptic changes and the accumulation of proteinaceous lesions in certain vulnerable areas in the brain (Giasson *et al.*, 2002).

A growing body of evidence indicates that the age-dependent changes are closely associated with increased oxidative stress, preceding amyloid β deposition and oxidative damage to neuronal cells (Harman, 1998; Bush, 2002; Giasson *et al.*, 2002). Increased lipid peroxidation has also been observed in brains of patients with dementia type of Alzheimer's disease, due to increased levels of malondialdehyde that doesn't correlate with age, (Delibas *et al.*, 2002; Giasson *et al.*, 2002). Oxidative damage to proteins, as ascertained by the generation of carbonyl functional groups, increases faster in brains from patients with Alzheimer's disease than in the normal ageing process. There is also substantial evidence for nitrative damage due to the presence of 3-nitrotyrosine (Giasson *et al.*, 2002).

Based upon the implication of amyloid β as the culprit protein in Alzheimer's disease, the major approaches for developing therapeutics for this disorder have attempted either to prevent amyloid β production (secretase inhibitors) or to clear amyloid β (vaccine) (Bush, 2002). Current drug therapies for Alzheimer's disease target symptomatic relief and do not interdict the underlying causal pathobiology (Bush, 2002). Clioquinol (5-chloro-7-iodo-8-hydroxyquinoline) (1) treatment may be able to restore homeostatic defects of normal metal

metabolism which may occur in Alzheimer's disease (Bush, 2002). Bush and colleagues hypothesized that a highly specific chelator of metal ions that bind to amyloid β would not only reduce amyloid load in a mouse model of Alzheimer's disease, but would also be therapeutic from the perspective of reducing oxidative stress within the brain (Melov, 2002a).

2.5.4 Parkinson's Disease

Parkinson's disease is the most common disease of motor system degeneration and, following Alzheimer's disease, the second most common neurodegenerative disease (Zheng *et al.*, 2005), and one of the most debilitating diseases in the United States (US) (Personal Health Lifestyles, Inc., 2002).

Parkinson's is highly age-dependent: it can manifest as early as the mid 30's, but becomes more common past the age of 50, with 57 being the average age of diagnosis in the United States. Typical sporadic Parkinson's disease has a prevalence of 0.6% at 65 years of age, but the risk of developing Parkinson's disease increases with age with a prevalence of 4-5% by the age of 85 (Giasson *et al.*, 2002). Ageing contributes to the progression of this disorder, perhaps because of its accumulative oxidative damage and steady decrease of antioxidant capacity (Kidd, 2000).

Parkinson's disease or "shaking palsy" is a terminal progressive neurodegenerative disorder whereby patients experience a debilitating loss of movement functionality. Initially Parkinson's disease becomes noticeable as tremor in a limb, and as it progresses motor symptoms including bradykinesia, rigidity, weakness, trembling muscles and posture instability arises (Alexi *et al.*, 2000; Przedborski and Vila, 2001; Personal Health Lifestyles, Inc., 2002; Sohmiya *et al.*, 2004). These symptoms worsen progressively as the neurodegeneration continue until patients are virtually unable to move (Alexi *et al*, 2000).

Parkinson's disease takes a heavy toll in mental anguish. The disease is not restricted to motor degeneration – as many as 35% of parkinsonian cases also develop dementia, and depression is also very common among these patients (Kidd, 2000; Personal Health Lifestyles, Inc., 2002). Parkinson's disease affects not only the central nervous system but also the peripheral and enteric systems (Kidd, 2000).

Normally, dopamine produced in the substantia nigra is moved to the caudate nucleus and the putamen, where it is involved in stimulating and coordinating the body's motor movements. The hallmark of Parkinson's disease is the dramatic and selective degeneration of dopamine-

producing neurons in the substantia nigra pars compacta, and it is to this profound decline in striatal dopamine to which most of the disabling abnormalities of Parkinson's disease are attributed (Kidd, 2000; Przedborski and Vila, 2001; Giasson *et al.,* 2002; Greenamyre, *et al.,* 2003).

Increased levels of iron and MAO-B, as well as subsequent production of free radicals due to the oxidation of dopamine, amplifying the oxidative burden within the brain, have been implicated to cause neuronal damage observed in Parkinson's disease. Mitochondrial dysfunction at complex I, resulting in a decreased level of energy production has also been observed in the substantia nigra of patients suffering from Parkinson's disease (Mizuno *et al.,* 1989; Schapira *et al.,* 1989; Jiménez-Jiménez *et al.,* 2000; Kidd, 2000). In addition, the electron leakage in the Parkinsonian brain was shown to be abnormally accentuated (Kidd, 2000; Greenamyre, *et al.,* 2003).

Furthermore, indicators of free radical damage, such as lipid peroxidation and oxidized DNA, are increased in post mortem parkinsonian brain, and nitrotyrosine, a marker of oxidative stress, is found in Lewy bodies in dopaminergic nigral neurons. (Alexi *et al.,* 2000; Giasson *et al.,* 2002; Shachar *et al.,* 2004; Zheng *et al.,* 2005).

Another pathological hallmark of Parkinson's disease is the accumulation of cytoplasmic proteinacious inclusions called Lewy bodies (Greenamyre, *et al.,* 2003). Among the many proteins identified in Lewy bodies, α-synuclein is a major component, not just in Parkinson's disease but also in dementia with Lewy bodies and in the Lewy body variant of Alzheimer's disease (Alexi *et al,* 2000; Kidd, 2000; Giasson *et al.,* 2002). Although Parkinsonism occurs commonly as a sporadic form, there are much rarer early-onset familial forms and growing evidence is consistent with a strong heritability component to this juvenile onset (Kidd, 2000). One such rare familial form is seen due to a dominant mutation in the α-synuclein gene (Alexi *et al,* 2000). Mutated α-synuclein has a tendency to misfold and aggregate, especially under oxidative stress.

Parkinson's disease has been linked to ten distinct chromosomal loci *(park*1-10) (Alexi *et al.,* 2000; Greenamyre, *et al.,* 2003). To date, seven loci on four chromosomes are reliably linked to neurodegeneration of the parkinsonian type, not always with the presence of the Lewy structures (Kidd, 2000).

Although several theories exist, the cause of Parkinson's disease is unknown (Personal Health Lifestyles, Inc., 2002). Cumulative evidence suggests this disorder be a multifactorial oxidative disease (Kidd, 2000). Exposure to noxious factors may orchestrate molecular events over a period of years or decades that lead to the formation of proteinaceous inclusions and cell loss that culminate into clinical symptoms (Giasson *et al.,* 2002). Evidence shows that an exogenous insult is needed to set the substantia nigra degenerative breakdown in motion, but no single agent has been confirmed beyond doubt (Kidd, 2000).

The pathology process that underlies Parkinson's disease typically is slow-paced but relentlessly progressive, the clinical symptoms tending to manifest relatively late in the pathological progression (Kidd, 2000).

Currently, Parkinson's disease is managed mainly through dopamine replacement therapy, with pharmaceutical agents aimed at replacing dopamine in the brain or mimicking its actions at dopamine receptors (Kidd, 2000).

The most potent treatment for parkinsonism remains the administration of the most immediate biochemical precursor to dopamine, L-3,4-dihydroxyphenylanaline, or levodopa (L-Dopa) in combination with carbidopa to prolong its retention, which by replenishing the brain with dopamine, alleviates the symptoms of Parkinson's disease (Alexi *et al*, 2000; Przedborski and Vila, 2001). However these drugs only improve clinical symptoms, but cannot mitigate the underlying progressive degeneration of dopamine-producing neurons in the substantia nigra (Kidd, 2000; Zheng *et al.*, 2005).

Patients experience benefits initially, but rarely do the benefits persist. Initially L-Dopa is effective against motor symptoms, but after 2-5 years of treatment, the patient's responses become erratic and the drug tends to cause motor fluctuations, featuring excessive and uncontrollable movements. Chronic L-Dopa treatment wears off of the palliative effects causing daily fluctuations called 'on-off' effects (Alexi *et al*, 2000; Kidd, 2000), which may be as debilitating as Parkinson's disease itself (Przedborski and Vila, 2001).

As Parkinson's disease progresses, in addition to the adverse effects accruing from L-Dopa therapy the ever-worsening loss of dopamine neurons causes progressively crippling damage to motor control circuits throughout the brain, shifting the control, so that the pathways that normally inhibit movement come to dominate those that activate movement (Kidd, 2000).

Other drugs used for symptom management include amantadine (Symmetrel®), selegiline, dopamine agonists (bromocryptine, pergolide, pramipexole, ropinirole), MAO-B inhibitors (rasagiline and selgiline (Eldepryl®), deprenyl, catechol-methyl transferase inhibitor, entacapone (Zheng *et al.*, 2005) and several anticholineric drugs (Kidd, 2000).

Alternative methods is underway to attenuate the progress of Parkinson's disease (Kidd, 2000), and neuroprotective strategies concentrate on the promotion of neuronal survival which involves improving the function of surviving cells or interfering with neurotoxic processes (Alexi et al., 2000).

One of the most promising neuronal rescue strategies is treatment with neurotrophic factors that has been found to rescue injured neurons in animal models of Parkinson's disease. Trophic factors have actions as anti-excitotoxins, antioxidants and can improve mitochondrial function. They upregulate calcium buffering proteins, antioxidant enzymes and anti-apoptotic signals, and have been shown to reverse dopaminergic deficits in animal models *in vivo*.

Anti-apoptotic strategies are a more recent approach and are technically more complex due to the gene-based mechanisms that occur during apoptosis and to the general role of apoptosis-related signals in normal physiological function. The advantage of anti-apoptotic therapy is that it is not necessary to determine the cause of the neurodegeneration in order to rescue neurons. Anti-apoptotic therapy successfully protects against neuronal cell death in animal models of Parkinson's disease and a number of small molecules inhibit the pro-apoptotic enzymes, caspases, and are effective in rescuing degenerating neurons (Alexi *et al.*, 2000).

Parkinson's disease exhibits numerous metabolic deficits and many bioenergetic compounds are already in use clinically. Bioenergetic supplementation may be one of the better approaches to treat this disease. Antioxidant therapy, with compounds such as CoQ_{10} and nicotinamide, which improve mitochondrial biochemistry, has proven effective in attenuating neuronal degeneration in animal models of parkinsonism as this disorder show evidence of oxidative stress (Alexi *et al.*, 2000). Patients suffering from Parkinson's disease, taking levodopa might also benefit from concomitant supplementation with folate and vitamin B_{12}, both of which help recycle plasma homocystein, which become elevated with the treatment of L-dopa (Kidd, 2000). With evidence that glutathione depletion is a central event in Parkinson's disease and that the degree of glutathione depletion is worse in the advanced disease, effective repletion of glutathione should be a therapeutic priority (Kidd, 2000).

Combinations of therapeutic approaches often show improved effectiveness. Combining metabolic supplements with antioxidants or anti-excitotoxins shows greater protection than does either strategy independently (Alexi *et al.*, 2000). Alternative approaches are still critically needed against these progressive neurodegenerative diseases (Zheng *et al.*, 2005).

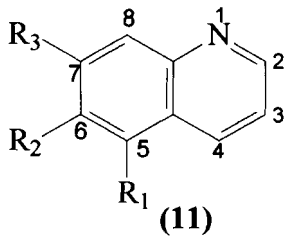
CHAPTER 3. QUINOLINES

3.1 Introduction

Compounds bearing a quinoline moiety are well known for a broad variety of biological activities ranging from cardiovascular, anti-inflammatory, antimicrobial activity and effects on the central nervous system (Musiol *et al.*, 2006).

Quinolines and their derivatives occur in numerous natural products, many of which possess interesting physiological properties (Narender *et al.*, 2006). Apart from the hydroxyl-substituted derivatives, mainly used as urinary tract antiseptics, quinolines have until recently only been studied for the treatment of diseases like HIV and as antibacterial and antimalarial agents (Kayirere *et al.*, 1998; Narender *et al.*, 2006). Substituted quinolines have also been reported to act as antagonists for endothelin, serotonin, and leucotriene receptors. They also function as inhibitors of gastric (H^+/K^+)-ATPase, dihydro-orotate dehydrogenase, and 5-lipoxygenase (Narender *et al.*, 2006).

Quinoline derivatives can be considered as the cyclic analogues of homoalkylamines **(11)** (Zwaans and Thomson, 1996; Musiol *et al.*, 2006). Quinolone drugs consist of two major types of ring structures, a naphtyridone nucleus, with nitrogens at positions 1 and 8, and a nucleus with only one nitrogen atom in position 1, referred to as the quinoline nucleus (Appelbaum and Hunter, 2000; Park *et al.*, 2002).



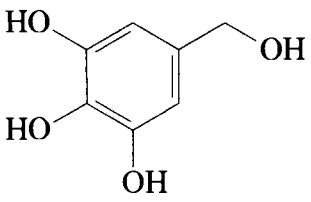
(11)

Many phenolic compounds have been found to be effective antioxidants in biological systems as it scavenges oxygen radicals and suppresses free radical chain oxidation (Laranjinha *et al.*, 1995; Liu *et al.*, 2002), and Zheng *et al.* (2005) showed that 8-hydroxyquinolines, just like flavonoids (section 2.2.2.1.a) and other phenolic compounds, act as radical scavengers due to the presence of the phenolic moiety. The

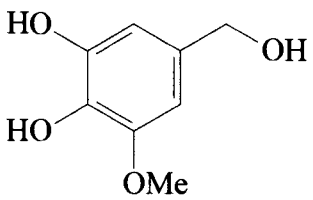
main structural feature responsible for the free radical scavenging activity, is the phenolic hydroxyl group, as it has the ability to neutralize lipid radicals by donating hydrogen atoms from the phenolic hydroxyl group to such radicals, forming resonance-stabilized, poorly reactive phenoxyl radicals ($ROO^{\bullet}$) (eq. 3.1); thereby stopping the propagation of the oxidation chain reaction (Zheng *et al*, 2005; De Pinedo *et al.*, 2006).

$$Phe\text{-}OH + ROO^{\bullet} \rightarrow Phe\text{-}O^{\bullet} + ROOH \qquad \textbf{Equation 3.1}$$

The effectiveness of phenolic compounds depends not only on the stability of the phenoxyl radical formed in the reaction, but substituents at different positions to the phenolic group may vary the properties of the antioxidants (Liu *et al.*, 2002; De Pinedo *et al.*, 2006). A hydroxyl- or methoxy-group at the *ortho* position [galloyl alchohol **(12)** and 5-methoxy-protocatechuyl alcohol **(13)**], giving a catechol ring, lowers the dissociation enthalpy of the O-H bond and increases the rate of hydrogen atom transfer (De Pinedo *et al.*, 2006). In this way the phenolic group can stabilize the phenoxyl radical by the transfer of an additional negative charge to the electron-deficient radical site (Liu *et al.*, 2002).



(12)



(13)

Quinolines with amino ($-NH_2$) groups as substituents allow introduction of a positive charge into the molecule by protonating to $-NH_3^+$ units. This increases the solubility in an aqueous solution (as usually present in living creatures) and hopefully its effectiveness as medicines. It will however, change the charge distribution in the substituent and may therefore affect the activity of the molecule (Zwaans and Thomson, 1996).

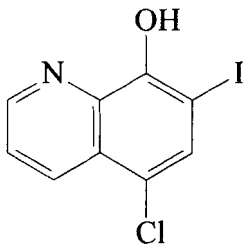
Cytotoxicity of quinolines is mainly affected by increased hydrophobicity and by the addition of electron withdrawing substituents to the quinoline ring (Smith *et al.*, 1997).

Quinolines have an excellent oral bioavailability, with good tissue penetration (Stern *et al.*, 2004).

3.2 Quinolines and Oxidative stress

Several researchers have shown that compounds containing a quinoline moiety have the potential to inhibit oxidative damage to biological systems due to antioxidative activities. Quinolines either scavenge induced free radicals or chelate iron; in this way quinolines prevent the formation of reactive oxygen species, with the possibility to be neuroprotective.

3.2.1 Clioquinol



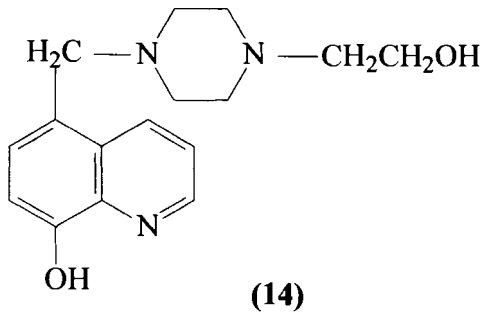
(1)

Clioquinol (5-chloro-7-iodo-8-hydroxyquinoline) **(1)**, previously used in the treatment of Alzheimer's disease, is a hydrophobic drug that freely crosses the blood brain barrier and acts on the amyloid by perturbing its metallo-chemistry, facilitating the dissolution of these aggregates and simultaneously inhibiting the production of neurotoxic hydrogen peroxide (Bush, 2002a; Melov, 2002a). In this way clioquinol possesses antioxidant properties as it has the ability to reduce the production of the pro-oxidant, hydrogen peroxide, and to chelate divalent zinc, copper and iron (Melov, 2002a, Shachar *et al.*, 2004; Zheng *et al.*, 2005). The chelation of these divalent metal ions prevents its participation in the Fenton reaction; hence reduce the production of hydroxyl radicals. In addition, the *in vivo* neuroprotective activity of this antibiotic iron chelator against MPTP-induced neurotoxicity has recently been reported (Shachar *et al.*, 2004; Zheng *et al.*, 2005).

3.2.2 Rebamipide

Rebamipide ([2-(4-chlorobenzoylamino)-3-[2-(1H) quinolinon-4-yl] propionic acid), a quinolone derivative, has the ability to protect the gastrointestinal mucosal integrity against reactive oxygen metabolites. Rebamipide provides gastroprotection through the scavenging of free radicals and prevents delayed wound repair induced by hydrogen peroxide. This protective effect of rebamipide appears to be due to its antioxidative properties (Banan *et al.*, 2001).

3.2.3 VK-28 (5-[4-(2-hydroxyethyl) piperazine-1-ylmethy]-quinoline-8-ol)

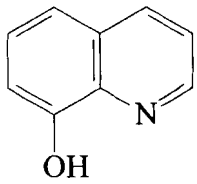


(14)

VK-28 **(14)** has a similar iron binding capacity to that of desferal, a prototype iron chelator which does not cross the blood brain barrier, but when injected intraventricularly, has neuroprotective activity against dopaminergic neurodegeneration as induced by 6-hydroxydopamine or MPTP in mice (Shachar *et al.*, 2004).

VK-28 protects against 6-hydroxydopamine-induced damage in the striatal dopaminergic neurons (Shachar *et al.*, 2004). The mechanism of VK-28's neuroprotective action is most likely related to its antioxidant properties as an iron chelator, since intranigral or intraventricular 6-hydroxydopamine initiates an increase in total iron in the substantia nigra and striatum, which may increase hydroxyl radical production via the Fenton reaction (Shachar *et al.*, 2004).

3.2.4 8-Hydroxyquinolines



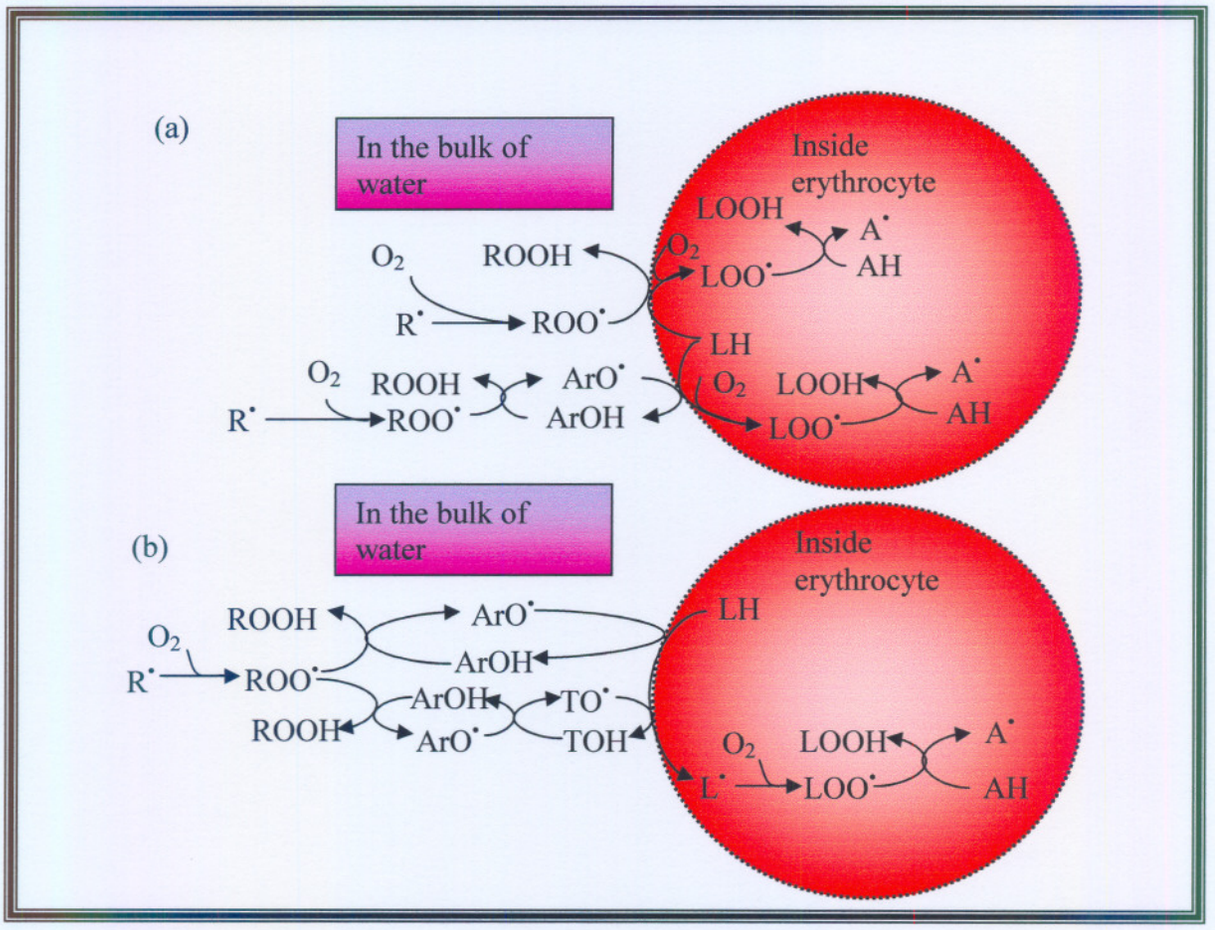
(2)

8-Hydroxyquinoline **(2)** is a strong iron chelator with antioxidative properties. It protects plants against oxidative damage caused by the herbicide paraquat via the blocking of the Fenton reaction. Moreover, 8-hydroxyquinolines has been shown to be able to cross the blood brain barrier (Zheng *et al.*, 2005).

3.2.5 4-Hydroxyquinolines

Studies have shown that 7-fluoro-4-hydroxyquinoline can protect erythrocytes from oxidative hemolysis and may thus possibly be used as antioxidant therapy (Liu *et al.*, 2002).

The synergistic effect of certain endogenous antioxidants in the erythrocyte, like α-tocopherol, significantly prolong the lag time of hemolysis, because it can trap initiating and/or propagating radicals to inhibit peroxidation of polyunsaturated fatty acids (LH) within the membrane of the erythrocytes (Liu *et al.*, 2002).



Scheme 3.1 Pro-oxidative mechanism of FQCE, CQCE, FQCA and CQCA used alone (a) and in combination with α-tocopherol (b) in initiated hemolysis, where ArOH designates FQCA, CQCA, FQCE and CQCE, and AH designates the endogenous antioxidants in erythrocytes (Liu et al., 2002).

Ethyl-7-fluoro-, ethyl-7-chloro-4-hydryoxyquinoline (FQCE; CQCE), 7-chloro- and 7-fluoro-4-hydroxyquinoline-3-carboxylic acid (CQCA; FQCA) packaged in the dipalmitoyl phosphatidylcholine (DPPC) vesicle, play a pro-oxidative role, either alone or in combination with α-tocopherol, in accelerating induced hemolysis of erythrocytes. These compounds can attack the membrane of the erythrocyte and initiate additional free radical propagation, oxidizing polyunsaturated fatty acids (LH) within the erythrocyte. In addition, in the absence of other antioxidants, these compounds can oxidize α-tocopherol (TOH) to the pro-oxidative tocopheroxyl-radical (TO•), which in turn may attack the polyunsaturated fatty acids (LH) on the membrane of erythrocyte to initiate still another

propagation of peroxidation. If these molecules are packaged in DPPC vesicle, the pro-oxidative role may be ascribed to the electron-attracting groups, i.e. $COOC_2H_5$ and COOH, at the *ortho* position to phenolic group, making these radicals unstable (eq. 3.2; 3.3 and eq 3.4) (Liu *et al.*, 2002).

$FQCA^{\bullet}$ ($CQCA^{\bullet}$) + LH → $L^{\bullet}$ + FQCA (CQCA) **Equation 3.2**

$FQCE^{\bullet}$ ($CQCE^{\bullet}$) + TOH → $TO^{\bullet}$ + FQCE(CQCE) **Equation 3.3**

$TO^{\bullet}$ + LH → TOH + $L^{\bullet}$ **Equation 3.4**

Alternatively, 7-chloro-4-hydroxyquinoline (CQ) and 7-fluoro-4-hydroxyquinoline (FQ) protect the erythrocyte against initiated hemolysis efficiently. 7-Chloro- and 7-fluoro-4-hydroxyquinolines recycle the endogenous antioxidant radical ($A^{\bullet}$), and the peroxyl radical of polyunsaturated fatty acids ($LOO^{\bullet}$) can be repaired by these hydroxyquinolines on the surface of the erythrocyte to form the polyunsaturated fatty acid (LOOH) (eq 3.5 and 3.6) (Liu *et al.*, 2002).

$A^{\bullet}$ + FQ (CQ) → AH + $FQ^{\bullet}$ ($CQ^{\bullet}$) **Equation 3.5**

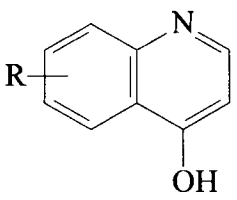
$LOO^{\bullet}$ + FQ (CQ) → LOOH + $FQ^{\bullet}$ ($CQ^{\bullet}$) **Equation 3.6**

The antioxidant activity of 7-fluoro-4-hydroxyquinoline either used alone or in combination with α-tocopherol is better than that of 7-chloro-4-hydroxyquinoline, probably because of the fluorine atom, of which the electron negativity, 4.00, is higher than that of the chlorine atom, 3.15 (Liu *et al.*, 2002). The tendency of the fluorine atom to combine with the surface of the erythrocyte by hydrogen bonding is thus larger than that of the chlorine. By forming a monomolecular 'coat' on the surface of the erythrocyte, with a hydrogen bond between the fluorine atom and the hydrogen atom of the erythrocyte, the peroxyl radical of polyunsaturated fatty acid ($LOO^{\bullet}$) on the surface of erythrocyte or the tocopheroxyl radical ($TO^{\bullet}$) in the bulk of water can be repaired. This protection of the 7-fluoro-compound is thus more efficient than that the 7-chloro-4-hydroxyquinoline. Therefore, the antioxidative effect of 7-fluoro-4-hydroxyquinoline, either used alone or in combination with α-tocopherol is higher than that of 7-chloro-4-hydroxyquinoline, having the potential to be an antioxidative drug (Liu *et al.*, 2002).

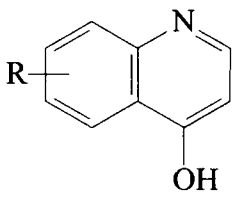
3.3 Free radical scavenging abilities of 4-Hydroxyquinolines

A large body of evidence has revealed that free radical initiated peroxidation of membrane lipids is associated with a variety of pathological events including a series of neurological pathologies (Liu *et al.*, 2002), hence it is necessary to identify compounds which may act as antioxidants within the brain, counteracting such peroxidation. In particular, a series of 4-hydroxyquinoline derivatives were introduced as antioxidants.

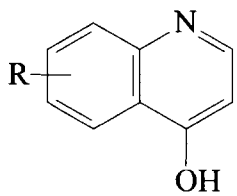
With the aim to clarify the antioxidative properties, a series of 4-hydroxyquinolines were selected as target compounds, synthesized and assayed. The following 4-hydroxyquinolines with a nitro, amino and dibuthylamino – substituent on the 6– or 7-position **(4-9)**, respectively, were synthesized and studied for their antioxidative properties.



(4) R = 6-NO_2
(5) R = 7-NO_2



(6) R = 6-NH_2
(7) R = 7-NH_2



(8) R = 6-$N(C_4H_9)_2$
(9) R = 7-$N(C_4H_9)_2$

3.4 Synthetic routes for quinolines

A large number of methods for the synthesis of quinoline derivatives are reported in the literature, but due to their interesting and important biological properties, the development of new, simple, convenient, and environmentally benign synthetic approaches using mild conditions remains an active area of research (Narender *et al.*, 2006).

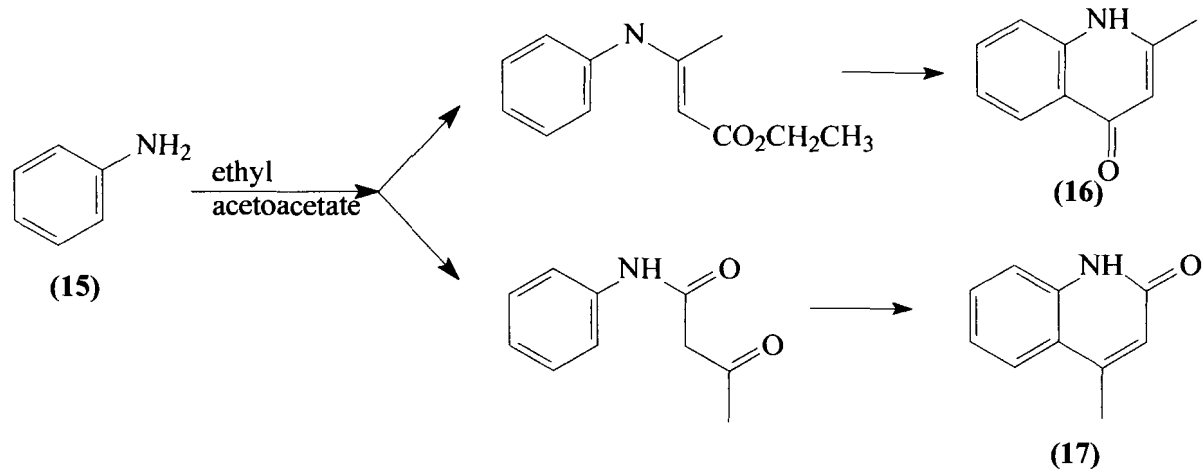
Methods for the synthesis of the quinoline nucleus can for the major part be divided into two main synthetic routes. The first involving cyclisation of a monosubstituted benzene (*N*-substituted aniline) or cyclohexane by reaction with a substituent on the side chain; of which the Skraup, Doebner-von Miller, Knorr and Conrad-Limpach synthesis are

examples. The second major route involves the intramolecular condensation of *o*-disubstituted benzene or the intermolecular condensation of the disubstituted benzene with a second carbon fragment of which the Friedlandänder and Pfitszinger synthesis are examples (Jones, 1977).

However, the Gould-Jacobs synthesis, a variation of the Conrad-Limpach synthesis, is the only synthesis route affording 4-hydroxyquinolines.

3.4.1 Knorr and Conrad-Limpach synthesis

Both the Knorr and Conrad-Limpach synthesis follow through an intermediate prepared by the condensation of an aromatic amine with a β-keto-ester. The intermediates used in the Conrad-Limpach synthesis, crotonates, are obtained by heating the two substances at low temperature in the presencre of an acid catalyst. Intermediates used in the Knorr synthesis, anilides, are obtained by heating the two substances at high temperature without an acid catalyst. As shown in scheme **3.2,** these two synthesis routes are widely used to prepare 2-quinolones (Knorr) and 4-quinolones (Conrad-Limpach). Aniline **(15)** and ethyl acetoacetate yield 2-methyl-4-quinlone **(16)** and 4-methyl-2-quinolone **(17)**, respectively (Campbell, 1976; Jones, 1977).

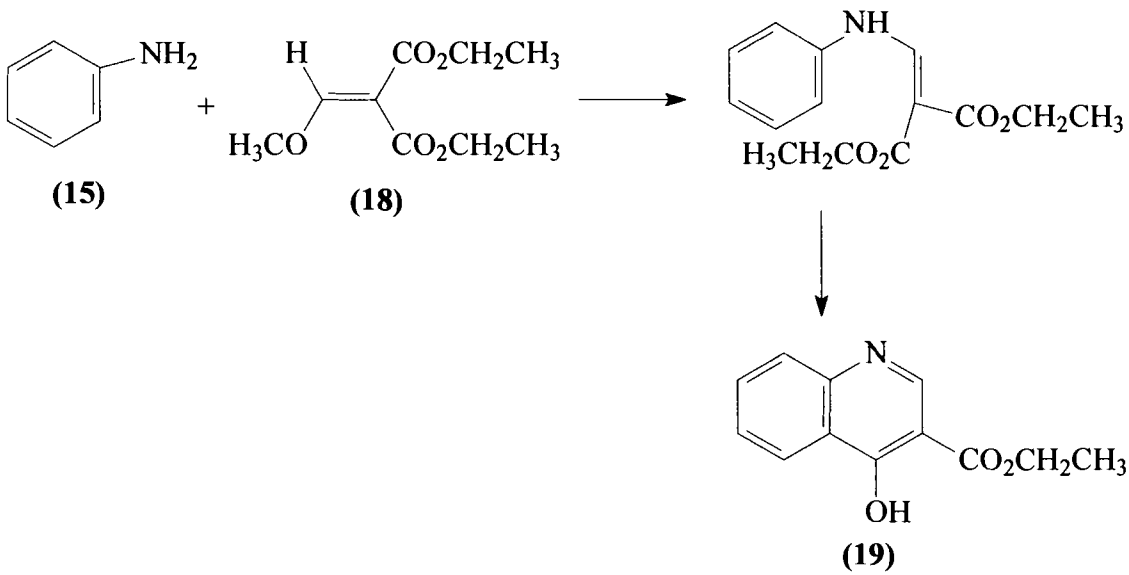


Scheme 3.2

Varying the substitution of the aniline used, with the exception of nitroanilines can achieve the substitution of the benzene ring. However, diamines give satisfactory yields.

3.4.2 Gould-Jacobs reaction

The Gould-Jacobs reaction (Gould and Jacobs, 1939; Baker and Bramhall, 1972; Reigel *et al.*, 1946), a variation to the Conrad-Limpach synthesis, widens the scope, including the synthesis of 4-hydroxyquinolines. As an alternative to the β-keto-ester, diethyl ethoxymethylenemalonate (DEEMM) **(18)** can be used in the condensation process. Anilinomethylene malonates, yielded may be thermally cyclised to 4-hydroxyquinoline-carboxylates (Campbell, 1976) as depicted in scheme **3.3**. Aniline **(15)** and DEEMM **(18)** yield ethyl-4-hydroxyquinoline-3-carboxylate **(19)**.



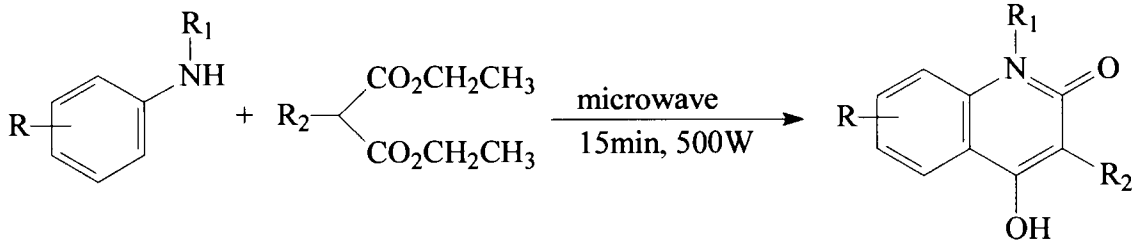
(15) **(18)** **(19)**

Scheme 3.3

Cyclising media such as Dowtherm®, diphenyl ether or mineral oils can be used., and varying the substitution of the used aniline, achieve substitutions in positions 5-, 6-, 7- or 8 (Jones, 1977)

Microwave-enhanced synthesis of 4-hydroxyquinolines **(20)** has been reported by Lange *et al.* (2001). A mixture of the preferred aniline **(21)** and malonic ester **(22)** is irradiated for 15 minutes in a microwave oven according to scheme **3.4**. The temperature reached

can be as high as 290°C. Work-up conditions for these reactions generally consist of simple filtration as the products often precipitate during the reaction (Lange *et al.*, 2001).



(21) **(22)** **(20)**

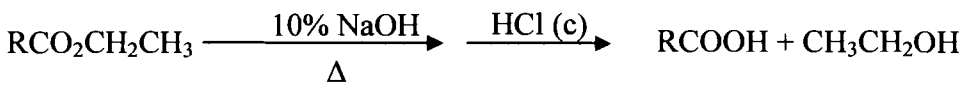
Scheme 3.4

3.5 Transformation reactions

Van Dyk (2001) successfully proposed a route for the synthesis of substituted 4-hydroxyquinolines, wherein the 4-hydroxyquinoline-carboxylate obtained from the Gould-Jacobs reaction, is subjected to a number of transformation reactions, including hydrolysis, decarboxylation, catalytic reduction and reductive alkylation, yielding the respective 4-hydroxyquinoline.

3.5.1 Hydrolysis

Carboxylates **(23)** can be converted to the corresponding carboxylic acids **(24)**, with hydrolysis. Hydrolysis is achieved by heating the carboxylates in 10% sodium hydroxide (NaOH) followed by precipitation of the free acid with concentrated hydrochloric acid (HCl (c)) (Baker and Bramhall, 1972; Shah and Coats, 1977), yielding the free acid as an amorphous substance (scheme **3.5**):

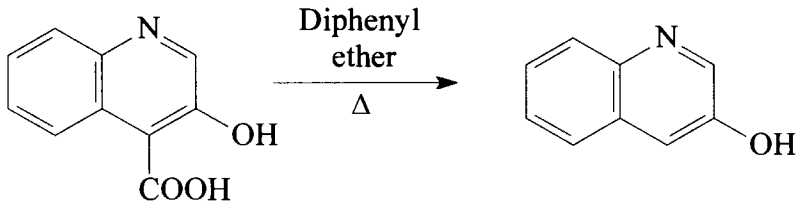


(23) **(24)**

Scheme 3.5

3.5.2 Decarboxylation

Heating hydroxyquinoline-carboxylic acids **(25)** in a solvent with a sufficiently high boiling point, for example diphenyl ether (Van Dyk, 2001) converts the carboxylic acids to hydroxyquinolines **(26)** (scheme **3.6**).

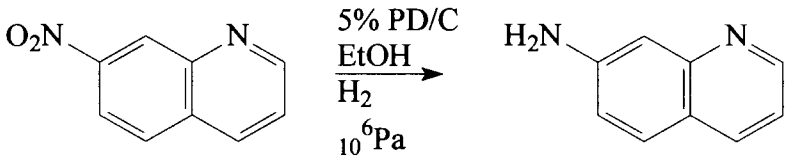


(25) **(26)**

Scheme 3.6

3.5.3 Reduction of nitro compounds

Catalytic hydrogenation with organic solvents attains the reduction of aromatic nitro- to aromatic amino-compounds (Hudlický, 1996). The reduction of nitroquinolines **(27)** to the corresponding aminoquinolines **(28)** can be achieved by hydrogenation of the nitroquinolines **(27)**, dissolved in ethanol in the presence of palladium on carbon as the catalyst (scheme **3.7**) (Van Dyk, 2001). Due to the large difference in the rate of hydrogenation between the nitro group and the aromatic ring, hydrogenation can be regulated in order not to reduce the aromatic ring.

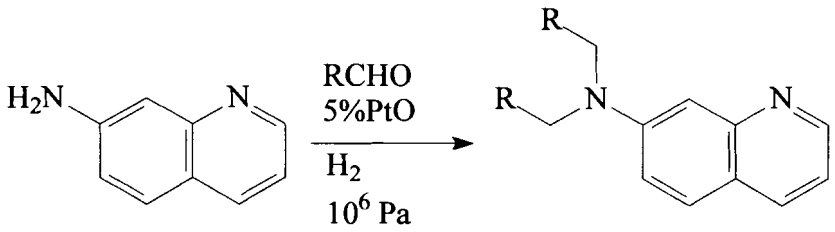


(27) **(28)**

Scheme 3.7

3.5.4 Reductive alkylation

Reductive alkylation is achieved by means of hydrogen along with a catalyst such as platinum oxide (Hudlický, 1996; Van Dyk, 2001) with yields ranging from low (23-35%) to very high (93%). According to scheme **3.8**; aminoquinolines **(28)** subjected to reductive alkylation with hydrogen over platinum oxide yield the corresponding *N,N*-dialkylamino compounds **(29)**. (Cliffe *et al.*, 1991)



(28) **(29)**

Scheme 3.8

3.6 Synthesis of 4-Hydroxyquinolines

The Gould-Jacobs reaction, involving the condensation of substituted aniline with diethyl ethoxymethylenemalonate followed by cyclisation to a quinoline and decarboxylation, was employed for the synthesis of 4-hydroxyquinolines. The final dibuthylamino compounds were derived from the quinolines by a number of transformation reactions, and the identity of the prepared compounds was confirmed by physical means (mainly NMR, MS and IR) and comparison to data reported by Van Dyk (2001).

3.6.1 Standard experimental techniques

3.6.1.1 Instrumentation

(a) Nuclear magnetic resonance spectroscopy

^{13}C and ^{1}H nuclear magnetic resonance (NMR) spectra were recorded on a Varian Gemini 300 or a Varian T60 spectrometer. ^{13}C spectra were recorded at a frequency of 75, 462 MHz and ^{1}H spectra at a frequency of 300,075 MHz or 60MHz. All chemical

shifts are reported in parts per million (ppm) relative to tetramethylsilane (δ = 0). The following abbreviations were used to describe multiplicity of ^{1}H signals: s = singlet, d = doublet, t = triplet, q=quartet and m = multiplet. Combinations of these abbreviations indicate corresponding multiplicity of the signals.

(b) Infrared absorption spectra

Infrared (IR) spectra were recorded on a Nicolet 550 series II spectrometer using KBr pellets.

(c) Mass spectrometry

Mass spectra (MS) were recorded on an analytical VG 7070E mass spectrometer using electron impact (EI) at 70 eV as ionisation technique.

(d) Melting point determination

Melting points (mp) were determined by differential scanning calorimetry (DSC). DSC thermograms were recorded with a Shimadzu DSC-50 instrument. Measurement conditions were as follows: sample weight of approximately 2 mg, an aluminium crimp cell sample holder, nitrogen gas flow at 40 ml/min and heating rate at 10°C/min.

3.6.1.2 Chromatography

(a) Thin layer chromatography

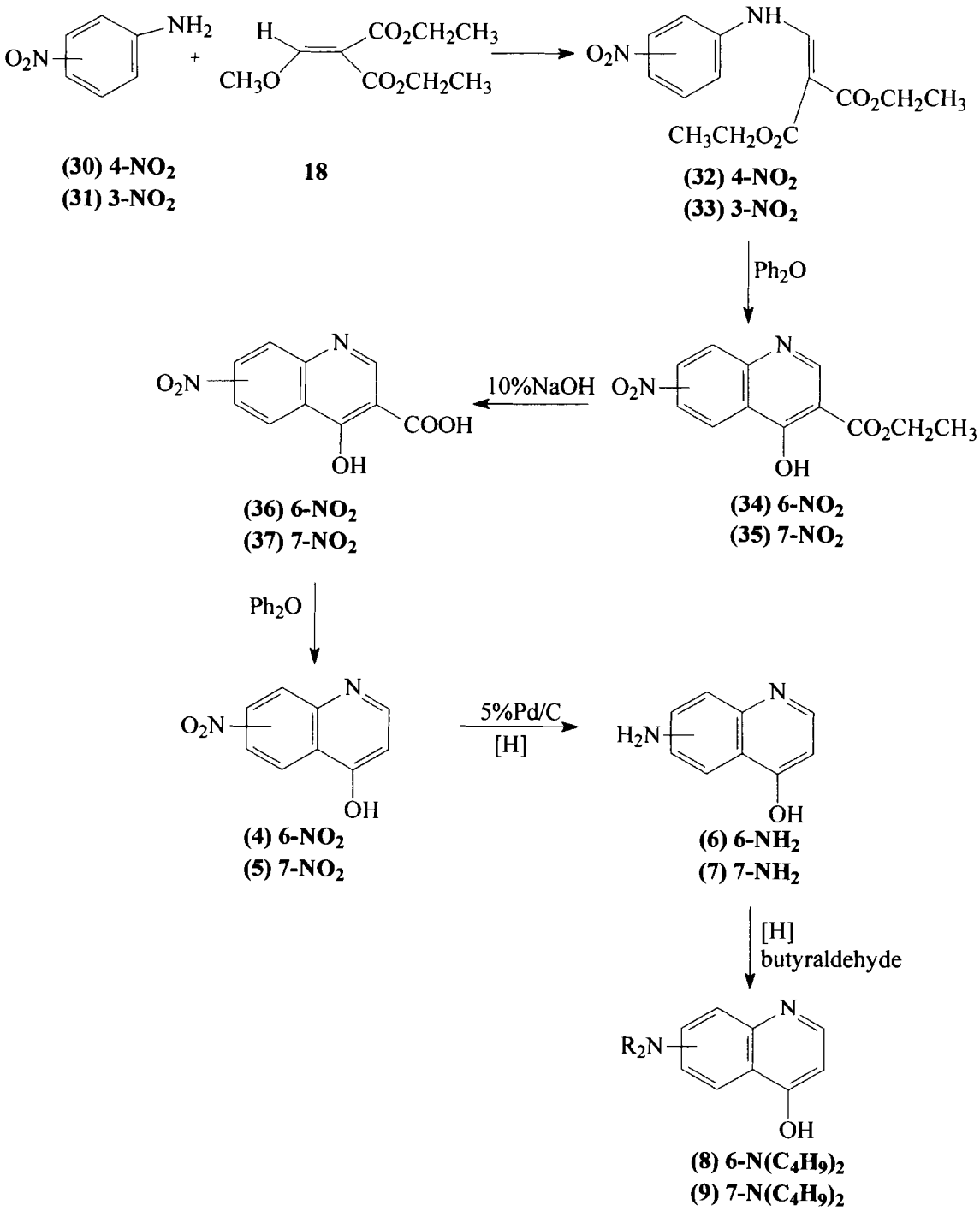
Analytical thin layer chromatography was performed on precoated Merk silica gel aluminium backed plates, thickness 0,25 mm (Merk® 5554 DC – Alufolien 60 F_{254}) with eluent systems as indicated for the individual compounds. Mobile phases were prepared by mixing solvents in a volume to volume ratio. Chromatograms were examined under UV-light (254 and 366 nm) for the detection of the individual compounds.

(b) Column chromatography

Column chromatography was performed using standard glass columns of length 600 mm and inner diameters of 150, 300 or 400 mm. The stationary phase used was Merk® Silica 60 (0,063 – 0,200 mm) silica gel, with eluents as indicated for the individual compounds. Mobile phases were prepared by mixing the solvents in a volume to volume ratio.

3.6.2 Gould – Jacobs reaction for the synthesis of 4-hydroxyquinolines

The total synthesis route, described by Van Dyk (2001) was executed for the preparation of the selected substituted 4-hydroxyquinolines and is depicted in scheme **3.9**.



(30) 4-NO_2
(31) 3-NO_2

18

(32) 4-NO_2
(33) 3-NO_2

(34) 6-NO_2
(35) 7-NO_2

(36) 6-NO_2
(37) 7-NO_2

(4) 6-NO_2
(5) 7-NO_2

(6) 6-NH_2
(7) 7-NH_2

(8) 6-$N(C_4H_9)_2$
(9) 7-$N(C_4H_9)_2$

Scheme 3.9

Diethyl-4-nitroanilinemethylenemalonate **(32)** and diethyl-3-nitroanilinemethylenemalonate **(33)** was synthesised by condensation of 4- and 3-nitroaniline **(30;31)**, respectively with DEEMM **(18)** (Section 3.4.2; Scheme 3.3). Cyclisation of the above to the respective ethyl nitro-4-hydroxyquinoline-3-carboxylates **(34;35)**, was achieved by heating the compounds in diphenyl ether at 260°C (Scheme 3.3). The carboxylates were hydrolyzed to the corresponding nitro-4-hydroxyquinoline-3-carboxylic acids **(36;37)** (Section 3.5.1; Scheme 3.5). Decarboxylation of the nitro-4-hydroxyquinoline-3-carboxylic acids to the nitro-4-hydroxyquinolines **(4;5)** respectively was achieved by heating the carboxylic acids at 260°C in diphenyl ether (section 3.5.2 Scheme 3.6), and catalytic hydrogenation (section 3.5.3; Scheme 3.7) was employed to reduce the nitro-4-hydroxyquinolines to the respective amino-4-hydroxyquinolines **(6;7)**, which was subsequently subjected to reductive alkylation with hydrogen over platinum oxide (section 3.5.4; Scheme 3.8) yielding the corresponding *N,N*-dibuthylamino-4-hydroxyquinolines **(8;9)** (Van Dyk, 2001).

3.6.2.1 Substituted nitroanilinemethylenemalonates

Following the method described by Van Dyk (2001), diethyl ethoxymethylenemalonate [DEEMM- **(18)**] and 4- nitro-and 3-nitroaniline **(30;31)**, respectively, were heated on a steam bath 6 hours resulting in solid lumps of crystals on cooling. Recrystallization of the products yielded 94% **(32)** as fine cream needles and 91% **(33)** as short thick, light yellow needles, and no further purification was needed.

(a) Diethyl-4-nitroanilinemethylenemalonate (32)

Mp 144,64°C (142 -143°C Reigel *et al.*, 1946); $C_{14}H_{16}N_2O_2$; M^+ 308; *m/z* (%) (spectrum 1): 308 (44), 262 (100), 235 (11), 206 (23), 206 (93), 189 (57), 186 (11), 162 (14); ν_{max} (spectrum 2, KBr, cm^{-1}): 748, 823, 864, 1024,1240, 1338, 1377, 1415, 1449, 1516, 1576, 1641, 1685, 2924; δ_H (spectrum 3, 300,075 MHz, $CDCl_3$): 1,35 (t; 3H; J=7,1; CH_3), 1,39 (t; 3H; J=7,1; CH_3), 4,28 (q; 2H;J=7,1; CH_2), 4,33 (q; 2H;J=7,1; CH_2), 7,23 (d; 2H; J=9,1; H-2', H-6'), 8,28 (d; 2H; J=9,1; H-3', H-5'), 8,41 (d; 1H; J=13,1; C=C**H**), 11.21 (d; 1H; J=13,0; N**H**) δ_C (spectrum 4, 75,462 MHz, $CDCl_3$):14.01 (CH_3), 14,17 (CH_3),

60,55 (CH_2), 60,86 (CH_2), 97,31 (-C=**C**), 116, 35 (C-2',C-6'), 126,01 (C-3', C-5'), 143,90 (C-4'), 144,59 (C-1'), 149,75 (-H**C**=C), 165,14 (CO_2), 168,63 (CO_2); HETCOR δ_H (spectrum 5, $CDCl_3$): 1,28 (t; CH_3), 1,31 (t; CH_3), 4,21 (q; CH_2), 4,26 (q; CH_2), 7,16 (d; H-2'; H-6'), 8,20 (d; H-3'; H-5'), 8,44 (d; C=C**H**); δ_C 13,96 (CH_3), 14.11 (CH_3) 60,47 (CH_2), 60,75 (CH_2), 116,21 (C-2'; C-6'), 125,88 (C-2'; C-5'), 149,56 (-H**C**=C).

(b) Diethyl-3-nitroanilinemethylenemalonate (33)

Mp 86,71°C (83-84°C Shah and Coats, 1977); $C_{14}H_{16}N_2O_2$; M^+ 308; *m/z* (%) (spectrum 6): 308 (53), 262 (87), 235 (8), 206 (100), 189 (42), 162 (13); ν_{max} (spectrum 7, KBr, cm^{-1}): 700, 800, 1000, 1100,1260, 1280, 1350, 1405, 1504, 1580, 1600, 1650, 1700, 2300; δ_H (spectrum 8, 300,075 MHz, $CDCl_3$): 1,35 (t; 3H; J=7,1; CH_3), 1,39 (t; 3H; J=7,1; CH_3), 4,28 (q; 2H;J=7,1; CH_2), 4,33 (q; 2H;J=7,1; CH_2), 7,45 (d; 1H; J=7,9; H-4'), 7,57 (t; 1H; J=7,9; H-5'), 8,0 (d; 1H; J=7.9; H-6'), 8,0 (s; 1H; H-2'), 8.53 (d;1H; J=13,1; **H**C=C), 11,2 (d; 1H; J=13,1; N**H**); δ_C (spectrum 9, 75,462 MHz, $CDCl_3$):14.0.3 (CH_3), 14,19 (CH_3), 60,36 (CH_2), 60,67 (CH_2), 96,17 (HC=**C**), 111,50(C-2'), 118.99 (C-6'), 122,61 (C-4'), 130.80 (C-5'), 140,67 (C-3'), 149,45 (H**C**=C), 150,62 (C-1'), 165,29 (CO_2), 168,82 (CO_2); HETCOR δ_H (spectrum 10, $CDCl_3$): 1,28 (t; CH_3), 1,31 (t; CH_3), 4,21 (q; CH_2), 4,26 (q; CH_2), 7,38 (d; H-4'), 7,50 (t; H-5'), 7.92 (d; H-6'), 7,94 (s; H-2'), 8,45 (d; **H**C=C); δ_C 13,99 (CH_3), 14.13 (CH_3) 60,30 (CH_2), 60,59 (CH_2), 111,40 (C-2'), 118,29 (C-6'), 122,50 (C-4'), 130,66 (C-5'), 150,49 (H**C**=C).

3.6.2.2 4-Hydroxyquinoline-3-carboxylates

According to Van Dyk (2001), the respective diethyl-nitroanilinemethylenemalonate **(32** and **33)** was heated in diphenyl ether at 260°C for 45 minutes and any products that had formed on cooling were collected by filtration, washed with petroleum ether and dried under vacuum. The respective ethyl nitro-4-hydroxyquinoline-3-carboxylates **(34;35)** were obtained in sufficiently pure products of **(34)** in a yield of 72% as light yellow crystals and 72% of **(35)** as a cream amorphous powder.

(a) Ethyl-6-nitro-4-hydroxyquinoline-3-carboxylate (34)

Mp 348,83°C (>320°C Reigel *et al.*, 1946); $C_{12}H_{10}N_2O_5$; M^+ 262; *m/z* (%) (spectrum 11): 262 (28), 216 (64), 186 (4), 170 (11); ν_{max} (spectrum 12, KBr, cm^{-1}): 600, 740, 800, 870, 1010, 1060, 1190, 1210, 1340, 1500, 1620, 1690, 3110, 3500; δ_H (spectrum 13, 60 MHz, TFA): 1,36 (t; 3H; J=7; CH_3), 3,50 (q; J=7; CH_2), 7,15 (d; 1H; J=11; H-8), 7,68 (dd; 1H; J=11; J=2; H-7), 8,25 (d; 1H; J=2; H-5), 8,25 (s; 1H; H-2).

(b) Ethyl-7-nitro-4-hydroxyquinoline-3-carboxylate (35)

Mp 333,10°C (325-326°C Shah and Coats, 1977); $C_{12}H_{10}N_2O_5$; M^+ 262; *m/z* (%) (spectrum 14): 262 (20), 216 (47), 191 (21), 186 (20), 170 (18), 160 (14), 145 (33), 133 (18); ν_{max} (spectrum 15, KBr, cm^{-1}): 510, 625, 750, 800, 860, 1020, 1140, 1200, 1290, 1360, 1470, 1530, 1570, 1620, 1700, 3100, 3450; δ_H (spectrum 16, 60 MHz, TFA): 1,47 (t; 3H; J=7; CH_3), 4,61 (q; 2H; J=7; CH_2), 8,50 (dd; 1H; J=2; J=9; H-6), 8,76 (d; 1H; J=9; H-5), 8,95 (d; J=2; H-8), 9,35 (s; 1H; H-2).

3.6.2.3 Hydrolysis to 4-hydroxyquinoline-3-carboxylic acids

The ethyl-nitro-4-hydroxyquinoline-3-carboxylate **(34** and **35)** was refluxed, respectively for 2 hours in ethanol and 10% NaOH. The nitro-4-hydroxyquinoline-3-carboxylic acids **(36;37)** were precipitated with HCl (c) subsequent to dilution with water, since direct addition of HCl (c) afforded gelatinous precipitates which was difficult to filtrate. Crystallization from ethanol yielded 74% of **(36)** as light yellow needles and 80% **(37)** as beige crystals.

(a) 6-Nitro-4-hydroxyquinoline-3-carboxylic acids (36)

Mp 287,34 and 322,61°C (>320°C Reigel *et al.*,1946); $C_{10}H_6N_2O_5$; M^+ 234; *m/z* (%) (spectrum 17): 234 (10), 216 (19), 190 (12), 170 (5); ν_{max} (spectrum 18, KBr, cm^{-1}): 520, 580, 632, 740, 848, 1060, 1160, 1260, 1350, 1410, 1480, 1510, 1550, 1620, 1710, 3070, 3350, 3450, δ_H (spectrum 19, 300,075, DMSO): 8,06 (d; 1H; J=9,1; H-8), 8,58 (dd; 1H; J=2,6; J=9,1; H-7), 8,85 (d; 1H; J=2,6; H-5), 8,90 (s; 1H; H-2); δ_c (spectrum 20, 75,462 MHz, DMSO): 108,96 (C-3), 121,31 (C-5), 121,86 (C-8), 124,24 (C-4a), 127,69 (C-7),

143,05 (C-6), 144,54 (C-8a), 146,95 (C-2), 165,64 (CO_2), 178,15 (C-4); HETCOR δ_H (spectrum 21, DMSO): 7,95 (d; H-8); 8,56 (dd; H-7), 8,85 (d; H-5), 8,97 (s; H-2); δ_C 121,2 (C-5), 121,8 (C-8) 127,6 (C-7), 147,3 (C-2).

(b) 7-Nitro-4-hydroxyquinoline-3-carboxylic acids (37)

Mp 303,08°C (282°C Shah and Coats, 1977); $C_{10}H_6N_2O_5$; M^+ 234; *m/z* (%) (spectrum 22): 234 (6), 216 (100), 190 (11), 170 (58),158 (12), 142 (17); ν_{max} (spectrum 23, KBr, cm^{-1}): 510, 620, 750, 800, 910, 1000, 1116, 1150, 1200, 1296, 1350, 1480, 1520, 1560, 1610, 1700, 2990, 3090, 3450, δ_H (spectrum 24, 300,075, DMSO): 8,13 (d; 1H; J=9,4; H-5), 8,76 (dd; 1H; J=2,7; J=9,4; H-6), 9,10 (d; 1H; J=2,7; H-8), 9,19 (s; 1H; H-2).

3.6.2.4 Decarboxylation to nitro-4-hydroxyquinolines

The nitro-4-hydroxyquinoline-3-carboxylic acids **(36;37)** was added respectively to boiling diphenyl ether, which was kept boiling for 3 hours. The reaction mixture was allowed to cool and the resulting precipitate was collected by filtration and washed with petroleum ether (Van Dyk, 2001). Purification by chromatography yielded 69% of **(4)** as light yellow needles and 87% of **(5)** as light yellow crystals.

(a) 6-Nitro-4-hydroxyquinoline (4)

R_f = 0,70 (dichloromethane:ethanol:ethyl acetate 1:1:1); mp 331,94°C; $C_9H_6N_2O_3$; M^+ 190; *m/z* (%) (spectrum 25): 190 (22), 160 (100), 144 (15), 132 (47), 116 (28); ν_{max} (spectrum 26, KBr, cm^{-1}): 520, 550, 740, 800, 840, 920, 1030, 1120, 1200, 1340, 1500, 1590, 1610, 1650, 3090, 3480; δ_H (spectrum 27, 300,075 MHz, DMSO): 6,15 (d; 1H; J=7,6; H-3), 7,70 (d; 1H; J=9,2; H-8); 8,01 (d; 1H; J=7,6; H-2), 8,37 (dd; 1H; J=2,7; J=9,2; C-7), 8,79 (d; 1H; J=2,7; H-5); δ_C (spectrum 28, 75,462 MHz, DMSO): 110,36 (C-3), 120,30 (C-8), 121,74 (C-5), 124,83 (C-4a), 125,92 (C-7), 140,89 (C-2), 142,70 (C-6), 143,97 (C-8a), 176,65 (C-4); HETCOR δ_H (spectrum 29, DMSO): 6,09 (d; H-3), 7,63 (d; H-8), 7,97 (d; H-2), 8,34 (dd; H-7), 8,76 (d; H-5); δ_C 110,2 (C-3), 120,1 (C-8), 121,6 (C-5), 125,8 (C-7), 140,7 (C-2).

(b) 7-Nitro-4-hydroxyquinoline (5)

R_f = 0,62 (dichloromethane:ethanol:ethyl acetate 1:1:1); mp 235,34°C; $C_9H_6N_2O_3$; M^+ 190; *m/z* (%) (spectrum 30): 190 (36), 170 (6), 144 (15),116 (12), 105 (22); ν_{max} (spectrum 31, KBr, cm^{-1}): 700, 750, 810, 880, 1040, 1090, 1160, 1240, 1390, 1490, 1540, 1600, 1650, 3200, 3450; δ_H (spectrum 32, 300,075 MHz, DMSO): 6,16 (d; 1H; J=7,4; H-3), 8,01 (dd; 1H; J=2,2; J=8,9; H-6), 8,08 (d; 1H; J=7,4; H-2), 8,27 (d; 1H; J=8,9; H-5), 8,42 (d; 1H; J=2,1; H-8), 12,14 (s, -OH); δ_C (spectrum 33, 75,462 MHz, DMSO): 110,32 (C-3), 114,58 (C-8), 116,65 (C-6), 127,44 (C-5), 128,99 (C-4a), 139,81 (C-8a), 141,29 (C-2), 149,10 (C-7), 176,10 (C-4); HETCOR δ_H (spectrum 34, DMSO): 6,11 (d; H-3), 8,00 (d; H-6), 8,06 (d; H-2), 8,26 (d; H-5), 8,41 (H-8); δ_C 110,23 (C-3), 114,52 (C-8), 116,62 (C-6), 127,39 (C-5), 141,20 (C-2).

3.6.2.5 Reduction to amino-4-hydroxyquinolines

The nitro-4-hydroxyquinolines **(4;5)** was reduced to the respective amino-4-hydroxyquinolines **(6;7)** employing hydrogenation with palladium on carbon, as a catalyst. Purification by means of chromatography offered a poor yield of 38% of **(6)** and 41% of **(7)** as beige amorphous powders.

(a) 6-Amino-4-hydroxyquinoline (6)

R_f = 0,44 (dichloromethane:ethanol:ethyl acetate 1:1:1); mp 202,26 and 237,11°C; $C_9H_8N_2O$; M^+ 160; *m/z* (%) (spectrum 35): 160 (41), 132 (15), 104 (10); ν_{max} (spectrum 36, KBr, cm^{-1}): 540, 590, 720, 770, 820, 890, 1150, 1200, 1240, 1320, 1490, 1550, 1590, 2940, 3070, 3210, 3340; δ_H (spectrum 37, 300,075 MHz, DMSO): 5,88 (d; 1H; J=7,2; H-3), 7,00 (dd; 1H; J=2,6; J=8,8; H-7), 7,23 (d; 1H; J=2,6; H-5), 7,30 (d; 1H; J=8,8; H-8), 7,69 (d; 1H; J=7,2; H-2); δ_C (spectrum, 75,462 MHz, DMSO): 105,35 (C-5), 106,56 (C-3), 119,16 (C-8), 121,17 (C-7), 127,53 (C-4a), 132,10 (C-8a), 137,36 (C-2), 145,16 (C-6), 176,39 (C-4); HETCOR δ_H (spectrum 39, DMSO): 5,87 (d; H-3), 6,98 (dd; H-7), 7,21 (d; H-5), 7,30 (d; H-8), 7,68 (d; H-2); δ_C 105,15 (C-5), 106,41 (C-3), 119,02 (C-8), 120,98 (C-7), 137,23 (C-2).

(b) 7-Amino-4-hydroxyquinolines (7)

R_f = 0,50 (dichloromethane:ethanol:ethyl acetate 1:1:1); mp 294,71°C; $C_9H_8N_2O$; M^+ 160; *m/z* (%) (spectrum 40): 160 (25), 132 (20), 104 (11); ν_{max} (spectrum 41, KBr, cm^{-1}): 500, 550, 590, 720, 800, 810, 850, 890, 960, 1150, 1170, 1220, 1300, 1400, 1490, 1510, 1550, 1610, 1650, 2820, 2870, 2950, 3190, 3310, 3450; δ_H (spectrum 42, 300,075 MHz, DMSO): 5,80 (d; 1H; J=7,3; H-3), 6,46 (d;1H; J=2,1; H-8), 6,58 (dd; 1H; J=2,1; J=8,8; H-6), 7,59 (d; 1H; J=7,3; H-2), 7,76 (d; 1H; J=8,8; H-5); δ_C (spectrum 43, 75,462 MHz, DMSO): 97,20 (C-8), 107,61 (C-3), 113,07 (C-6), 117,05 (C-4a), 126,28 (C-5), 138,17 (C-2), 142,39 (C-8), 152,13 (C-7), 176,66 (C-4); HETCOR δ_H (spectrum 44, DMSO): 5,77 (d; H-3), 6,45 (d; H-8), 6,57 (dd; H-6), 7,58 (d; H-2), 7,75 (d; H-5); δ_C 97,23(C-8), 107,63 (C-3), 113,08 (C-6), 126,27 (C-5), 138,13 (C-2).

3.6.2.6 Reductive alkylation to *N,N*-dibuthylamino-4-hydroxyquinolines

The amino-hydroxyquinoline **(6;7)** was dissolved in ethanol with addition of platinum oxide as a catalyst. Butyraldehyde was added to the solution for the preparation of the corresponding *N,N*-dibuthylamino-4-hydroxyquinolines. The hydrogenation was conducted at 10^6 Pa hydrogen pressure for 24 hours. Purification with chromatography yielded *N,N*-dibuthylamino-4-hydroxyquinolines 74% **(8)** and 79% **(9)** as brownish oils.

(a) 6-N,N-dibuthylamino-4-hydroxyquinoline (8)

R_f = 0,63 (dichloromethane:ethanol:ethyl acetate 1:1:1); $C_{17}H_{24}N_2O$; M^+ 272; *m/z* (%) (spectrum 45): 272 (50), 229 (100), 187 (57), 173 (47), 149 (23); ν_{max} (spectrum 46, KBr, cm^{-1}): 830, 1070, 1100, 1170, 1215, 1260, 1370, 1415, 1500, 1540, 1585, 1740, 2340, 2370, 2860, 2885, 2915, 2960, 3430; δ_H (spectrum 47, 300,075 MHz, $CDCl_3$): 0,88 (t; 6H; J=7,4; 2 x CH_3), 1,29 (m; 4H, J=7,4; 2 x CH_2), 1,52 (m; 4H; J=7,4; 2 x CH_2), 3,28 (t; 4H; J=7,4; 2 x CH_2), 6,29 (d; 1H; J=7,0; C-3), 7,09 (dd; 1H; J=2,9; J=9,2; H-7), 7,41 (d; 1H; J=2,9; H-5), 7,56 (d; 1H; J=9,2; H-8), 7,64 (d; 1H; J=7,0; C-2); δ_C (spectrum 48, 75,462 MHz, $CDCl_3$): 13,79 (2 x CH_3), 20,16 (2 x CH_2), 29,20 (2 x CH_2), 50,80 (2 x CH_2), 103,10 (C-5), 106,80 (C-3), 120,07 (C-8), 120,20 (C-7), 127,24 (C-4a), 132,23 (C-8a), 145,92 (C-6), 177,56 (C-4).

(b) 7-N,N-dibuthylamino-4-hydroxyquinoline (9)

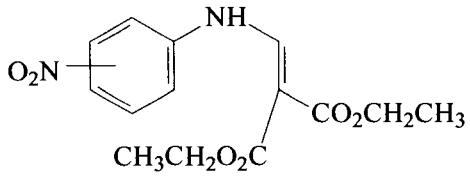
R_f = 0,74 (dichloromethane:ethanol:ethyl acetate 1:1:1); $C_{17}H_{24}N_2O$; M^+ 272; *m/z* (%) (spectrum 49): 272 (5), 229 (20), 216 (9), 173 (34), 149 (18); ν_{max} (spectrum 50, KBr, cm^{-1}): 800, 985, 1230, 1370, 1415, 1460, 1540, 1630, 1660, 1715, 2360, 2370, 2860, 2885, 2930, 2970, 3300; δ_H (spectrum 51, 300,075 MHz, $CDCl_3$): 0,83 (t; 6H; J=7,4; 2 x CH_3), 1,22 (m; 4H, J=7,4; 2 x CH_2), 1,48 (m; 4H; J=7,4; 2 x CH_2), 3,22 (t; 4H; J=7,4; 2 x CH_2), 6,17 (d; 1H; J=7,1; H-3), 6,59 (d; 1H; J=2,0; H-8), 6,72 (dd; 1H; J=2,0; J=9,2; H-6), 7,65 (d; 1H; J=7,1; H-2), 8,11 (d; 1H; J=9,2; H-5); δ_C (spectrum 52, 75,462 MHz, $CDCl_3$): 13,79 (2 x CH_3), 20,07 (2 x CH_2), 29,25 (2 x CH_2), 50,67 (2 x CH_2), 96,22 (C-8), 107,41 (C-3), 111,59 (C-6), 116,32 (C-4a), 126,45 (C-5), 139,13 (C-2), 143,16 (C-8a), 151,07 (C-7), 178,21 (C-4).

3.7 Discussion

A series of 4-hydroxyquinoline compounds **(4-9),** with substituents in position 6- or 7 was selected as target compounds.

The Gould-Jacobs reaction (Gould and Jacobs, 1939), a diethyl ethoxymethylenemalonate variation of the Conrad-Limpach syntheses (Campbell, 1976), was used as it is best suited for the synthesis of the proposed 4-hydroxyquinolines **(4-9)** (Van Dyk, 2001). Hydroxyquinoline-carboxylates, the product from this reaction was subjected to a number of transformation reactions as descried by Van Dyk (2001) producing the proposed substituted 4-hydroxyquinolines **(4-9)**. Identification of the synthesized compounds was achieved by means of physical data and compared to that obtained by Van Dyk (2001).

3.7.1 Diethyl-nitroanilinemethylenemalonate (32;33)

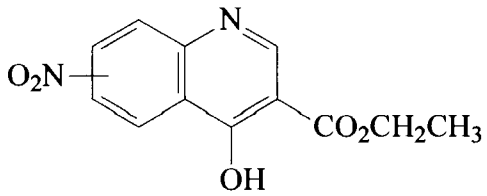


(32) 4-NO_2
(33) 3-NO_2

The molecular ion of **(32)** was confirmed by mass spectrometry (spectrum 1) at *m/z* 308, corresponding to a molecular formula of $C_{14}H_{16}N_2O_2$. NMR signals (spectra 3 and 4) confirmed the structure of **(32)** with triplets at δ_H 1,35 ppm and δ_H 1,39 ppm (spectrum 3), and resonances at δ_C 14,01 ppm and δ_C 14,17 ppm (spectrum 4), which are representative of the methyl groups of the malonate moiety, whereas quartets at δ_H 4,28 ppm and δ_H 4,33 ppm (spectrum 3) and resonances at δ_C 60,55 ppm and δ_C 60,86 ppm (spectrum 4) represented the CH_2 groups. Doublets at δ_H 7,23 ppm and δ_H 8,28 ppm (spectrum 3) and resonances at δ_C 116,35 ppm and δ_C 126,01 ppm (spectrum 4), represent the protonated carbon atoms of the 1,4-substituted benzene ring. A doublet at δ_H 8,41 ppm (spectrum 3) and resonance at δ_C 149,75 ppm (spectrum 4) is indicative of the methylene carbon and proton and a doublet at δ_H 11,21 ppm (spectrum 3) confirms the aniline proton. Resonances at δ_C 165,14 ppm and δ_C 168,63 ppm (spectrum 4) indicate the CO_2 functions of the malonate moiety. Stretching vibration at 1685 cm^{-1} in the IR spectrum (spectrum 2) was evidence of the C=O function.

Corresponding to a molecular formula of $C_{14}H_{16}N_2O_2$, mass spectrometry data (spectrum 6) confirmed the molecular ion of **(33)** at *m/z* 308. Resonances characterising the methylmalonate moiety of **(33)** followed a similar pattern to that of seen for **(32)** (spectra 8 and 9). Substitution on the benzene ring made it possible to distinguish between compounds **(32)** and **(33).** 1,3-Disubtitution of **(33)** was ascertained by a singlet at δ_H 8,0 ppm representing H-2', a doublet at δ_H 8,0 ppm for H-6', a triplet at δ_H 7,57 ppm for H-5' and a doublet at δ_H 7,45 ppm for H-4' (H^1 NMR; spectrum 8). For **(33)**, the C=O stretching vibration was evident at 1700cm^{-1} (spectrum 7).

3.7.2 Ethyl-nitro-4-Hydroxyquinoline-3-carboxylates (34;35)



(34) 6-NO_2
(35) 7-NO_2

Due to poor solubility properties of compounds **(34)** and **(35)** in deuterated solvents available for NMR only H^1 NMR spectra were recorded.

The molecular ion observed at *m/z* 262, by mass spectral data of **(34)** and **(35)** (spectra 11 and 14), correspond to the molecular formula of $C_{12}H_{10}N_2O_5$. Stretching vibrations in the IR spectra (spectra 12 and 15) at 3500 and 3450 cm^{-1}, respectively, signifies the 4-hydroxy function. According to H^1 NMR, a triplet at δ_H 1,36 ppm representing the CH_3 and a quartet at δ_H 3,50 ppm representing the CH_2 for **(34)** (spectrum 13) and a triplet at δ_H 1,47 ppm and a quartet at δ_H 4,61 ppm for **(35)** (spectrum 16), indicated the carboxylate moiety of these two compounds.

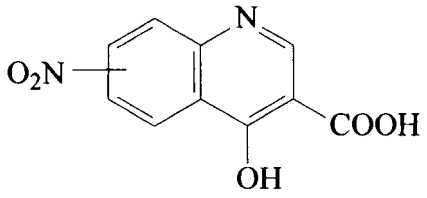
These two compounds were distinguished from each other in terms of proton spin systems in the aromatic region (low field resonance).

A typical ABX-spin system with 2 doublets and a doublet of doublets indicative of the hydrogens on the benzene ring of the quinoline function could be distinguished for **(34)** and **(35)** (spectra 13 and 16). In spectrum 13, a doublet at δ_H 7,15 ppm representative of H-8, a doublet of doublets at δ_H 7,68 ppm indicating H-7 and a doublet at δ_H 8,25 ppm for H-5 confirmed **(34).** Similar spin systems were observed for the rest of the 6-substituted compounds **(36; 4; 6 and 8)** (spectra 19; 27; 37 and 47).

Spectrum 16 showed a singlet at δ_H 8,25 ppm representing H-2; a doublet of doublets at δ_H 8,50 ppm for H-6, a doublet at δ_H 8,76 ppm indicating H-5 and a doublet at δ_H 8,95 ppm representing H-8, for **(35)**. This spin system was also noted for the compounds substituted in the 7-position **(37; 5; 7;** and **9)** (spectra 24; 32; 42 and 51). H-2 was represented with a singlet at δ_H 9,35 ppm.

The IR spectra for **(34)** and **(35)** (spectra 12 and 15) showed broad OH stretching vibrations at 3500 cm^{-1} for **(34)** and 3450 cm^{-1} for **(35)** and C=O stretching vibrations at 1690 cm^{-1} for **(34)** and 1700 cm^{-1} for **(35).**

3.7.3 Nitro-4-hydroxyquinoline-3-carboxylic acids (36;37)



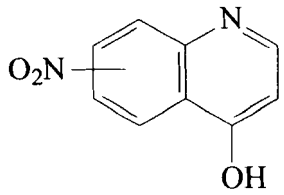
(36) 6-NO_2
(37) 7-NO_2

Mass spectral data (spectra 17 and 22) established the molecular ion of **(36)** and **(37)** at *m/z* 234, corresponding to a molecular formula $C_{10}H_6N_2O_5$. The absence of the triplet and quartet at high field resonance in the H^1 NMR spectra (spectra 19 and 24) and of resonance at approximately δ_C 14,00 ppm and δ_C 60,00 ppm in the C^{13} NMR spectrum (spectrum 20) provided evidence of hydrolysis from the carboxylates **(34;35)** to the corresponding carboxylic acids **(36;37).**

Only H^1 NMR spectrum was recorded for **(37)** owing to poor solubility thereof in deuterated solvents available for NMR. Resonance spin patterns on the benzene moiety following a similar pattern as the spin systems of **(34** and **35)** enabled the distinction between these two compounds **(36** and **37).**

Broad OH stretching vibrations at 3450 cm^{-1} and C=O stretching vibrations at approximately 1700 cm^{-1} was observed in the IR spectra of both these compounds (spectra 18 and 23), providing evidence of the presence of the 4-hydroxy functions as well as the carboxylic acid groups.

3.7.4 Nitro-4-hydroxyquinolines (4;5)



(4) 6-NO_2
(5) 7-NO_2

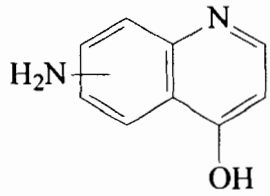
The molecular ions of **(4)** and **(5)** at *m/z* 190, corresponding to the molecular formula of $C_9H_6N_2O_3$, were respectively confirmed by the mass spectral data (spectra 25 and 30).

The decarboxylation of **(36)** and **(37)** to **(4)** and **(5)**, was demonstrated respectively by the appearance of a doublet at δ_H 6,15 ppm (J=7,6) representing H-3 (spectrum 27) and resonance at δ_C 110,36 ppm representing C-3 (spectrum 28) as well as the absence of resonance in the region of δ_C 165,00 ppm indicative of the CO_2 (spectra 28 and 33). The coupling constant of J=7,6 was reciprocated at δ_H 8,01 ppm signifying H-2 (spectrum 27). Resonance indicative for C-2 was observed at δ_C 140, 89 ppm and that of C-3 at δ_C 110, 36 ppm. This is in agreement with the general rule stating that the resonance for C-2 of nitrogen containing rings is found further downfield than that of C-3 (Silverstein and Webster, 1998).

Signals in the NMR spectra which ascertained the structure of **(5)** were the doublet at δ_H 6,16 ppm, (J=7,4) for H-3 (spectrum 32) and resonance at δ_C 110,23 ppm for C-3 (spectrum 33). The reciprocated coupling constant of J=7,4 at δ_H 8,08 ppm represented H-2 (spectrum 32).

To differentiate between compounds **(4)** and **(5)**, the same resonance spin pattern on the benzene moiety, as described previously, could be used (spectra 27 and 32).

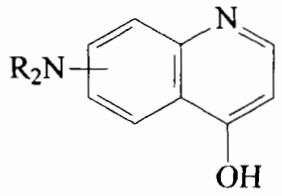
3.7.5 Amino-4-hydroxyquinolines (6;7)



(6) 6-NH_2
(7) 7-NH_2

Mass spectra (spectra 35 and 40) ascertained the molecular ion of **(6)** and **(7)** at *m/z* 160, corresponding to molecular formula of $C_9H_8N_2O$. To distinguish between these two compounds the typical resonance spin patterns observed in the H^1 NMR spectra (spectra 37 and 42) could be applied.

3.7.6 *N,N*-dibuthylamino-4-hydroxyquinolines (8;9)



(8) 6-$N(C_4H_9)_2$
(9) 7-$N(C_4H_9)_2$

The molecular ion of **(8)** and **(9)** was confirmed by mass spectrometry (spectra 45 and 49) at *m/z* 272 corresponding to the molecular formula of $C_{17}H_{24}N_2O$. The typical resonance spin patterns seen in the H^1 NMR spectra, as described previously was used to distinguish between the substitution patterns of 6-*N,N*-dialkylamino-**(8)** and 7-*N,N*-dialkylamino-compounds **(9)** (spectra 47 and 51).

Amines disubtituted with butyl groups **(8;9)** were characterized according to the triplet at δ_H 0,88 ppm (2 x CH_3), the multiplets at δ_H 1,29 ppm (2 x CH_2), and at δ_H 1,52 ppm (2 x CH_2) and the triplet at δ_H 3,28 (2 x CH_2) with a coupling constant J=7,4 for **(8)** (spectrum 47), and corresponding resonances were noted at δ_C 13,79 ppm (2 x CH_3), δ_C 20,16 ppm (2 x CH_2), δ_C 29,20 ppm (2 x CH_2) and δ_C 50,80 ppm (2 x CH_2) (spectrum 48).

NMR signals confirming the butylation of **(9)** were a triplet at δ_H 0,83 ppm, a multiplet at δ_H 1,22 ppm, a multiplet at δ_H 1,48 ppm and a triplet at δ_H 3,22 ppm with a coupling

constant of J=7,4 (spectrum 51) and corresponding resonances at δ_C 13,76 ppm, δ_C 20,07 ppm, δ_C 29,25 ppm and 50,67 (spectrum 52). The identity of **(8)** and **(9)** were also confirmed by means of the IR spectra (spectra 46 and 50) with stretching vibrations evident in the region 2850- 3000 cm^{-1} .

Confirmed identities of the synthesized compounds, with the physical data recorded were evidence that the followed synthetic route, established by Van Dyk (2001) and depicted in scheme 3.9, was successfully implemented.

CHAPTER 4. OXYGEN RADICAL ABSORBANCE CAPACITY

4.1 Introduction

The involvement of oxidative stress in the pathogenesis of various diseases as well as its role in the ageing process have become an area of active investigation and considerable controversy (Glazer, 1988; Prior *et al.,* 2003), and at present there is major interest in the reactive oxygen species – mediated events in biochemical processes (Cao *et al.,* 1996).

Antioxidants counteracting the deleterious effects of reactive oxygen species prevent the formation of these oxidants from their unstable precursors (inhibitory effect) and/or trap these species, by interrupting radical chain reactions (scavenger effect) (Ou *et al.*, 2001; Gaboriau *et al.*, 2002).

Several methods for measuring antioxidant capacity *in vitro* have been developed and reviewed, however, the oxygen radical absorbance capacity (ORAC) assay, with some modifications is the only method that takes the free radical action to completion, using an area under the curve technique to combine both inhibition time and the degree of inhibition into a single quantity (Cao *et al.,* 1996; Cao and Prior, 1999). This provides a unique and complete assessment in which these parameters are measured as the reaction goes to completion (Ou *et al.,* 2001). Because a number of methods measuring antioxidant activity are conducted in aqueous systems, and thus not suitable for lipophilic antioxidants, Huang and co-workers, 2002, introduced randomly methylated β-cyclodextrin (RMCD) as a molecular host to enhance the solubility of lipophilic antioxidants in aqueous solution (Huang *et al.,* 2002).

Cyclodextrins are a group of naturally occurring cage molecules, built up from α-D-glucose units, and depending on the number of glucose moieties in the ring, they are named α-, β- and γ-cyclodextrin. Cyclodextrins are doughnut shaped containing a relatively hydrophobic (fatlike) central cavity and hydrophilic (waterlike) outersurface and can bind a wide variety of organic "guest" compounds inside the apolar cavity within an aqueous solution. The main driving force for this binding is hydrophobic interactions

(Huang *et al.*, 2002). The use of 10-40% methylated-β-cyclodextrin results in the 1000-fold enhancement of the aqueous solubility of lipophilic compounds, making it increasingly popular as a vehicle for enhancing the solubility of lipophilic compounds in an aqueous environment in pharmaceutical and food industries (Huang *et al.*, 2002). Randomly methylated β-cyclodextrin (RMCD) itself consists of hydroxyl- and methoxyl-functional groups and does not possess any antioxidant activity nor does it prevent a complexed antioxidant molecule from functioning as an antioxidant, making RMCD an ideal solubility enhancer (Huang *et al.*, 2002).

Originally developed by Cao and Prior, 1999, and significantly improved in 2001 by Ou and co-workers, the ORAC assay, based on the work by Glazer, 1988, measures the ability of compounds to scavenge peroxyl radicals, induced by thermal decomposition of azo-compounds at 37°C (Ou *et a.*, 2001; Prior *et al.*, 2003), therefore inhibiting oxidation, and thus the decline in fluorescence, of a certain fluorescent probe. The assay depends on the unique properties of the fluorescent probe (Glazer, 1988; Prior and Cao, 1999; Kuti, 2004). The method relates a lag phase or a rate constant of the fluorescence decay to antioxidant capacity of an added antioxidant and under appropriate conditions, the loss of fluorescence in the presence of reactive oxygen species is an index of oxidative damage to the protein (Cao and Prior, 1999). In the ORAC assay, all reactive components of the antioxidative defence, including non-proteic and most of the proteic antioxidants, are oxidized (Gaboriau *et al.*, 2002).

The fast reaction of antioxidants with the peroxyl radicals protects a fluorescent indicator from attack (Gaboriau *et al.*, 2002). β-Phycoerythrin was previously used as a fluorescent probe. Limitations in the use of β-phycoerythrin as a fluorescent probe consisted of inconsistency from lot to lot, resulting in variable reactivity to peroxyl radicals and photoinstability, as after exposure to excitation light for a certain time, it can be photobleached. It was also found that β-phycoerythrin interacted with polyphenols, a major class of antioxidants, causing falsely low ORAC values (Ou *et al.*, 2001).

However, more recently the ORAC method has been adapted to use fluorescein (3',6'-dihydrospiro[isobenzofuran-1[3H], 9'9[9H]-xanthe]-3-one) as the fluorescent probe, instead of the original β-phycoerythrin, as it also has distinct excitation and emission wavelengths, high fluorescence yield and sensitivity to reactive oxygen species and water solubility (Aruoma, 2003; Prior *et al.*, 2003). Fluorescein (pK_a = 6.4) is a small organic compound with a simple structural skeleton, high quantum yield of fluorescence at pH>7 and long wavelengths (492/515nm, excitation/emission) (Ou *et al.*, 2001). Fluorescein does not interact with other compounds, is very stable in a 96 well plate reader and shows excellent photostability without photobleaching (Ou *et al.*, 2001). However, the fluorescence intensity of fluorescein is pH sensitive and when pH drops below 7, its intensity decreases greatly (Ou *et al.*, 2001). The oxidation mechanism of fluorescein has been described by Ou *et al.* (2001). The reaction mechanism for the ORAC assay proceeds as a classic hydrogen atom transfer mechanism of which the driving force is the formation of a delocalized stable radical that does not continue the chain reaction or continues the chain reaction with only a low efficiency (Ou *et al.*, 2001). Chain breaking antioxidants donate labile hydrogen atoms to peroxyl radicals much more rapidly than the peroxyl radicals react with the substrate, forming stable radicals not able to continue the autooxidation of the chain (Ou *et al.*, 2001).

The ORAC assay uses a biologically relevant radical source, namely the water-soluble 2,2'-azo-bis(2-amidinopropane)hydrochloride (AAPH), as a radical azo-initiator (Pajero *et al.*, 2003). AAPH undergoes spontaneous thermal decomposition, yielding molecular nitrogen and two carbon radicals ($R^{\bullet}$), which rapidly react with molecular oxygen to produce peroxyl radicals ($ROO^{\bullet}$) (Cao and Prior, 1999; Gaboriau *et al.*, 2002; Pajero *et al.*, 2003). These peroxyl radicals are produced at a constant rate, primarily determined by temperature (Cao and Prior, 1999; Gaboriau *et al.*, 2002). The analyzed antioxidant samples are not likely to affect this rate, particularly when the chemical structure of AAPH and its very high molar ratio (more than 2000) to an antioxidant sample are considered; therefore, the ORAC assay measures the capacity of an antioxidant to directly quench peroxyl radicals, and the high molar ratio between the free radical generator and an antioxidant indicates a high specificity (Cao and Prior, 1999). Increased AAPH quantities increase the reaction rate and thus decrease the run time and the

corresponding area under the fluorescence decay curve (Prior *et al.*, 2003). However, too little AAPH extend the length of the reaction such that it would be impractical (Prior *et al.*, 2003).

Protection efficiency provided by an antioxidant defence system depends not only on the antioxidant capacity of the system but also on the amount of the substances that need protection (Cao *et al.*, 1996). The ORAC value is a kinetic value as two elements need to be considered in measuring the inhibition of a reactive oxygen species' action by an added antioxidant: the time that the inhibition lasts and the percentage that the inhibition displays at different times (Cao and Prior, 1999). In contrast with the observation by Glazer, 1988, the loss of fluorescence in the presence of reactive oxygen species does not follow zero-order kinetics (Fig. 4.1), and any method based on the assumption of zero-order kinetics will inevitably have technical difficulties in measuring the lag phase in the loss of fluorescence (Glazer, 1988; Cao and Prior, 1999). Relating the percentage inhibition observed at a specific time point to the antioxidant capacity of an antioxidant is not ideal either, because two compounds having the same inhibition percentage at one time point may exhibit different inhibition percentages at another time point (Cao and Prior, 1999). Thus, an area under the fluorescence decay curve (AUC) technique was developed for quantification of the results in the ORAC assay by integrating inhibition percentages over the whole inhibition time period, into a single quantity (Cao and Prior, 1999). In this way the ORAC assay successfully overcomes all related problems in quantification of the antioxidant capacity of an antioxidant (Cao and Prior, 1999). The protective effect of an antioxidant is measured by assessing the area under the fluorescence decay curve of the sample as compared to that of a blank in which no antioxidant is present (Ou *et al.*, 2001, Gaboriau *et al.*, 2002).

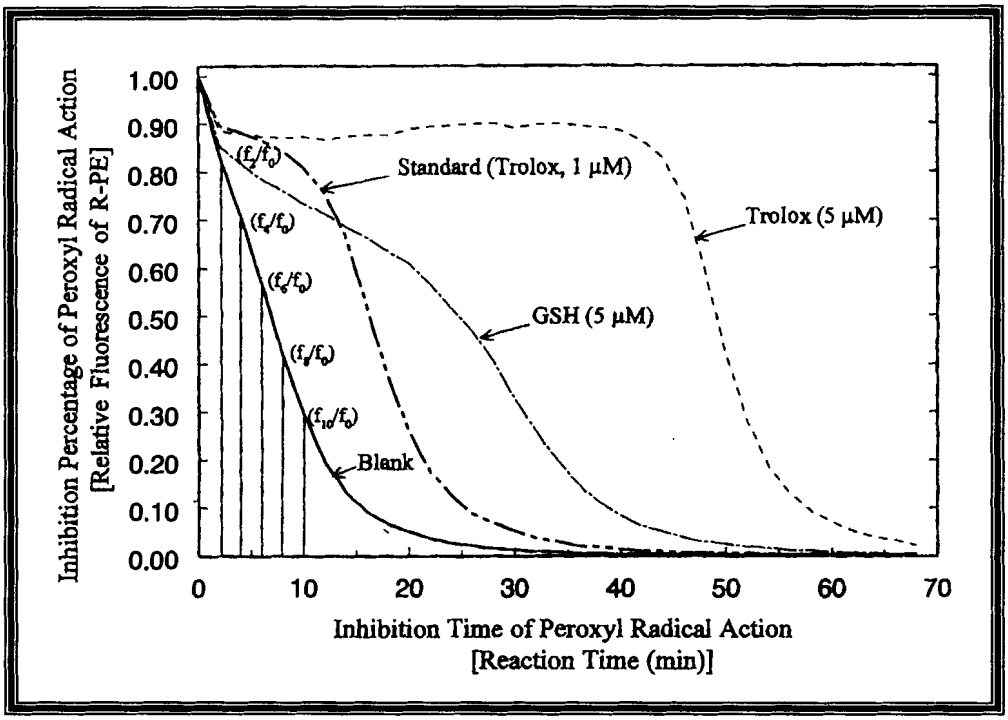
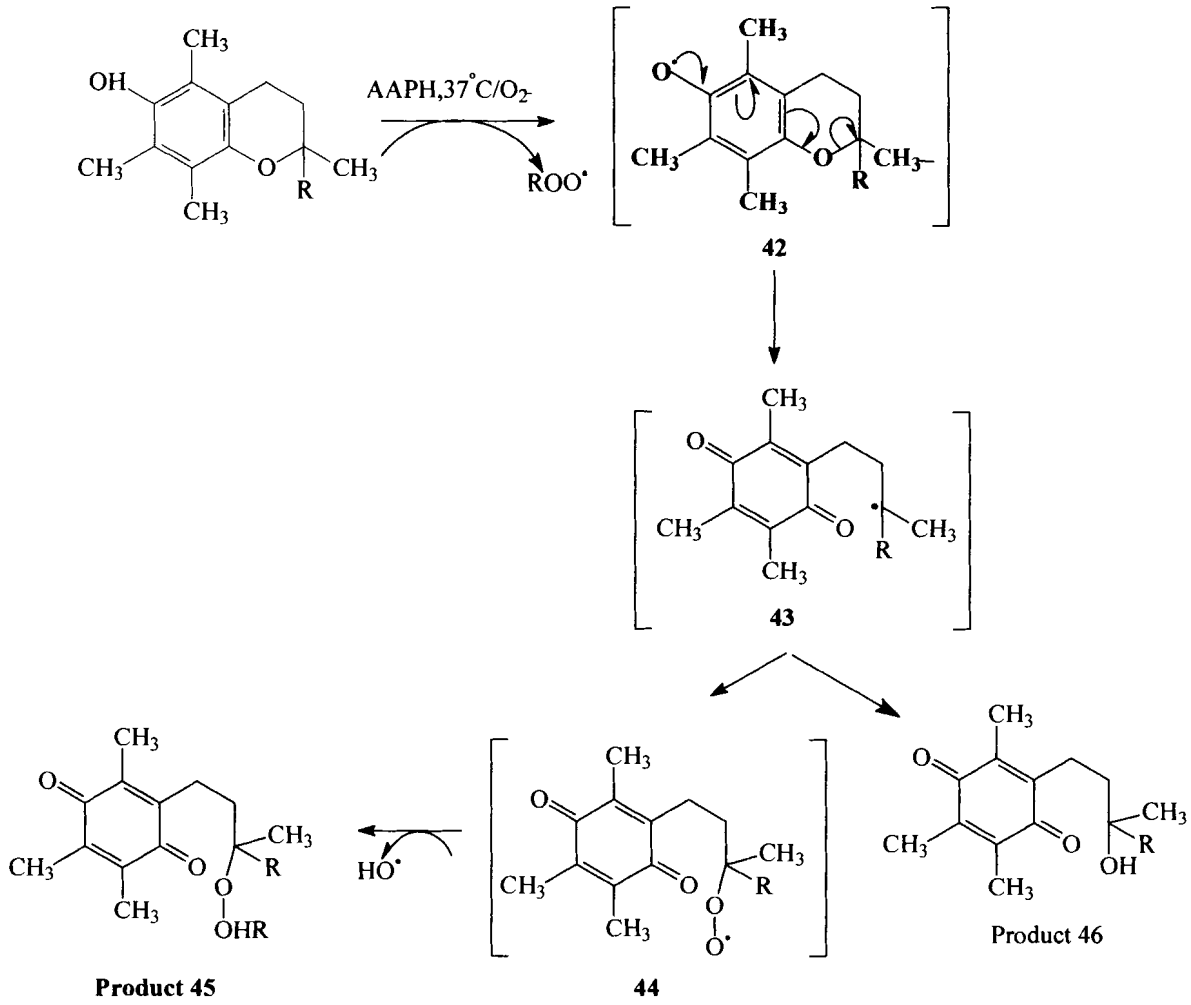


Figure 4.1 Principle of the ORAC assay with β-phycoerytrhin as a target for free radical action and AAPH as a peroxyl radical generation (Cao and Prior, 1999).

The water-soluble vitamin E analogue, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) is an efficient antioxidant and provides complete protection, in as much as one molecule of Trolox traps two peroxyl radicals (Glazer, 1988). Scheme 4.1 illustrates a proposed reaction mechanism for Trolox and α-tocopherol in the presence of AAPH (Huang *et al.*, 2002). As shown, the reaction is initiated by the formation of a phenoxyl radical **(42)** due to a hydrogen atom being abstracted from the phenol group by the peroxyl radical (Huang *et al.*, 2002). This phenoxyl radical **(42)** can further undergo intermolecular arrangement to form an intermediate **(43)**, a tertiary carbyl radical and in the presence of oxygen (O_2), this intermediate **(43)** is peroxidized to yield another intermediate **(44)**, a peroxyl radical that may abstract a hydrogen from the water molecule to yield product 45 and a highly reactive hydroxyl radical (**OH**$^{\bullet}$). Meanwhile, the intermediate **(43)** can couple with the generated hydroxyl radical (**OH**$^{\bullet}$) to produce product 46 (Huang *et al.*, 2002).



Scheme 4.1 Proposed oxidation mechanism for Trolox and α-tocopherol in the presence of AAPH (Huang et al., 2002).

Huang and co-workers (2002) demonstrated that the phenol group is the key functional group for antioxidant activity and the steric hindrance around the phenol group decreases ORAC values of tocopherols and other phenolic lipophilic antioxidants.

ORAC values have shown to be linearly correlated to the concentrations of antioxidants in solution or in plasma samples (Gaboriau *et al.*, 2002). The ORAC assay has also proven to be specific for antioxidants and to be sensitive, precise and robust within accepted criteria (Ou *et al.*, 2001).

The antioxidant capacity of the selected 4-hydroxyquinolines was determined by the ORAC assay described by Cao and Prior (2003), using AAPH as a peroxyl radical ($ROO^{\bullet}$) generator, and fluorescein as the target of free radical attack and fluorescent probe.

4.2 Experimental

4.2.1 Materials and Methods

4.2.1.1 Chemicals and reagents

Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), 2,2'-azobis(2-amidino-propane) dihydrochloride (AAPH) and fluorescein were purchased from Sigma Chemical Co., St Louis, MO. Randomly methylated cyclodextrin (RMCD) was a generous gift from Prof. F.H van der Westhuizen (Department of Biochemisty, NWU, Potchefstroom campus). All other chemicals were of analytical grade.

4.2.1.2 Reagents

Phosphate buffer (75mM, pH 7,4): 1M K_2HPO_4 and NaH_2PO_4, (61.6:38.9, v/v) were diluted with deionised water (1:9, v/v) to make up 75mM phosphate buffer. The pH was ascertained to be 7.4. The 0.75*M* K_2HPO_4 and 0.75*M* NaH_2PO_4 can be stored at 4°C for several months.

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

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

Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, 250µM, in phosphate buffer was aliquoted in vials and kept at -70°C for 4 months. A new set of stock Trolox

vials were removed from the freezer daily for use. A solution of 20μM was prepared by diluting the stock solution with buffer (1:4, v/v). In the standard assay, 40 μl Trolox calibration solutions (2.5; 5; 10; 15 and 20μM) in phosphate buffer (75mM, pH 7.4) were pipetted into appropriate wells.

4.2.2 Sample preparation

10 mg of the nitro-and amino-compounds or 10 μl of the dibuthylamino-4-hydroxyquinoline oils, respectively were accurately weighed and dissolved in 125 μl acetone and diluted with 375 μl of 7% RMCD dissolved in acetone: water (50:50 v/v) solution to enhance solubility (the solution was kept in the fridge until needed). After dilution to a concentration of 5 mg/ml, the tubes were vortexed for 30 seconds, followed by sonication at 37°C for 5 min. The tubes were inverted once in the middle of the sonication step to suspend the samples. The tubes then remained at room temperature for 10 min with occasional shaking, followed by centrifugation at 3500 rpm for 15 min and the supernatant was removed and diluted to 12.5 ml volume for analysis. Further dilutions were made in phosphate buffer.

4.2.3 Instrumentation

All ORAC analyses were preformed on a BioTek FL600 plate reader using an excitation wavelength of 485 nm and an emission filter 520 nm.

4.2.4 ORAC assay

KC4 software on the BioTek FL600 plate reader was used for the analyses of the oxygen radical absorbance capacity of the compounds. A 96- well opaque microtitre plate was used and wells containing appropriate aliquots of phosphate buffer and standard solution were prepared with a final volume of 200 μl.

The first row of the opaque microtitre plate was reserved for the Trolox standards. Trolox was added in a manner similar to the samples, described below, giving final a concentration of 20μM. The calibration curve was generated by measuring the

absorbance of fluorescence decay in the presence of increasing concentration of Trolox in increments of 5μM.

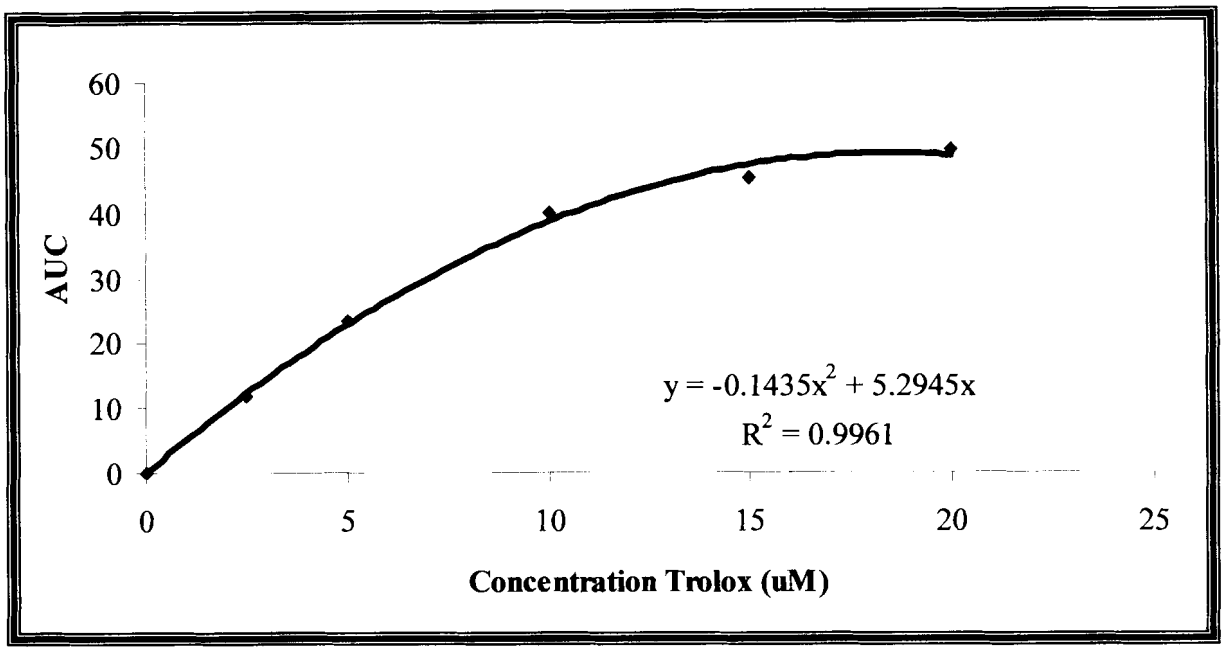


Figure 4.2 Regression of net area under fluorescence decay curve of Trolox standards in increasing concentration.

The rest of the plate wells contained 20 μl of the diluted (1:10) test compound samples, 60 μl fluorescein solution #2 plus 20 μl RMCD. Analyses of all the test samples were replicated four times. The sensitivity was adjusted that it read ±146 and after the first reading at excitation and emission wavelengths of 485 and 520 nm, respectively, the first readings should be approximately 65000 relative fluorescence units (RFU). The reaction was started by adding 100 μl AAPH (48mM) and the reader was initialized to record fluorescence with the same excitation and emission (static mode) every 5 min for 3 hours, or until the fluorescence of the last reading has declined to less than 5% of the initial reading. If the fluorescence of the last reading does not decline to less than 5% of the first reading, the dilution of the sample analyzed can be adjusted accordingly and the sample is reanalyzed (Cao and Prior, 1999). Relative fluorescence unit - data was exported to a disk.

Samples and Trolox calibration solutions were analyzed in a "forward-then-reverse" order as follows: 2.5μM Trolox, 5μM Trolox, 10μM Trolox, 15μM Trolox, 20μM Trolox sample 1. . . sample 1, 20μM Trolox, 15μM Trolox, 10μM Trolox, 5μM Trolox, 2.5μM Trolox. This arrangement can correct possible errors due to the signal drifting associated with the different positions of the same sample (Ou *et al.*, 2001).

4.2.5 Data Collection

The basis for the ORAC value calculation is presented schematically in figure 4.1 (Cao and Prior, 1999). The ORAC value refers to the net protection area under the quenching curve of fluorescein in the presence of an antioxidant (Kuti, 2004), and were calculated based on the area under the fluorescence decay curve of fluorescein in the presence of the test compound compared to that of Trolox, using a regression equation between the Trolox concentration and the net area under the fluorescence decay curve, figure 4.2. All measurements were expressed relative to the initial reading.

Data were analysed using Mirosoft Excel and the results were calculated according to the formulas described by Cao *et al.* (1999) (equations 4.1; 4.2 and 4.3):

The net area under the fluorescence decay curve (AUC) was calculated as:

$$AUC = (0.5 + f_1/f_0 + f_2/f_0 + f_3/f_0 + f_4/f_0 \ldots\ldots f65/f0 + fi/f0) \times CT \qquad \textbf{Equation 4.1}$$

where f_0 is the initial fluorescence reading at 0min, f_i is the fluorescence reading at time i and CT is the cycle time in minutes.

A Trolox standard was used to draw a graph with a second order polynomial slope with eq. 4.2 (Fig. 4.2):

$$y=ax^2+bx+c \qquad \textbf{Equation 4.2}$$

Hence, equation 4.3 was mathematically derived accordingly to determine the ORAC values (μM Trolox equivalents):

$$\text{ORAC value} = -b + \sqrt{\frac{b^2 - 4a(c-y)}{2a}} \text{ x dilution factor} \qquad \textbf{Equation 4.3}$$

The final results are expressed as ORAC units (micromoles of Trolox equivalents per litre of sample [μmole Trolox equivalents/l].

4.2.6 Statistical analysis

Samples were analyzed using four replicates and the data are presented as the mean of 4 values ± S.E.M., and descriptive statistics were done using Graphpad Instat. Data were subjected to one way analysis of variance (ANOVA), and to determine whether there were any significant differences between the radical absorbing capacity of the proposed 4-hydroxyquinolines, analysis of variation was performed with the Student-Newman-Keuls Multiple Range test, where a p-value less than 0.05 were considered significant (Zar, 1974).

4.3 Results

Six different 4-hydroxyquinolines were analyzed for oxygen radical absorbance capacity on a 96 well microtitre plate. The obtained fluorescence decay curves are presented in Appendix B, and the ORAC units were calculated in reference to Trolox. Equation 4.1 was used to obtain the respective area under the fluorescence decay curves (y-value) and this value was incorporated into equation 4.3 to determine the ORAC value (x-value). The relative ORAC values obtained from this assay are presented in Table 4.1 and Figure 4.3.

Table 4.1 Relative ORAC values of the nitro-4-hydroxyquinolines

Total Oxygen Radical Absorbance Capacity			
Compounds	**μM TE***	**±**	**SEM**
6-Nitro-4-hydroxyquinoline (4)	537	±	201
7-Nitro-4-hydroxyquinoline (5)	109	±	17
6-Amino-4-hydroxyquinoline (6)	7463	±	2509
7-Amino-4-hydroxyquinoline (7)	1332	±	88
6-Dibuthylamino-4-hydroxyquinoline (8)	1754	±	379
7-Dibuthylamino-4-hydroxyquinoline (9)	838	±	337

ORAC values (μM TE) are expressed as micromole Trolox equivalents per litre (n=4)

The total ORAC values measured in terms of Trolox equivalents ranged from as low as 106 ± 17 μM/l sample (7-nitro-4-hydroxyquinoline **(5)**) to as high as 7463 ± 2509 μM/l sample (6-amino-4-hydroxyquinoline **(6)**) (Table 4.1).

4.4 Discussion

Defined as molecules that intercept free radicals, antioxidants exhibit scavenging properties on reactive oxygen species (Gaboriau *et al.*, 2002). The total antioxidant capacity of the selected 4-hydroxyquinolines has been determined by the oxygen radical absorbance capacity assay, measuring the ability of a compound to scavenge peroxyl radicals, thereby inhibiting induced decline in the fluorescence of damaged fluorescein (Kuti, 2004). The ORAC method was claimed to be highly specific with an excellent response to a wide range of antioxidants, robust, reliable and sensitive (Lee, 2000; Ou *et al.*, 2001; Huang *et al.*, 2002).

Curves of fluorescence decay (Appendix B), illustrate the peroxyl radical damage to fluorescein (Lee, 2000). When Trolox is added, the rate of fluorescence decay was delayed resulting in a distinctive plateau region, the length of which was directly related to the Trolox concentration. The fluorescence decreased rapidly following the plateau phase in a similar fashion to the blanks that contained no antioxidant. In the presence of Trolox, the net protection area under the quenching curve of fluorescence decay

increased proportionally to an increment of the Trolox concentration. Curves of fluorescence decay are thus an indication of the absorption of peroxyl radicals by an added compound (Lee, 2000).

6-Amino-4-hydroxyquinoline **(6)** showed the highest oxygen radical absorbing capacity compared to the other compounds tested ($p<0.001$) (Fig. 4.3). Because amino-hydroxyquinolines allow the introduction of a positive charge by protonating to $-NH_3^+$ (section 3.1), this substituent may donate a hydrogen atom to the induced peroxyl radical, thereby forming less reactive species, and consequently prevent the radical to react with fluorescein, and thus inhibiting the fluorescence decay.

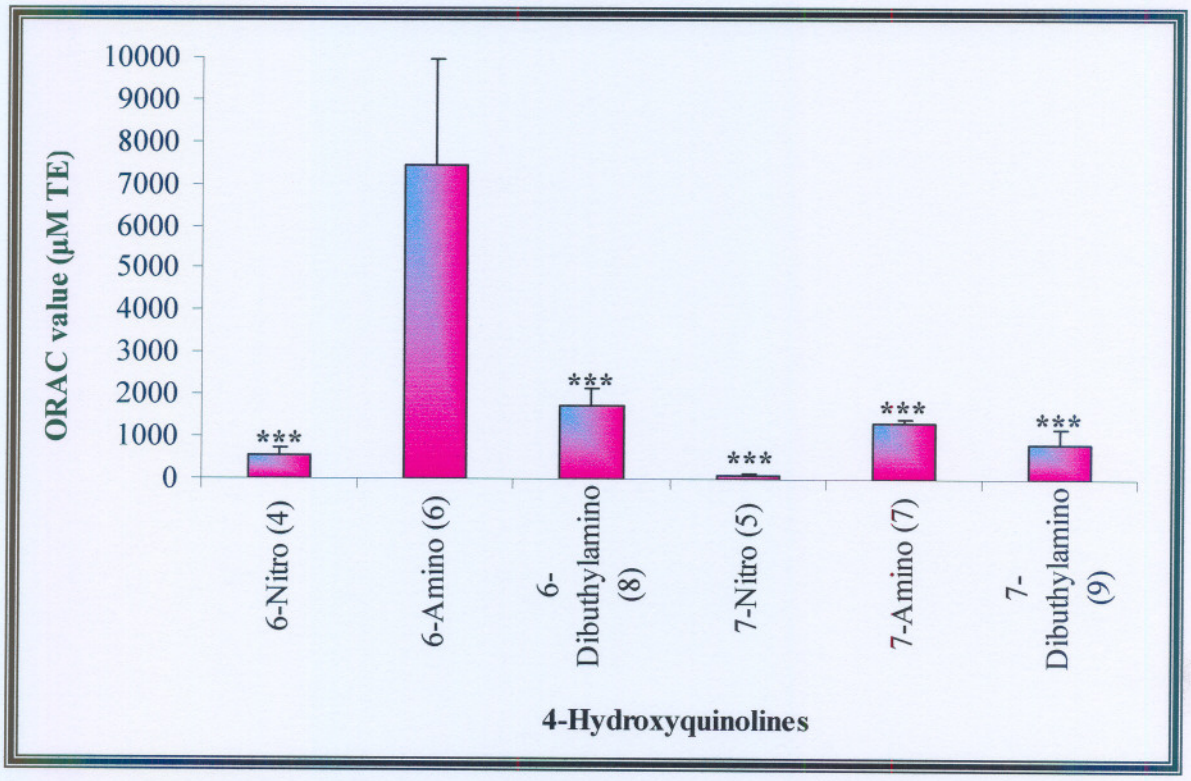


Figure 4.3 Total ORAC values of the 4-hydroxyquinolines (expressed as µmoles Trolox equivalents/l). Data are presented as means ±S.E.M, *n=4.* *** $p<0.001$ (vs. 6-amino-4-hydroxyquinoline).

Although there weren't any significant difference between the rest of the tested compounds, the hydroxyquinolines with the substituent in the 6-position, appeared to show more oxygen radical absorbing capacity than with the substituent in the 7-position. Data presented indicate that the antioxidant capacity of the different compounds differs by more than 70-fold.

These compounds may be important antioxidants, as all the compounds tested had antioxidant capacity in comparison to the antioxidant Trolox, because it delayed the fluorescence decay, thus showing that to some extent, it has an ability to absorb the peroxyl radicals generated by AAPH. Overall, irrespective of the position of the substituent, it appeared that the amino-compounds had more absorbing capacity than the corresponding dibuthylamino-compounds, which in turn was more effective than the nitro-hydroxyquinolines.

CHAPTER 5. FERRIC REDUCING/ANTIOXIDANT POWER

5.1 Introduction

Harmful reactive oxygen species, produced as a consequence of normal aerobic metabolism are usually removed or inactivated *in vivo* by an organism's antioxidative system (Benzie and Strain, 1996). The oxidizing species react with the antioxidant instead of the biomolecular substrate, and in this way the antioxidant reduces the oxidant (Benzie and Strain, 1996). Therefore, antioxidant power may be referred to as reducing ability (Benzie and Strain, 1996). In recent years the implication of iron dependent oxidative stress in neurodegenerative processes and ageing have become an area of active investigation (Pulido *et al.*, 2000; Zheng *et al.*, 2005).

The human body contains iron present in haemoglobin, enzymes and various iron carriers and storage proteins (Halliwell and Gutteridge, 1984). A pool of non-protein-bound iron moving between transferrin, cell cytoplasm, mitochondria and ferritin provides iron as a catalyst for the Fenton reaction generating highly toxic hydroxyl radicals from hydrogen peroxide (Halliwell and Gutteridge, 1984; Prior and Cao, 1999; Ou *et al.*, 2001; Zheng *et al.*, 2005). However, neither Fe^{2+} nor Fe^{3+} is able to directly cause oxidative damage to biomolecules itself (Prior and Cao, 1999) (Section 2.1.2.1*e*).

Iron and hydrogen peroxide can provoke a series of radical reactions (Halliwell and Gutteridge, 1984), and it has been reported that the antioxidative properties of iron chelators, such as desferal and clioquinol, are due to iron chelation activity, forming inert complexes, thereby interfering with the Fenton reaction leading to a decrease in hydroxyl radical production (Zheng *et al.*, 2005) (Section 2.2.2.3).

Because a number of iron chelators, such as desferal and clioquinol, have shown to possess neuroprotective activity in animal models, a new neuroprotective strategy has been proposed, that is, neuroprotection may require a drug combining iron chelating- with antioxidant capacity (Zheng *et al.*, 2005).

A number of methods have been developed to measure the efficiency of antioxidative compounds focussing on different mechanisms of the antioxidant defence system, including scavenging of reactive oxygen species, reduction of peroxyl radicals, inhibition of lipid peroxidation, or chelation of metal ions (Pulido *et al.*, 2000). These tests assess antioxidant power by measuring the ability of a compound to withstand oxidative effects purposefully induced in the reaction mixture (Benzie and Strain, 1996).

In most cases irrespective of the stage in the oxidative chain in which the antioxidant action is assessed, a common mechanism involving redox reactions takes place (Pulido *et al.*, 2000). On this basis, Benzie and Strain (1996) developed a methodology to determine the reducing ability of a compound as a direct measure of its antioxidant power (Benzie and Strain, 1999; Pulido *et al.*, 2000).

The ferric reducing antioxidant power (FRAP) assay uses antioxidants as reductants in a redox-linked colorimetric method, employing an easily reduced oxidant present in stoichiometric excess (Benzie and Strain, 1999). The FRAP assay presumably relies on the hypothesis that the redox reactions proceed so fast that all reactions are complete within a short period of time (Benzie and Strain, 1999; Ou *et al.*, 2001). Unless an antioxidant, by definition prevents the generation of an oxidizing species, delaying or preventing oxidation of a biological substrate, a redox reaction occurs. Reactive oxygen species reacts with the antioxidant instead of the substrate, and therefore the antioxidant reduces the oxidant. Thus, electron-donating antioxidants can be described as reductants, and the subsequent inactivation of oxidants, redox reactions (Benzie and Strain, 1999).

In the FRAP assay stoichiometric factors are constant, absorbance change - linearity is maintained over a wide range of antioxidant concentration mixtures, reproducibility is excellent and sensitivity is high (Benzie and Strain, 1999). In addition, the FRAP assay is inexpensive, simple and quick, and offers a putative index of antioxidant, or reducing potential of a sample (Benzie and Strain, 1996).

The FRAP assay involves neither a pro-oxidant, oxygen radical nor an oxidizable substrate and there are no oxidants applied in the assay (Prior and Cao, 1999; Ou *et al.*, 2001).

The FRAP assay of Benzie and Strain (1996) determines the specific oxidant-reducing power by measuring the reduction of a ferric tripyridyltriazine (Fe^{3+}-$TPTZ_2$) to the ferrous tripyridyltriazine (Fe^{2+}-$TPTZ_2$), which has an intense blue colour with an absorption maximum at 593 nm, by an antioxidant at the non physiological low pH of 3.6, (Benzie and Strain, 1996; Prior and Cao, 1999; Pulido *et al.*, 2000; Aruoma, 2003).

The standard redox potential of Fe^{3+}/Fe^{2+} is 0.77 V; thus the reaction is non-specific in that any half-reaction that has a lower redox potential under the same reaction conditions as that of the ferric/ferrous half-reaction will drive the ferric to ferrous reduction. In this assay an excess of Fe^{3+} is used and the rate-limiting factor of Fe^{2+}- TPTZ formation, thus change in colour, is the reducing ability of the compound tested. The change in absorbance, therefore, is directly related to the "total" reducing power of the electron donating antioxidant present in the reaction mixture (Benzie and Strain, 1999).

FRAP follows a single electron-transfer mechanism, where the antioxidants are oxidized by oxidants (Fe^{3+}) and as a result, a single electron is transferred from the antioxidant molecule to the oxidant (eq. 5.1) (Ou *et al.*, 2001; Ou *et al.*, 2002):

$M(n) + A - H \rightarrow M(n-1) + A - H^+$ **Equation 5.1**

M represents the iron metal ion.

The reaction with ascorbic acid is assumed to be very fast, reaching completion in less than 1 minute. In the FRAP assay, ascorbic acid has a constant stoichiometric factor of 2.0, i.e., direct reaction of Fe^{2+} gives a change in absorbance, half that of an equivalent molar concentration of ascorbic acid. An ascorbic acid standard of 1000μM therefore is equivalent to 2000μM FRAP power (Benzie and Strain, 1996).

FRAP values are obtained by comparing the absorbance change at 593 nm in test reaction mixtures with those containing ferrous ions in known concentration.

The present work was aimed at studying the ferric reducing efficiency of the proposed 4-hydroxyquinolines with a modification of the FRAP assay described by Benzie and Strain (1999). Ascorbic acid was used for comparison.

5.2 Experimental

5.2.1 Materials and Methods

5.2.1.1 Chemicals and reagents

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

5.2.1.2 Reagent preparation

The FRAP reagent, used during the experiment, consisted of 300mM acetate buffer, pH 3.6 (sodium acetate trihydrate in 8 ml glacial acetic acid, made up to 500 ml with distilled water); 10mM tripyridyltriazine (TPTZ) solution in 40mM HCl; and 20mM $FeCl_3.6H_2O$ solution in water, in the ratio a 10:1:1.

Freshly prepared aqueous solutions of a pure antioxidant, ascorbic acid, in the range of 0-1000μM were used for the calibration curve of the FRAP assay.

All the solutions were prepared fresh, as was required and ascorbic acid was prepared and used within the hour.

5.2.1.3 Sample preparation

10 mg of the nitro-and amino compounds powders or 10 μl of the dibuthylamino-4-hydroxyquinoline oils were diluted in ethanol, to give an effective concentration of 10mg/ml in the reaction mixture.

5.2.2 Instrumentation

The FRAP assay was preformed on a BioTek FL600, spectrophotometer, measuring absorbance at 593 nm.

5.2.3 FRAP assay

The reaction was preformed on a transparent 96- well microtitre plate, as this is a colorimetric assay, and the first row was reserved for the ascorbic acid standards. The antioxidant potential of the compounds was determined using a standard curve of increasing concentrations (0-1000 μM) of ascorbic acid. Ascorbic acid was added in a similar manner to the samples, described below.

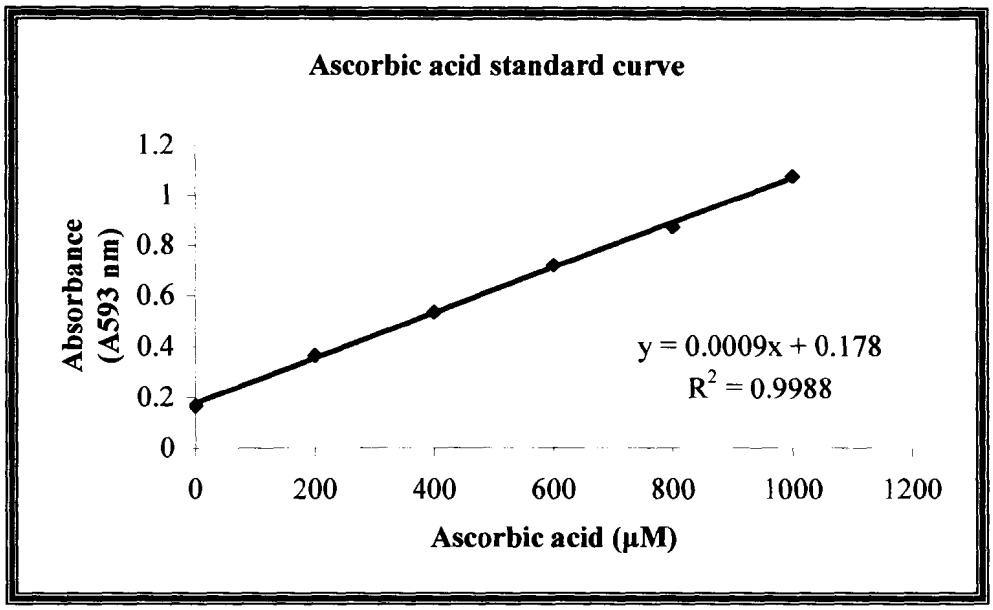


Figure 5.1 Regression of absorbance with increasing concentration of ascorbic acid.

The rest of the wells on the transparent microtitre plate each contained 10 μl of the respective hydroxyquinoline solution, tested in triplicate, 90 μl water and 250 μl FRAP reagent. The final dilution of sample in reaction mixture was therefore 1/34. The reaction was carried out at 37°C, the samples were mixed and the absorbance of each well was measured at 593 nm.

5.2.4 Data Collection

The FRAP value refers to the ability of a compound to reduce Fe^{3+}-TPTZ to a blue Fe^{2+}-complex. Absorbance readings were translated to μM Ascorbic acid equivalents (as the FRAP value) by relating the absorbance value of the reaction mixture consisting of test compound to that of the standard ascorbic acid solution, with a known FRAP value.

Data were analyzed with Microsoft Excel according to the regression equation obtained from the calibration curve of the ascorbic acid standard solution, tested in parallel (y=mx+c; fig 5.1).

The final results are expressed as FRAP value (µM Ascorbic acid equivalents) in regard to the concentration iron reduced per 10 mg test compound.

5.2.5 Statistical Analysis

Samples were analyzed in triplicate and data are presented as the mean of 3 values ± S.E.M., and descriptive statistics were done using Graphpad Instat. Data were subjected to one way analysis of variance (ANOVA), and to determine whether differences between the various compounds existed, analysis of variation was performed with the Student-Newman Keuls Multiple Range test, with significance accepted at $p<0.05$ (Zar, 1974).

5.3 Results

The various hydroxyquinolines were assayed *in vitro* for its ferric reducing ability at a non physiological pH of 3.6, and the FRAP values were calculated using the regression equation obtained from the standard curve of increasing concentrations of ascorbic acid (Fig. 5.1). The FRAP values of the tested compounds are presented in Table 5.1 and figure 5.2:

Table 5.1 FRAP values of the tested 4-hydroxyquinolines

Ferric Reducing/Antioxidant Power			
Compounds (10mg.ml^{-1})	**µM AA***	**±**	**S.E.M.**
6-Nitro-4-hydroxyquinoline (4)	29.259	±	0.7407
6-Amino-4-hydroxyquinoline (6)	3950.370	±	88.604
6-Dibuthylamino-4-hydroxyquinoline (8)	604.444	±	9.686
7-Nitro-4-hydroxyquinoline (5)	77.407	±	7.21
7-Amino-4-hydroxyquinoline (7)	1574.815	±	115.91
7-Dibuthylamino-4-hydroxyquinoline (9)	57.407	±	5.185

* FRAP values (µM AA*) are expressed as micromolar ascorbic acid equivalents (n = 3)

Results of this study show that the FRAP value of the 4-hydroxyquinolines are from as high as 3950.370 ● 88.604μM Ascorbic acid equivalents [6-amino-4-hydroxyquinoline (**6**)] to as low as 29.259 ± 0.7407μM Ascorbic acid equivalents [6-nitro-4-hydroxyquinoline (**4**)]

5.4 Discussion

In the body, oxidative stress occurs as a result of imbalance between free radical production and the antioxidant defence system (Skibska *et al.*, 2006). Defined as molecules that may prevent chain reactions associated with oxidative stress some antioxidants act by exhibiting a chelating effect on metal ions involved in the Fenton reaction (Gaboriau *et al.*, 2002). Therefore, antioxidant capacity may be defined as the ability to reduce pro-oxidants (Prior and Cao, 1999). The FRAP assay, developed to determine the ferric reducing ability of pure compounds in solution (Pulido *et al.*, 2000), in a redox-linked colorimetric assay (Grimm, 2004), was employed to determine the ability of the synthesized 4-hydroxyquinolines to reduce free chelatable iron.

The FRAP assay is quick, reproducible and sensitive, and will facilitate experimental and clinical studies investigating the relationship among antioxidant status (Benzie and Strain, 1999), with results comparable to those obtained with more complex methodologies (Pulido *et al.*, 2000).

Regardless of the substitution pattern, amino-4-hydroxyquinolines (**6;7**) had the highest iron reducing power in comparison to the other compounds tested (Table 5.1 and fig 5.2). In addition, 6-amino-4-hydroxyquinolines (**6**) had significantly more reducing ability than 7-amino-4-hydroxyquinolines (**7**) ($p<0.001$). Also, with the dibuthylamino-group in the 6 position (**8**), the hydroxyquinolines had significantly more activity than the nitro compounds (**4;5**) ($p<0.001$).

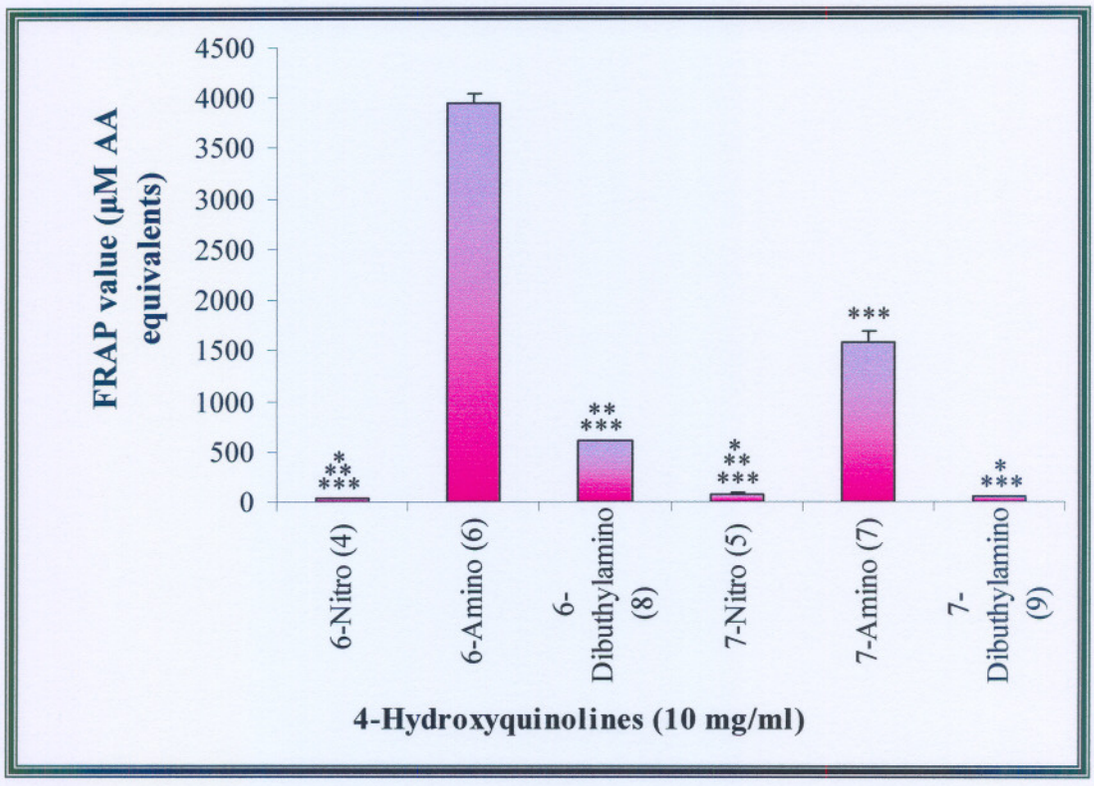


Figure 5.2 FRAP values (expressed as µM Ascorbic acid equivalents) of the tested 4-hydroxyquinolines. Data are presented as the mean of 3 values ± S.E.M., n = 3. ***p<0.001 vs. 6-amino-4-hydroxyquinoline **(6)**;**p<0.001 vs. 7-amino-4-hydroxyquinoline **(5)**; *p<0.001 vs. 6-dibuthylamino-4-hydroxyquinoline **(8).**

Although there were no significant difference between the 6- and 7-nitro-4-hydroxyquinolines **(4;5)**, the nitro group in the 7 position **(5)** seemed to have more reducing ability than in the 6 position **(4)**.

Overall, it was clear that the compounds containing a substituent in position 6 were more potent in terms of iron reducing ability, than those with a substituent in position 7. The results show a 2.5 fold difference between 6-amino-4-hydroxyquinoline **(6)** and its corresponding 7-amino compound **(7)**, whereas the difference between the dibuthylamino group in the 6 and 7 position **(8;9)** was 10 fold, in favour of the 6 position. In contrast, the nitro group in the 7 position **(5)** was 2.6 fold more potent than in the 6 position **(4)**.

The following order of reducing capacity was observed with the various substituents: Amino > Dibuthylamino > Nitro. Compounds with the substituent in the 6 position were significantly more potent than those with substitution in the 7 position. Amino-4-

hydroxyquinolines **(6;7)** exhibited statistically more antioxidant activity in the redox-linked colorimetric assay, with 6-amino-4-hydroyxquinoline **(6)** being significantly more potent than all the other compounds tested.

In addition, because these compounds reduce iron, it may also attenuate the initiation or propagation of lipid peroxidation induced by reactive oxygen species resulting from the iron dependent Fenton reaction associated with oxidative stress (Skibska *et al.*, 2006).

CHAPTER 6. SUPEROXIDE ANION SCAVENGING ACTIVITY

6.1 Introduction

Although aerobic life styles are advantageous in many ways, the utilization of oxygen results in the formation of highly reactive free radical products, which can cause reversible and irreversible damage to basically all the macromolecular structures within the cell (Ottino and Duncan, 1997).

The toxicity of the superoxide anion is demonstrate by its ability to inhibit certain enzymes, thereby attenuating vital metabolic pathways, its effects on major classes of biological molecules, such as DNA, the role it plays in lipid peroxidation, the ability to mediate inflammation and the formation of the highly toxic hydroxyl radical (McCord, 2000). The superoxide theory of oxygen toxicity states that formation of the superoxide radical *in vivo* plays a major role in the toxic effects of oxygen and has been given an enormous boost by the accumulation of evidence showing that superoxide dismutase enzymes, removing superoxide, are of great importance in allowing organisms to survive in the presence of oxygen and to tolerate increased oxygen concentrations (Halliwell and Gutteridge, 1984).

Under physiological conditions, the major source of superoxide radicals is the mitochondria, resulting from electron "leakage" from the electron transport chain, as well as by activation of certain enzymes, where the one electron reduction of oxygen yields the superoxide radical (McCord; 2000) (Section 2.1.2.1.*d*). Other sources of superoxide include cytochrome P450 in the endoplasmic reticulum, lipoxygenases, cyclooxygenases, xanthine oxygenases and NADPH oxidase (Curtin *et al.*, 2002). Although, the main mitochondrial defect observed in the neurodegeneration of Parkinson's disease concerns complex I of the respiratory chain (nicotinamide adenine dinucleotide coenzyme Q reductase), figure 6.1 diagrammatically illustrates the different sources of superoxide anions (Curtin *et al.*, 2002).

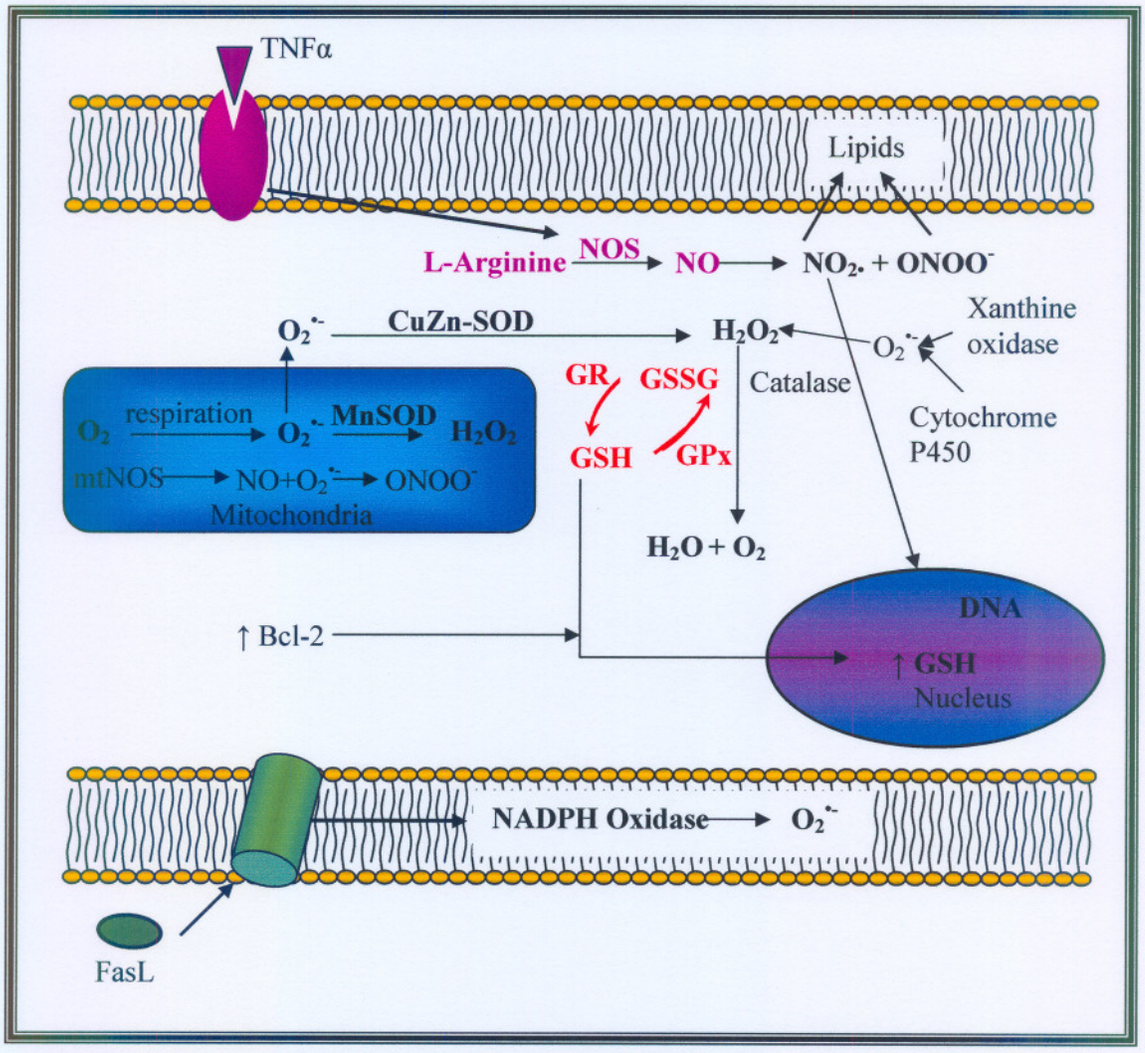


Figure 6.1 Diagrammatic representation of the intracellular sources of reactive oxygen species and principle defence mechanisms (Curtin et al., 2002).

At least two sites have been identified in the electron-transport chain (complex I and ubisemiquinone) where electrons may leak out to waiting oxygen molecules, resulting in generation of superoxide radicals (McCord, 2000). These sites are represented in figure 6.2 as the primary sources of intracellular superoxide generation (McCord, 2000).

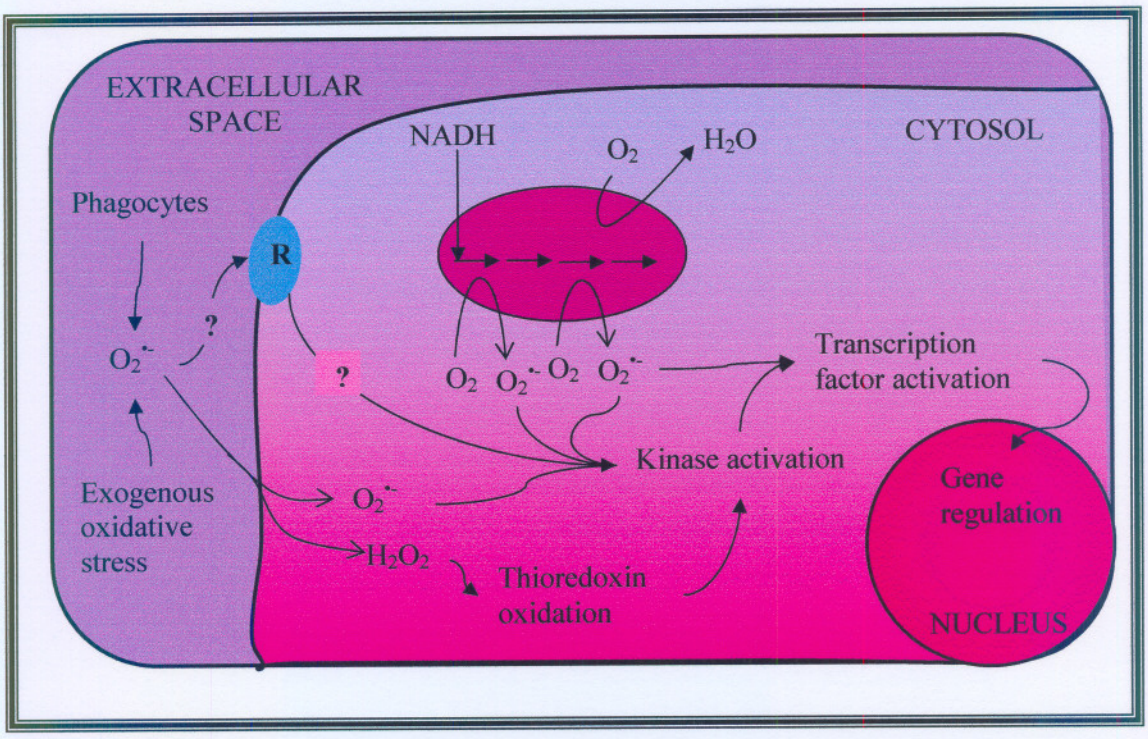


Figure 6.2 A schematic representation of signal transduction pathways for superoxide radicals (McCord, 2000).

Low levels of the superoxide radical may modulate various kinases, or may activate transcription factors directly to effect gene regulation in the nucleus. It is speculated on the existence of a cell surface receptor for superoxide (R), which might tranduce various responses within the cell by means of kinase activation, for example. However, there is presently no direct evidence for such a receptor (McCord, 2000).

In non-polar environments superoxide is a powerful base (proton acceptor), nucleophile and reducing agent (Halliwell and Gutteridge, 1984). However, superoxide can also act as an oxidizing agent, as seen in the presence of antioxidants. When superoxide becomes protonated, the substrate radical is oxidized by the resulting hydroperoxyl radical ($HO_2^{\cdot}$) or by molecular oxygen (Halliwell and Gutteridge, 1984). For example, the oxidation of α-tocopherol (TOH) can be written as follows (Halliwell and Gutteridge, 1984):

$O_2^{\cdot-} + TOH \rightarrow TO^{-} + HO_2^{\cdot}$

$O_2^{\cdot-} + HO_2^{\cdot} \rightarrow HO_2^{-} + O_2$

$O_2 + TO^{-} \rightarrow O_2^{\cdot-} + TO^{\cdot}$

$2TO^{\cdot} \rightarrow$ dimer $\rightarrow$ other products

In aqueous solution the basic properties, nucleophilicity and oxidizing capacity of superoxide are greatly reduced. Superoxide then reduces Fe^{3+} ions at the active site of cytochrome *c*, hence acting as a reducing agent (Halliwell and Gutteridge, 1984).

The interior of biological membranes are hydrophobic, and superoxide produced in this environment can be extremely damaging, for example destroying phospholipids due to nucleophilic attack upon the carbonyl groups of the ester bonds linking fatty acids to glycerol (Halliwell and Gutteridge, 1984).

The brain is the primary target for cyanide toxicity which may lead to a Parkinsonian condition (Utti *et al.*, 1985). This toxic attributes can be ascribed to the production of cellular anoxia in the brain, hence oxidative stress (Ardelt *et al.*, 1989; Maharaj *et al.*, 2003). This neurotoxin exerts its primary toxic effect by inhibiting the mitochondrial complex IV resulting in severe depletion of cellular ATP and cell death (Southgate and Daya, 1999). Thus cyanide augments the production of reactive oxygen species, and is therefore used during this study to induce superoxide generation *in vitro,* within whole rat brain homogenates (Section 2.3.4.5)

The MPTP model of Parkinson's disease is well characterized and has been extensively used in the screening of anti-parkinsonian drugs (Beal, 2001; Maharaj *et al.*, 2004). MPP^+, the toxic metabolite, is selective for nigrostriatal dopaminergic neurons and can be made available in the striatum or the substantia nigra to make animals used in a study, parkinsonian (Maharaj *et al.*, 2004). MPP^+ inhibits the activity of NADH-ubiquinone (complex I), altering the electron transport chain activity which contributes to both nigral cell loss and the generation of reactive oxygen species (Maharaj *et al.*, 2006). In particular, superoxide play a pivotal role in the neurotoxicity of MPTP, and because the MPTP model is regarded to be the best experimental model for Parkinson's disease (Przedborski and Vila, 2001), this model was used during this study (Section 2.3.4.3).

The nitro-blue tetrazolium (NBT) assay is generally accepted as a simple and reliable method for assaying the superoxide free radical (Ottino and Duncan, 1997; Maharaj *et al.*, 2003). In this method the superoxide anion gradually reduces yellow nitro-blue tretrazolium chloride (NBT^{2+}) to an insoluble diformazan, i.e. nitro-blue diformazan (purplish blue) during incubation at a pH of 7.4 (Das *et al.*, 1990; Sagar *et al.*, 1992; Wang *et al.*, 1998; Grimm *et al.*, 2004). The diformazan complex is subsequently

extracted with glacial acetic acid and measured spectrophotometrically at 560 nm (Parejo *et al.*, 2003). If radical scavenging compounds are present in the solution, less diformazan is formed, decreasing the absorption at 560 nm (Grimm *et al.*, 2004). Therefore, antioxidants are able to inhibit the formation of purple diformazan (Parejo *et al.*, 2003).

Studies, conducted by Wang and co-workers in 1998, have shown that although nitric oxide synthase may account for reducing NBT; the NBT assay is specific for superoxide anion rather than hydrogen peroxide (Wang *et al.*, 1998).

It is of great importance that novel agents, that have the ability to cross the blood brain barrier, are found in order to prevent the toxic effects of free radicals, curtailing the progression of neurodegeneration. The present study sought to determine whether the proposed quinolines are capable of preventing induced generation of superoxide anions, by investigating the effect of the neurotoxin, cyanide, on the production of superoxide anions in rat brain homogenate *in vitro* and to determine the ability of the quinolines to reduce cyanide-induced $O_2^{\bullet-}$ generation, *in vitro*. Secondly, a comparative study was conducted to determine whether the quinolines could cross the blood brain barrier in order to prevent or reduce MPP^+ -induced superoxide generation, *in vivo* in rat striatal regions.

6.2 Experimental

The well established nitro-blue tetrazolium (NBT) assay described by Ottino and Duncan (1997) is a modification of the assay described by Sagar *et al.* (1992) and Das *et al.* (1990) and was used in this set of experiments. The NBT assay was used to quantify the levels of superoxide anions induced by neurotoxins KCN and MPP^+ in rat brain homogenate *in vitro*, and in rat striatal regions *in vivo,* respectively. Hence the ability of the six proposed hydroxyquinolines to scavenge such induced radicals *in vitro* and *in vivo* was assayed, seeing whether these compounds were able to cross the blood brain barrier.

6.2.1 Materials and methods

6.2.1.1 Chemicals and reagents

Potassium cyanide (KCN), nitro-blue tetrazolium (NBT), nitro-blue diformazan (NBD) and 1-methyl-4-phenylpyridinium iodide (MPP^+) were purchased from Sigma Chemical Corporation, St. Louis, MO, USA. Glacial acetic acid was purchased from Saarchem (PTY) Ltd., Krugersdorp, South Africa. Chemicals used in the protein determination: Folin & Ciocalteu's reagent and diethyl ether were purchased from Saarchem (PTY) Ltd, Krugersdorp, South Africa. Bovine serum albumin (BSA) was supplied by Sigma Chemical Corporation, St. Louis, MO, USA, and all other chemicals and reagents were obtained locally and were of the highest available purity.

6.2.1.2 Animals

Three week old, adult male albino rats of the Wistar strain, weighing between 250 and 300 g were used throughout this study and were purchased from the South African Institute for Medical Research (Johannesburg, South Africa). The animals were chosen at random and assembled into groups of five. They were housed in separate opaque plastic cages with metal grid floors and covers, under a diurnal lighting cycle of 12 hours light and 12 hours dark, and were given access to standard laboratory food and water *ad libitum*. The animal room was windowless and well ventilated with automatic temperature and lighting controls. Lights were turned on at 6 a.m. daily. The intensity of the light illumination during the 12 hour phase was approximately 300 μWatts/cm^2, and the temperature of the animal room was maintained at 20 ± 2°C while an extractor fan ensured the constant removal of stale air. The cages were cleaned daily and all animals were given a one-week acclimatisation period before the onset of experimentation.

All the experimental protocols followed the guidelines for the care and use of laboratory animals and were approved by the Rhodes University Ethics Committee.

6.2.1.3 Reagents

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

determine whether cyanide induced superoxide anion in rat brain homogenate *in vitro*, hence determining the concentration of cyanide that causes the most significant superoxide generation.

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

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

6.2.1.4 Sample preparation

Stock solutions of the respective hydroxyquinoline were prepared by dissolving the quinoline in absolute ethanol, and subsequently diluting it with Milli-Q water so that the final concentration of ethanol in the brain homogenate was 0.5%. Stock solutions were prepared so that on addition of 250 µl of the compound, the reaction mixture would contain the correct incubation concentration (0.25; 0.5 and 1mM).

KCN (1mM) was dissolved in Milli-Q water, for the *in vitro* experiments, whereas MPP^+ (32nmol/1µL) was dissolved in 0.9% saline solution for the *in vivo* studies.

6.2.2 Preparation of standards

6.2.2.1 BSA standard

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

Bovine serum albumin (BSA), in increasing concentrations was used as a standard for determining the protein content of each brain. Protein standards containing 0-300 µg/ml of BSA at intervals of 60 µg/ml were used to generate a standard curve (Fig 6.3). These standards were assayed in the same manner as described by Lowry *et al.*, 1951 (Section 6.2.6).

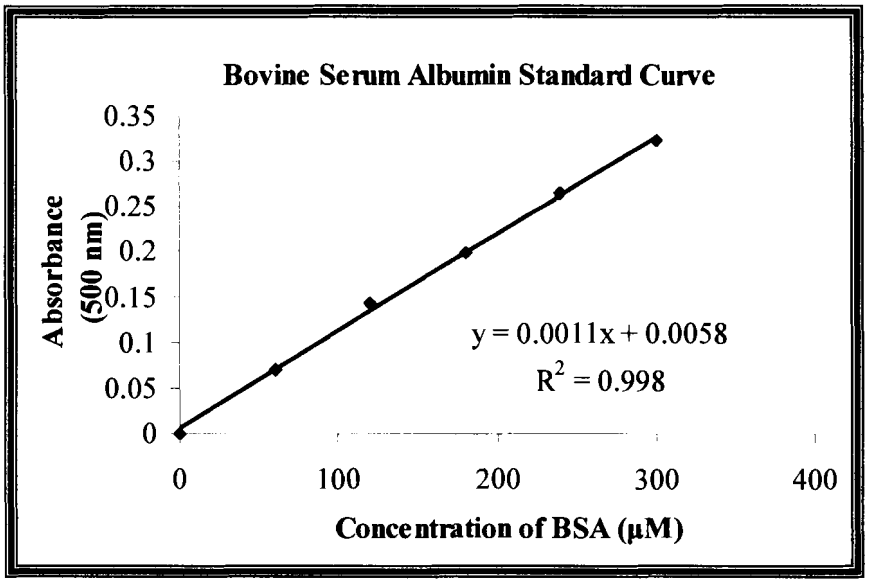


Figure 6.3 Protein Standard Curve generated from bovine serum albumin.

6.2.2.2 NBD standard

Nitro-blue diformazan was used as a standard measuring the level of induced superoxide anions with the NBT assay. A series of reaction tubes, each containing appropriate aliquots of NBD dissolved in glacial acetic acid was prepared to a volume of 1 ml. A calibration curve was generated by measuring the absorbance at 560 nm in 100 µmole/ml increments to the range of 0-400µM.

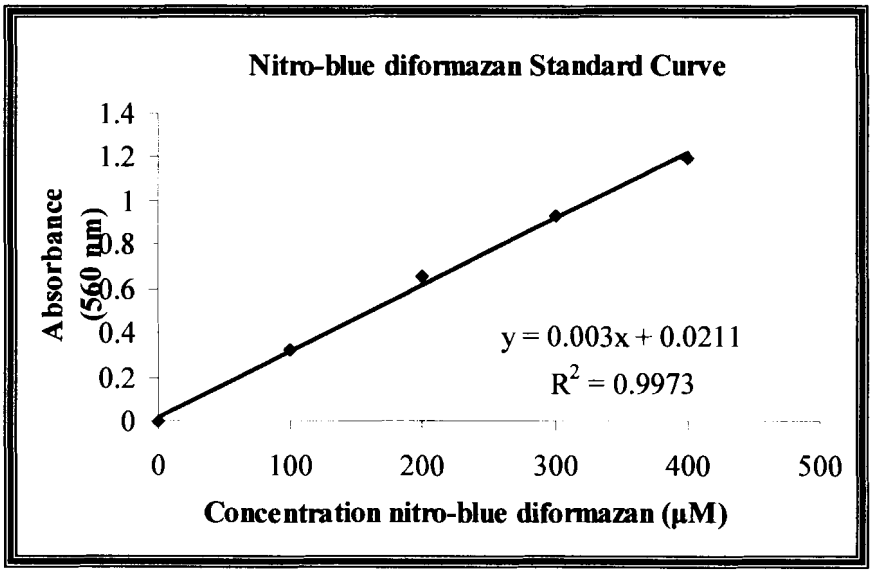


Figure 6.4 Nitro-blue diformazan Standard Curve.

6.2.3 Tissue preparation

6.2.3.1 Whole rat brain homogenate for *in vitro* studies:

Rats were sacrificed by cervical dislocation and the whole brain of each rat was rapidly excised by removing the top of the skull. An incision was made through the bone on either side of the parietal suture from the foramen magnum to near the orbit. Using forceps the calvarium was lifted and removed, exposing the brain which was easily removed. All adhering tissue and visible traces of blood was eliminated by washing the tissue in 0.9% saline solution. The whole brain was homogenized using a glass Teflon homogenizer in 0.1M phosphate buffered saline (PBS), pH 7.4 so as to give a final concentration of 10% (w/v). This is necessary to prevent lysosomal damage of the tissue. PBS buffer was used as it has been shown not to scavenge free radicals (Anoopkumar-Dukie *et al.*, 2001; Maharaj *et al.*, 2004).

6.2.3.2 Preparation of the striatum for *in vivo* study:

(a) Dosing of animals (Drug treatment)

The animals were randomly divided into eight groups (I-VIII), each group containing five animals. Groups I and II acted as control and toxin (MPP^+) groups, respectively. The treatment regime was chosen according to the procedure described by Maharaj (2004).

On the first day, 30 minutes prior to the intrastriatal injection of MPP^+ (32 nmoles/ 1 µl) (Maharaj *et al.*, 2004), the control and MPP^+ groups received ethanol:water solution (40:60), the vehicle for the test compounds, while the animals in groups III-VIII received a dose of 14 mg/kg of the respective hydroxyquinoline injected intraperitoneally. This dose was decided upon; since Shachar and co-workers (2004) have shown that treatment with quinoline derivatives, with this concentration, significantly protected the brain against induced oxidative damage (Shacar *et al.*, 2004). The animals in group I (control group) each received an intrastriatal injection of 0.9% saline. Four hours after intrastriatal injection, each rat was injected intraperitoneally with either ethanol:water solution (groups I and II) or the respective 4-hydroxyquinoline (groups III-VIII).

All the animals were treated for four days following surgery, with intraperitoneal injections of ethanol:water solution (40:60), to control groups I and II, while the animals in groups III-VIII each received a daily dose of 14 mg/kg of the respective 4-hydroxyquinoline, administered in the same way. The injections of the ethanol solution and of the respective hydroxyquinolines were administered at the same time each day. On the fourth day, when maximum toxicity has been reached, the animals were sacrificed by decapitation 1 hour following the intraperitoneal injection.

(b) Surgical Procedures

(i) Anaesthesia

Diethyl ether is a desirable anaesthetic to use because the mortality rate of the animals is lower than with halothane or phenobarbitone. Diethyl ether is also easy to administer and it is easy to monitor the depth of anaesthesia, and was therefore employed for all surgical procedures carried out during this study.

Animals were placed, one at a time, in a desiccator containing cotton wool soaked in diethyl ether. Once the animal reached unconsciousness, the animal was removed and placed on the operating surface as shown in figure 6.5. A small conical flask containing cotton wool soaked in ether was placed approximately 3 cm from the rats' nose, and remained in this position throughout surgery, except in cases where respiration became too weak. A good indication of the depth of anaesthesia was monitoring the colour of the limbs and tail, which displayed a faint, almost pale pinkness, indicative of the optimum level of anaesthesia, meaning a satisfactory rate and depth of respiration with good narcosis. A purple colour of the limbs was an indication of cyanosis.

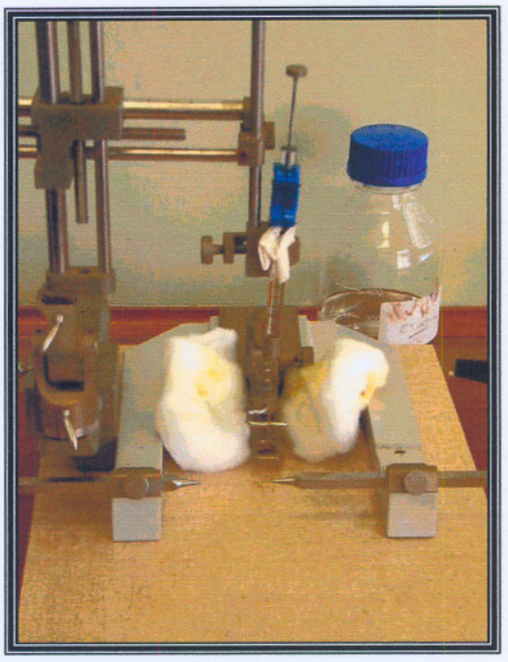


Figure 6.5 A view of the stereotaxic apparatus and Hamilton syringe used for the unilateral intrastriatal injection of MPP^+ (Stoelting, IL, USA).

(ii) Unilateral intrastriatal injections

The neurotoxin, MPP^+ was unilaterally infused into the substantia nigra compacta region employing rat brain stereotaxic apparatus (Fig 6.5) (Stoelting, IL, USA). The skull was orientated according to König and Klippel stereotaxic atlas (1963), and after a saggital cut in the skin of the skull, the lambda and bregma sutures were located and holes were drilled with a Bosch electrical drill fitted with a drill bit of 0.5 mm in diameter at the following coordinates: 0.30 mm anterior, 0.24 mm lateral and 0.78 mm ventral of the dura, from the lambda point (Fig 6.6) (Paxinos and Watson, 1998). Care was taken not to lesion the meninges. A Hamilton syringe, with a cannula of diameter 0.3 mm held rigidly in the stereotaxic micromanipulator, was used to inject 32 nmoles of MPP^+ in 1 µl saline into the striatum.

The injection was administered at a rate of 1 µl per minute and the cannula was left *in situ* for a further 3 minutes following the drug injection, to allow for passive diffusion away from the cannula tip and to minimize spread into the injection tract. The cannula was then slowly removed and the wound was disinfected with ethanol. Subsequently the scalp was closed with sutures. Animals recovered completely from the anaesthesia after approximately 30 minutes.

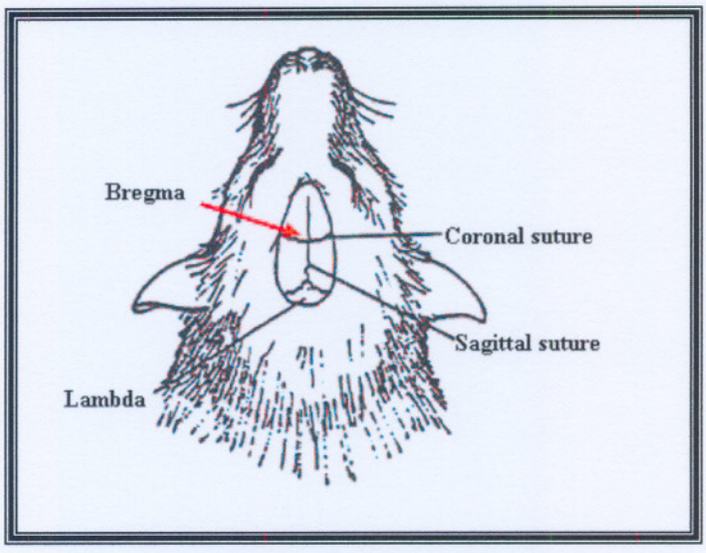


Figure 6.6 A view of the rat skull after the skin has been cut. The sutures shown are used as a reference point for the measurement of the coordinates for the intrastriatal injection (König and Klippel stereotaxic atlas, 1963).

(iii) Sham Lesioned Rats

The rats used as controls were subjected to the same surgical procedures described. However, stereotaxic injections into the striata were free of MPP^+ and comprised solely of saline.

(iv) Dissection of the striatum

On the fourth day following the intrastriatal injection of MPP^+, and treatment with the respective 4-hydroxyquinolines, the rats were swiftly sacrificed by cervical dislocation and decapitated. The whole brain was rapidly excised as described in section 6.2.3.1, and was blotted dry on ash-free filter paper. Only the striata were dissected according to a modified method of Glowinsky and Iversin, 1966, as shown in figure 6.7. Briefly, the rhombencephalon is separated by a transverse section from the rest of the brain. A transverse section is then made at the level of the optic chiasma, which delimits the anterior part of the hypothalamus and passes through the anterior commissure (section 2). This section separates the cerebrum into two parts, part B and C. Part B is divided into five fractions. The easiest way to reach the striatum is to first dissect the cortex, revealing the striatum (Fig 6.7). The striatum is then gently separated from the remaining part of the brain.

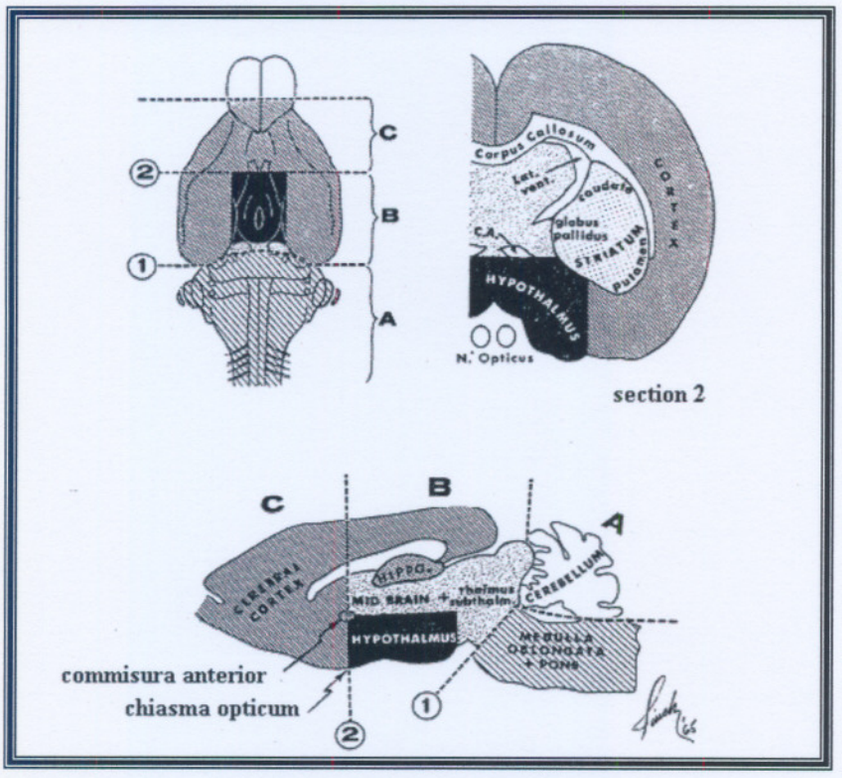


Figure 6.7 Diagrammatic representation of the dissection procedure for rat brain (Glowinsky and Iversen, 1966).

After removal of the striata, the striata were homogenized in PBS, pH 7.4, to yield a 10% (w/v) homogenate as described for *in vitro* studies. Striata were either used immediately or immediately frozen in liquid nitrogen and stored at -70°C.

6.2.4 Instrumentation

Absorbance values for the NBT assay were analysed at 560 nm, using a Shimadzu UV-160A-visible recording spectrophotometer.

6.2.5 NBT assay

6.2.5.1 KCN

In preliminary studies to determine the effective concentrations, 1 ml rat brain homogenate containing varying concentrations of KCN (0; 0.25; 0.5; 1mM) was incubated with 0.4 ml of a 0.1% NBT solution in an oscillating water bath for 1 hour at 37±2°C. Termination of the assay and extraction of reduced NBT was carried out by centrifugation of the suspensions at 2000 x *g* for 10 min. The supernatant was decanted and the pellet was resuspended with 2 ml glacial acetic acid, into which the reduced NBT dye was extracted. The absorbance of the glacial acetic acid fraction was measured at 560 nm and converted to µmoles diformazan using a standard curve generated from NBD (Fig 6.4).

6.2.5.2 *In vitro* exposure of rat brain to 4-hydroxyquinolines

To determine the superoxide scavenging ability of the proposed 4-hydroyxquinolines *in vitro*, homogenate (1 ml) containing 1mM KCN, inducing the superoxide anions, in the presence of varying concentrations of the proposed test compounds (0, 0.25, 0.5 and 1mM) was incubated with 0.4 ml 0.1% NBT solution for 1 hour at 37 ± 2°C in an oscillating water bath. The NBT assay was carried out as described for KCN. Final results are expressed as µmoles/mg protein.

All experiments were done with 5 replicates and repeated 3 times to ensure reproducibility.

6.2.5.3 *In vivo* exposure to 4-hydroxyquinolines

After intrastriatal infusion of MPP^+, the animals were treated post-operative with the proposed compounds for four days, killed and decapitated. A single dose of MPP^+ was assayed, merely to determine whether these proposed hydroxyquinolines posses the ability to cross the blood brain barrier and would indeed scavenge superoxide anions *in vivo*. The brains were removed followed by the dissection of the striata. The experiments were conducted as described for the *in vitro* determination using a 10% (w/v) homogenate of the striata (Das *et al.*, 1990), with n=5.

6.2.6 Protein assay

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

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

6.2.7 Data Collection

Absorbance values of the protein assay were converted to mg protein, using the calibration curve generated from increasing concentrations of bovine serum albumine (Fig. 6.3). These values were used in expressing the superoxide anion scavenging results.

Absorbance values of each NBT assay was converted to µmoles diformazan produced using the standard curve generated from increasing concentrations of NBD (Fig. 6.4). Final results are expressed as µmoles diformazan/mg protein.

6.2.8 Statistical analysis

Data from the *in vitro* studies are presented as the mean ± S.E.M. from 5 determinations in each run; the experiments were repeated 3 times. Data of the *in vivo* studies are presented as the mean ± S.E.M. of five animals per group. Statistical significance was evaluated using one way analysis of variance (ANOVA) followed by the Student-Newman Keuls Multiple Range test for comparison of different means. A p value of less than 0.05 was considered significant (Zar, 1974).

6.3 Results

The final results, obtained from the NBT assay *in vitro* and *in vivo*, expressed as µmol diformazan/mg protein are presented in tables 6.2 and 6.3 and figures 6.8, 6.9 and 6.10. *In vitro* results for the individual compounds are presented in Appendix C.

6.3.1 Results *–in vitro*

In favour of simplicity, all statistical differences regarding *in vitro* results are not shown graphically, but differences between the tested concentrations of the individual compounds are shown in Appendix C.

Table 6.1 The *in vitro* effect of the 4-hydroxyquinolines on KCN-induced superoxide generation in rat brain homogenates.

Test Compound	Concentration (mM)	Diformazan (µMole/mg protein)	±	S.E.M.
Control		14.905	±	0.119
KCN	0.25	28.116	±	0.641
	0.5	38.106	±	1.588
	1.	44.339	±	1.372
6-Nitro-4-Hydroxyquinoline **(4)** + 1mM KCN	0.25	14.095	●	2.158
	0.5	15.317	●	2.509
	1	30.314	±	0.648
6-Amino-4-Hydroxyquinoline **(6)** + 1mM KCN	0.25	17.472	±	1.750
	0.5	29.449	±	1.699
	1	34.611	±	0.946
6-Dibuthylamino-4-hydroxyquinoline **(8)** + 1mM KCN	0.25	17.537	±	0.768
	0.5	20.511	±	0.744
	1	25.184	±	1.907
7-Nitro-4-Hydroxyquinoline **(5)** + 1mM KCN	0.25	10.625	±	1.278
	0.5	7.670	±	1.348
	1	4.763	±	0.658
7-Amino-4-Hydroxyquinoline **(7)** + 1mM KCN	0.25	22.713	±	2.139
	0.5	29.697	±	1.557
	1	30.298	±	2.108
7-Dibuthylamino-4-hydroxyquinoline **(9)** + 1mM KCN	0.25	25.912	±	0.670
	0.5	23.609	±	0.537
	1	23.726	±	0.742

As evident from figure 6.8, *in vitro* exposure of whole rat brain homogenate to different concentrations of KCN caused a significant concentration-dependent increase in superoxide generation, furthermore, 1mM KCN induced the most significant generation of these reactive oxygen species, as it caused an increase of superoxide anions of almost 3 fold in comparison to the control. Maharaj (2003) showed that no further increase in superoxide generation can be observed for concentrations of KCN higher than that of 1mM, hence 1mM KCN was used in the ensuing experiments.

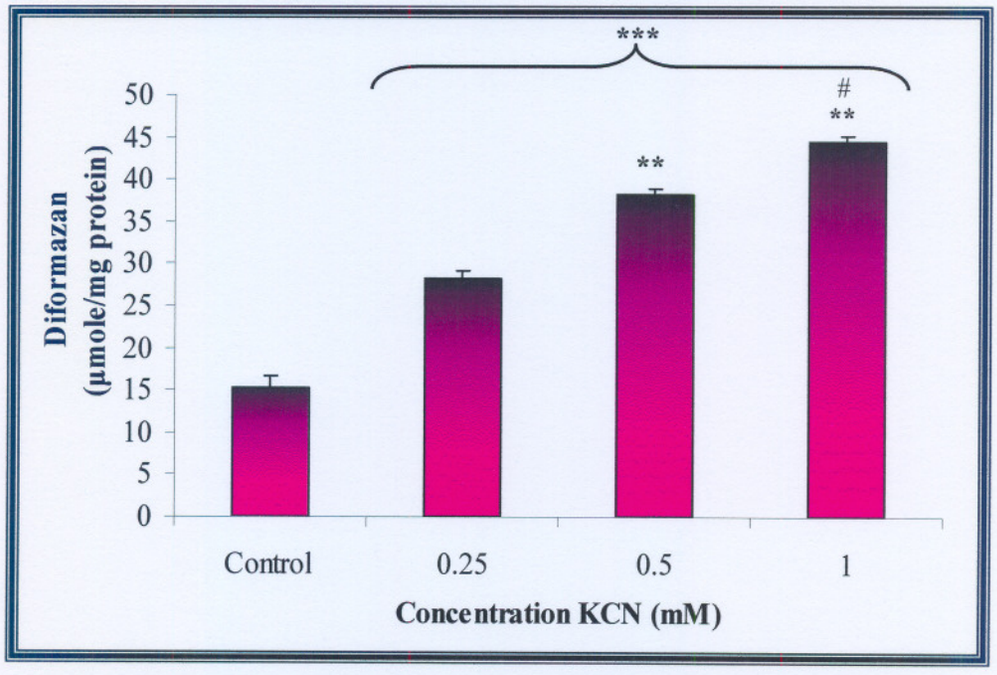


Figure 6.8 The effect of increasing concentrations KCN on the generation of superoxide anions in rat brain homogenate. Each bar represents the mean ± S.EM.; n=5; ***p<0.001 vs. control; **p<0.001 vs. 0.25mM KCN; #p<0.001 vs. 0.5mM KCN.

The *in vitro* exposure of whole rat brain homogenate to 1mM KCN caused a rapid increase in superoxide anion generation ($p<0.001$) and although no predictable trend could be observed, all the tested compounds significantly curbed this induced increase of superoxide anions ($p<0.001$) at all the concentrations (0.25-1mM) tested. 7-Nitro-4-hydroxyquinoline **(5)** seemed to be the best scavenger of superoxide anions, compared to the other compounds, as it was the only compound able to reduce the induced level of superoxide anions, in a concentration-dependent manner, to a level below that of the control, even at the lowest concentration tested (0.25mM) (Fig 6.9).

This was the only assay in which the nitro-compounds showed significantly more activity than the other compounds tested.

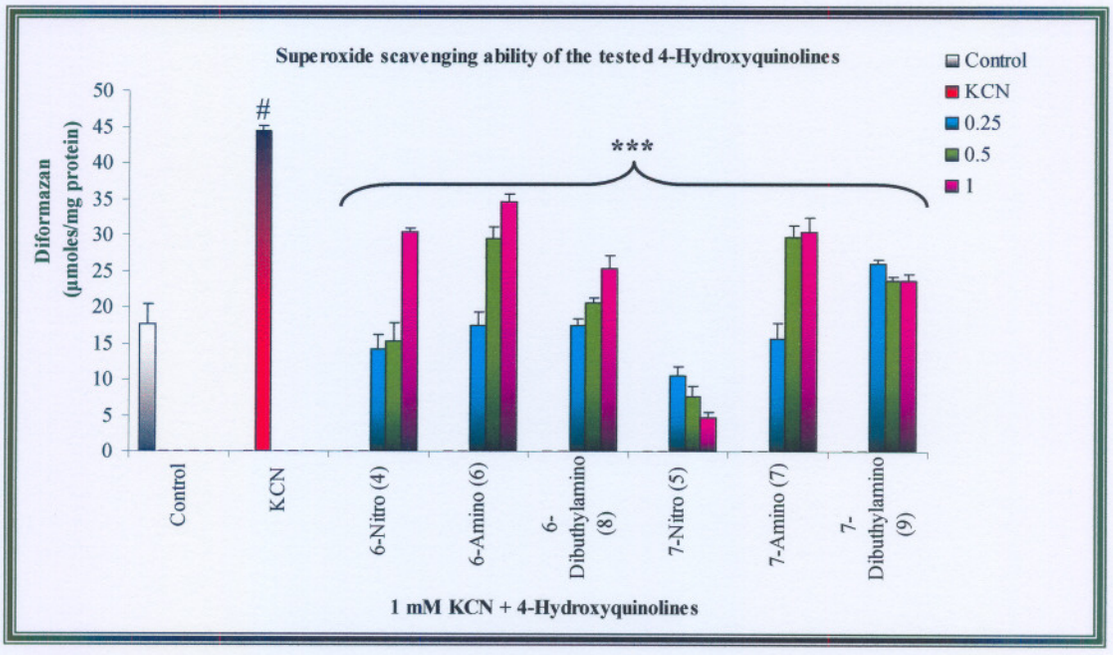


Figure 6.9 The superoxide scavenging properties of increasing concentrations of the 4-hydroxyquinolines in the presence of 1mM KCN in rat brain homogenate. Each bar represents the mean ± S.E.M. n=5. #$p<0.001$ vs. control; ***$p<0.001$ vs. 1mM KCN.

6.3.2 *In vivo* results

Table 6.2 The effect of the in vivo administration of the selected 4-hydroxyquinolines on intrastriatally injected MPP^+-induced superoxide generation in rat striatal homogenate.

Compounds Administered	Diformazan (µMoles/mg protein)	± S.E.M.
Control	44.409	± 2.170
MPP^+	89.581	± 7.480
6-Nitro-4-Hydroxyquinoline **(4)** + MPP^+	52.004	± 0.5334
6-Amino-4-Hydroxyquinoline **(6)** + MPP^+	53.4800	± 0.9912
6-Dibuthylamino-4-Hydroxyquinoline **(8)** + MPP^+	45.752	± 1.864
7-Nitro-4-Hydroxyquinoline **(5)** + MPP^+	54.027	± 2.795
7-Amino-4-Hydroxyquinoline **(7)** + MPP^+	51.765	± 1.186
7-Dibuthylamino-4-Hydroxyquinoline **(9)** + MPP^+	45.003	± 0.6670

As illustrated in figure 6.10, an intrastriatal injection of MPP^+ significantly enhances the levels of superoxide anions in rat striatal homogenate in comparison to the control group ($p<0.001$). Although there wasn't any significant difference between the *in vivo* activity of the tested compounds, all the compounds injected intraperitoneally significantly reduced the MPP^+-induced rise in superoxide generation ($p<0.001$ vs. MPP^+) to a level not statistically different from that of the control (Fig 6.10). Furthermore, the dibuthylamino-compounds **(8;9)** were the most effective of the quinoline treatment regimens used, in attenuating the effects of MPP^+. Because this study was conducted merely to evaluate whether these compounds are able to cross the blood brain barrier and reduce the generation of superoxide anions *in vivo*, a single dose of the test compounds were used.

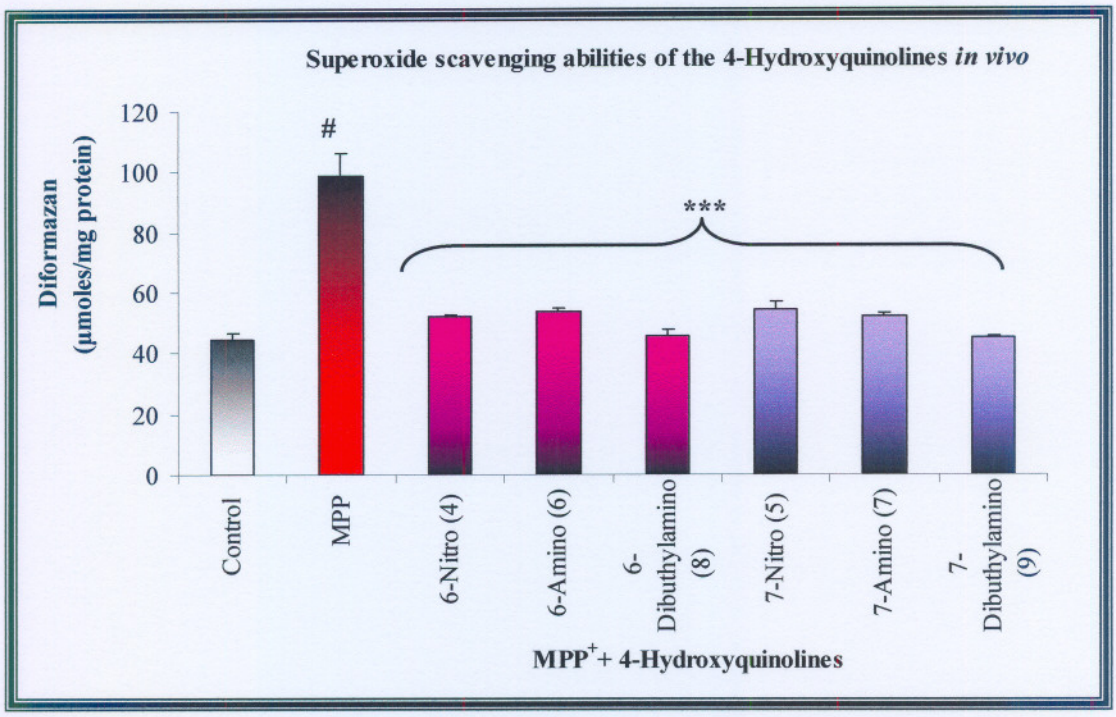


Figure 6.10 The superoxide scavenging effect of 4-hydroxyquinolines in the presence of MPP^+ *in vivo*. Each bar represents the mean ± S.E.M., n=5. #p<0.001 vs. control; ***p<0.001 vs. MPP^+.

6.4 Discussion

The brain is especially susceptible to damage induced by free radicals and during normal respiration reactive oxygen species are continuously produced. In the case of oxidative stress, defence mechanisms are overwhelmed and these species can damage essential biomolecules. Also, the generation of free radicals is considered to play a major role in the pathogenesis and progression of neurodegeneration (Maharaj *et al.*, 2006). Superoxide anions are known to exert destructive effects on cellular components with lipid peroxidation being one such consequence (Maharaj *et al.*, 2004).

Free radical scavengers are becoming increasingly popular as a means of reducing or preventing the hazardous effects of free radicals and their inducers. The antioxidant properties of the proposed 4-hydroxyquinolines in its ability to scavenge induced superoxide radicals was determined with the well established nitroblue-tetrazolium (NBT) assay *in vitro*, using whole rat brain homogenates, as well as its neuroprotective effects *in vivo* using rats treated intranigrally with MPP^+.

It is well known that superoxide radical production occurs primarily within complex I and III and cyanide inhibits complex IV, therefore inhibiting the defence mechanism of the mitochondria (Waypa and Schumacker, 2002). Results from this study aptly demonstrate that potassium cyanide is a potent neurotoxin which induces rapid generation of superoxide anions in rat brain homogenates, in a concentration dependent manner (Fig 6.8). 1mM KCN lead to the most significant superoxide anion generation.

In vitro, all the 4-hydroxyquinoline compounds tested, significantly scavenged the superoxide radicals generated by 1mM potassium cyanide, at all concentrations tested.

The concentration dependent effect of 7-nitro-4-hydroxyquinoline **(5)** on superoxide generation induced by KCN is evident in figure 6.9 and appendix C, figure 4. All the concentrations tested (0.25-1mM) reduced the KCN induced superoxide anion generation ($p<0.001$) to a level lower than that of the basal control (control vs. 0.5mM and 1mM $p<0.001$ and control vs. 0.25mM $p<0.05$). In addition, the 7-nitro compound, in the lowest concentration had significantly more activity than all the concentrations of the other 7-substituted hydroxyquinolines **(7;9)** ($p<0.001$) tested, and 0.5 and 1mM had significantly more activity than all the concentrations evaluated for all the other test compounds ($p<0.001$). In contrast, only the lower concentrations (0.25 and 0.5mM) of 6-nitro-4-hydroxyquinolines **(4)** reduced the level of induced superoxide anions to a level significantly lower than that of the control ($p<0.01$) (Fig 6.9 and appendix C, Fig 1). Although there were no significant differences in the activity of 0.25 and 0.5mM concentrations, both were significantly more active than 1mM for this compound ($p<0.01$) (appendix C Fig 1). Because both the nitro-compounds reduced the level of superoxide anions to a level below that of the control, it is suggested that nitro-4-hydroxyquinoline may offer complete protection against the effects of potassium cyanide.

As evident from figure 6.9 and appendix C, figures 2 and 5, 0.25mM of the 6 and 7-amino-4-hydroxyquinoline **(6;7)** reduced the induced level of superoxide anions to a level not significantly different to that of the control value, and in both cases 0.25mM of the amino-4-hydroxyquinoline had significantly more activity than 0.5 and 1mM [6-amino-4-hydroxyquinoline **(6)**: $p<0.001$ and 7-amino-4-hydroxyquinoline (7): $p<0.01$].

None of the tested concentrations for either 6 or 7-dibuthylamino-4-hydroxyquinoline **(8;9)** were able to reduce the induced level of superoxide anions below that of the control. The 0.25mM concentration of the 6-substituted compound **(8)** was significantly more active than 1mM ($p<0.05$), and superoxide scavenging ability appeared in a reversed dose-dependant manner (Fig. 6.9 and appendix C, Fig 3). In contrast, 0.25mM of the 7-dibuthylamino-compound **(9)** showed slightly less superoxide anion scavenging ability than in the higher concentrations. However, at all concentrations used, 7-dibuthylamino-4-hydroxyquinoline **(9)** showed significantly more activity than 1mM 6-amino-4-hydroxyquinolines **(6)** ($p<0.001$).

Overall, the nitro-4-hydroxyquinolines exhibited statistically the best superoxide radical scavenging ability compared to all the other compounds tested, *in vitro*. In addition, it is significantly more effective with the nitro group in the 7 position, as the 7 nitro compound in the concentration of 0.25mM had 1.3 fold more activity than its 6-nitro isomer, the 0.5mM twice as much, and 1mM showed a 6 fold increase in activity. It is worth noting that the NBT assay is the only assay evaluated during the course of this study in which the nitro-4-hydroxyquinolines had more activity than the rest of the compounds. Furthermore, it's also the only assay in which the hydroxyquinolines with the substituent in the 7 position are more active. Therefore, it may be concluded that the nitro group, especially in the 7 position may be essential for scavenging superoxide anions, implying a different antioxidant mechanism than those with the amino-substituents.

MPP^+ is a potent inhibitor of mitochondrial complex I, causing impairment of neuronal energy metabolism, leading to decreased levels of cellular ATP, and subsequent increase in cytoplasmic calcium and excitotoxicity, ultimately augmenting the formation of reactive oxygen species (Di Monte *et al.*, 1986; Turski *et al.*, 1991). Results from the *in vivo* study, illustrated in figure 6.10 indicate that intrastriatal injection of MPP^+ significantly increased the generation of superoxide anions *in vivo*, as compared to the control group. Furthermore, it is evident that postoperative treatment of the rats with the suggested 4-hydroxyquinolines combined with the MPP^+ significantly attenuated the level of MPP^+-induced superoxide anions in the striatum. In contrast to the *in vitro* results, the dibuthylamino-4-hydroxyquinolines, irrespective of the position of the substituent, have more superoxide anion scavenging activity. These results provide novel

data, firstly indicating the ability of these agents to cross the blood brain barrier, as it was injected intraperitoneally, and secondly highlighting its potent antioxidant properties in the ability to scavenge superoxide radicals generated by MPP^+ *in vivo*. In addition, the illustrated antioxidant properties of these agents are independent of MAO inhibitory activity since MPP^+ was administered directly into the rat striatum.

The test compounds have the potential to limit the deleterious effects of KCN- and/or MPP^+-induced superoxide generation, *in vitro* and *in vivo*, respectively. This study therefore, establishes the potential and novel neuroprotective activity of 4-hydroxyquinoline derivatives as strong antioxidants.

CHAPTER 7. LIPID PEROXIDATION

7.1 Introduction

The role of oxidative stress, including consequential lipid peroxidation, has been implicated in many conditions involving neurodegeneration, as well as the process of ageing (Glazer, 1988; Anoopkumar-Dukie and Daya, 2000; Young and Woodside, 2001; Giasson *et al.*, 2002; Bellè *et al.*, 2004).

Membranes of living cells, have a remarkable molecular architecture, and display a variety of functions (Seeley *et al.*, 2000). The modern concept of plasma membranes, described as the "fluid mosaic model", suggests that the plasma membrane is neither a rigid nor static boundary that segregates regions, but rather highly dynamic and flexible systems. Plasma membranes function as important barriers, enclosing all living cells, including neurons, to separate the cell interior from the extracellular environment, protecting the cells from possible harmful compounds in the surrounding medium (Seeley *et al.*, 2000). Membranes form permeable barriers for the selective transport of molecules into and out of the cell, compartmentalize internal structures of the cell which is essential for cellular function. Membranes are also responsible for the production of ATP and the binding of regulatory molecules such as hormones, growth factors and neurotransmitters mediating neurotransmission (Seeley *et al.*, 2000). Oxidative damage may cause changes in membrane fluidity, altering membrane functions and ultimately destroying the integrity of the cell membrane leading to increased permeability to ions such as Ca^{2+}, which in turn augments a cascade of events resulting in cell destruction.

Biological membranes are believed to be irregular lipid mixtures of phospholipids and cholesterol, with globular proteins embedded within the membrane, determining their function (Seeley *et al.*, 2000). A particularly important consequence of free radical damage is the oxidation of polyunsaturated fatty acids (Halliwell and Gutteridge, 1989; Ottino and Duncan, 1997; Akyol *et al.*, 2002; Granot and Kohen, 2004). Lipid peroxidation includes an oxidant abstracting a hydrogen atom from a polyunsaturated lipid, leaving the lipid in a radical form, which stabilizes by rearranging to a conjugated diene that in turn reacts with oxygen generating a peroxyl radical. This peroxyl radical

itself can abstract a hydrogen from a neighbouring lipid, resulting in a new lipid radical (Halliwell and Gutteridge, 1984; Granot and Kohen, 2004). This chain reaction is only terminated when the lipid peroxyl undergoes further oxidation, via its interaction with reduced metals (Fe^{2+}) (Granot and Kohen, 2004). Due to the self-perpetuating nature, lipid peroxidation can be extremely damaging (Granot and Kohen, 2004) (Section 2.3.3.3).

Characteristically, lipid peroxidation, give rise to a variety of products including short chain aldehydes, conjugated dienes and a variety of hydroxides and lipid peroxides, which in turn can amplify the oxidative cell injury, causing extensive damage to membrane structure and integrity (Glazer, 1988; Ottino and Duncan, 1997; Young and Woodside, 2001; Granot and Kohen, 2004) (Section 2.3.2.3a).

Lipid peroxidation is an inevitable accompaniment of cell death from any cause (Young and Woodside, 2001), and is implicated in several neurodegenerative disorders (Morel *et al.*, 1998), as the levels of lipid peroxidation products are increased in areas of severe neurodegeneration (Giasson *et al.*, 2002).

The brain is the most active tissue in the body (Bernheim *et* al., 1948) and is particularly susceptible to free radical damage, (Akyol *et* al., 2002; Arivazhagan *et al.*, 2002; Bellè *et al.*, 2004). In addition, the striatum and substantia nigra, are part of the brain regions most sensitive to the effects of free radical damage, as it lies deeper and is more subject to acidification, it is rich in dopaminergic neuronal endings and iron (Arivazhagan *et al.*, 2002; Giasson *et al.*, 2002; Barc *et al.*, 2004) (Section 2.3.3).

Cyanide induces neurotoxicity due to the production of cellular anoxia in the brain, resulting in oxidative damage and consequentially extensive lipid peroxidation of neuronal membranes (Maharaj *et al.*, 2003). Increased intracellular calcium after cyanide exposure also generates reactive oxygen species leading to lipid peroxidation and subsequent neuronal damage (Maharaj *et al.*, 2003) (Section 2.3.4).

According to the MPTP animal model of Parkinson's disease, MPP^+ increases the generation of reactive oxygen species, due to inhibition of mitochondrial respiration,

displacement of dopamine vesicular storage sites, *in vivo*, resulting in increased dopamine oxidation and enhanced iron (II) content within the brain (Giasson *et al.*, 2002). Oxidative damage, ultimately resulting in lipid peroxidation and thus neurodegeneration has been shown to play an important role in toxicity of MPP^+ (Shachar *et al.*, 2004).

In chapter 6 it was shown that cyanide and MPP^+ induce the production of superoxide anions, which itself play a major role in the process of lipid peroxidation (McCord, 2000). Both compounds are capable, via the Haber-Weiss reaction, to produce the toxic hydroxyl radical ($OH^{\bullet}$) in the presence of H_2O_2, increasing the risk of lipid peroxidation (Young and Woodside, 2001).

The measurement of putative elevated end products of lipid peroxidation in animal material is probably the evidence most frequently quoted in support of oxidative tissue damage (Anoopkumar-Dukie and Daya, 2000).

Malondialdehyde (MDA), a three-carbon compound, is formed due to peroxidation of fatty acids with three or more double bonds, and is a commonly used marker of lipid peroxidation (Draper and Hadley, 1990; Skibska *et al.*, 2006). The determination of malondialdehyde appears to offer a facile means of assessing lipid peroxidation in biological materials (Draper and Hadley, 1990). Although the validity of malondialdehyde as an index of lipid peroxidation has been clouded by controversy regarding its formation as an artefact of analysis and as a product of enzyme reactions, some investigators have found that malondialdehyde is a reliable indicator of peroxidation in many samples and that difficulties in its determination can be overcome by appropriate modifications in methodology (Draper and Hadley, 1990).

First introduced by Kohn and Liversedge (1944), the thiobarbituric acid (TBA) assay was a measurement for lipid rancidity in the food industry (Kohn and Liversedge, 1944). Currently, the thiobarbituric acid test is the most widely used assay for measuring lipid peroxidation, in terms of malondialdehyde (Draper and Hadley, 1990; Anoopkumar-Dukie and Daya, 2000; Granot and Kohen, 2004).

The classic procedure referred to as the thiobarbituric acid (TBA) test, involves heating a trichloroacetic acid extract of the sample with thiobarbituric acid (TBA) and measuring the absorbance of the crude mixture in a spectrophotometer at 532-535 nm (Draper and Hadley, 1990). However, certain aldehydes other than malondialdehyde, if present in the reaction mixture, can also react with TBA to produce the coloured chromogenes (Wilbur *et al.*, 1949), hence the term thiobarbituric acid –reactive substances (TBARS) assay, expressed as malondialdehyde equivalents, came into use (Draper and Hadley, 1990).

The foundation of the TBARS assay is based on the reaction of one molecule of malondialdehyde (MDA), the degraded lipid product (L'H) from cell membranes, with two molecules of thiobarbituric acid (TBA) at 95°C, yielding a pink coloured chomogen with an absorption maximum at 532 nm (Ottino and Duncan, 1997; Anoopkumar-Dukie and Daya, 2000; Akyol *et al.*, 2002). Figure 7.1 outlines the reaction that occurs during the TBA test (Gutteridge, 1987).

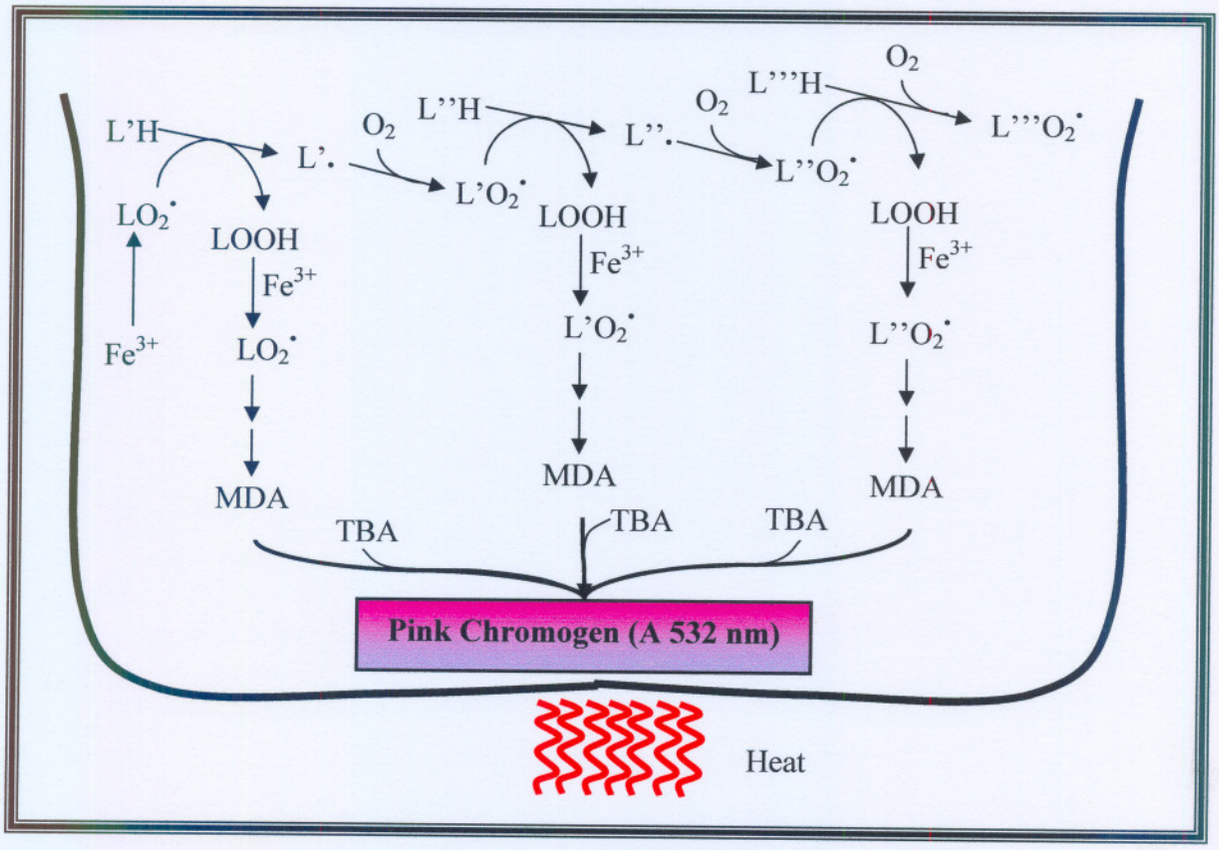


Figure 7.1 The formation of malondialdehyde by lipid peroxidation during the acid-heating stage (Gutteridge, 1987).

In biological materials, malondialdehyde occurs not only in the free state, but also in various covalently bound forms, as it forms adducts to proteins and nucleic acids (Draper and Hadley, 1990). Materials of animal origin usually contain large amounts of protein, to which the TBA-malondialdehyde complex may adsorb, imparting to it a pink colour and compromising the assessment of lipid peroxidation based on the determination of free malondialdehyde (Draper and Hadley, 1990; Anoopkumar-Dukie and Daya, 2000). Protein precipitates should thus be removed before carrying out the TBA reaction, and it is imperative to release the protein-bound malondialdehyde (Draper and Hadley, 1990; Anoopkumar-Dukie and Daya, 2000). Addition of trichloroacetic acid (TCA), facilitates the hot acid hydrolysis (pH 2-3), which presicipitate the protein from the protein bound complex. Subsequently this protein can be centrifuged off to yield a clear protein free solution (Bernheim *et al.*, 1948; Draper and Hadley, 1990; Anoopkumar-Dukie and Daya, 2000).

Oxidation of polyunsaturated fatty acids during the assay can interfere with the results, and hence should be eliminated by adding butylated hydroxyanisole or methanolic butylated hydroxytoluene (BHT) to the reaction mixture prior to tricholoroacetic acid (TCA) precipitation (Draper and Hadley, 1990).

In contrast to other methods, which use Tris-HCl buffer to stabilize membranes, PBS is used during this set of experiments, as unlike Tris-HCl buffer, PBS does not scavenge free radicals to consequently reduce lipid peroxidation values (Anoopkumar-Dukie and Daya, 2000).

One of the objectives of the present study was to investigate the relationship of the proposed 4-hydroxyquinolines as antioxidants, and thiobarbituric acid reactive substances, as products of lipid peroxidation. This study determined the possible manner in which varying concentrations of the proposed 4-hydroxyquinolines protect against oxidatively induced lipid peroxidation (TBARS) in rat brain homogenates after incubation with cyanide. Subsequently, a comparative study was conducted to determine whether the 4-hydroxyquinolines would cross the blood brain barrier to exert

neuroprotective effects *in vivo* in rats treated intranigrally with MPP^+. Intrastriatal infusion of MPP^+ avoided any influence of MAO activity (Maharaj *et al.*, 2004).

7.2 Experimental

The TBARS assay used in this set of experiments was described by Ottino and Duncan (1997), and is a modification of the method as proposed by Draper and Hadley (1990) (Draper and Hadley, 1990; Ottino and Duncan, 1997). The method measures the extent of lipid peroxidation in terms of malondialdehyde equivalents.

7.2.1 Materials and methods

7.2.1.1 Chemicals and reagents

Potassium cyanide (KCN), 2-thiobarbituric acid (98%) (TBA), 1,1,3,3-tetraethoxypropane (98%), buthylated hydroxytoluene (BHT) and MPP^+ were purchased from Sigma Chemical Corporation, St. Louis, MO, USA. Trichloroacetic acid (TCA) and buthanol was purchased from Saarchem (PTY) Ltd., Krugersdorp, South Africa. All other bench reagents were purchased from Merck, Darmstadt, Germany, and were of the highest chemical purity.

7.2.1.2 Animals

The study was performed on adult male Wistar rats weighing between 250 and 300 g. The rats were maintained in separate cages in a controlled environment as described in section 6.2.1.2. All experimental protocols were approved by the Rhodes University Ethics Committee.

7.2.1.3 Reagents

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

A stock solution was prepared so that on addition of 100 µl of the toxin, the stock solution would be diluted to the correct incubation concentration. Potassium cyanide was tested in concentrations of 0; 0.25; 0.5 and 1mM.

Butylated hydroxytoluene (BHT) (0.5 g/ℓ) was dissolved in methanol; trichloroacetic acid (TCA) (10%) and thiobarbituric acid (0.33%) were prepared in Milli-Q water. Because thiobarbituric acid is light sensitive, it was always prepared fresh and protected from light by covering the container with aluminium foil.

For *in vitro* studies, a stock solution of KCN was prepared with Milli-Q water, so that on addition of 100 µl of KCN, the stock solution would be diluted to the incubation concentration of 1mM. MPP^+ (32 nmoles/1 µl) was dissolved in 0.9% saline solution for the intrastriatal injections, in the *in vivo* studies.

7.2.1.4 Sample

Stock solutions of the proposed hydroxyquinolines were prepared by dissolving the respective compound in absolute ethanol, and subsequently diluting with Milli-Q water so that the final concentration of the ethanol in the brain homogenate was 0.5%. The following concentrations of the test compounds were assayed: 0, 0.25, 0.5, 1mM.

7.2.2 Preparation of standard

1,1,3,3-Tetraethoxypropane (TEP) was used as a standard. A series of reaction tubes each contained appropriate aliquots of water and standard solution, prepared with Milli-Q water to a final volume of 1 ml (Sagar *et al.*, 1992). The procedure described in section 7.5.5.1 was followed. A calibration curve was generated by measuring the absorbance at 10 nmoles/ml intervals, in the range of 0-50 nmoles/ml at a detection wavelength of 532 nm. The absorbance of the TBA/MDA-complex was plotted against the known concentration of malondialdehyde (Fig 7.2).

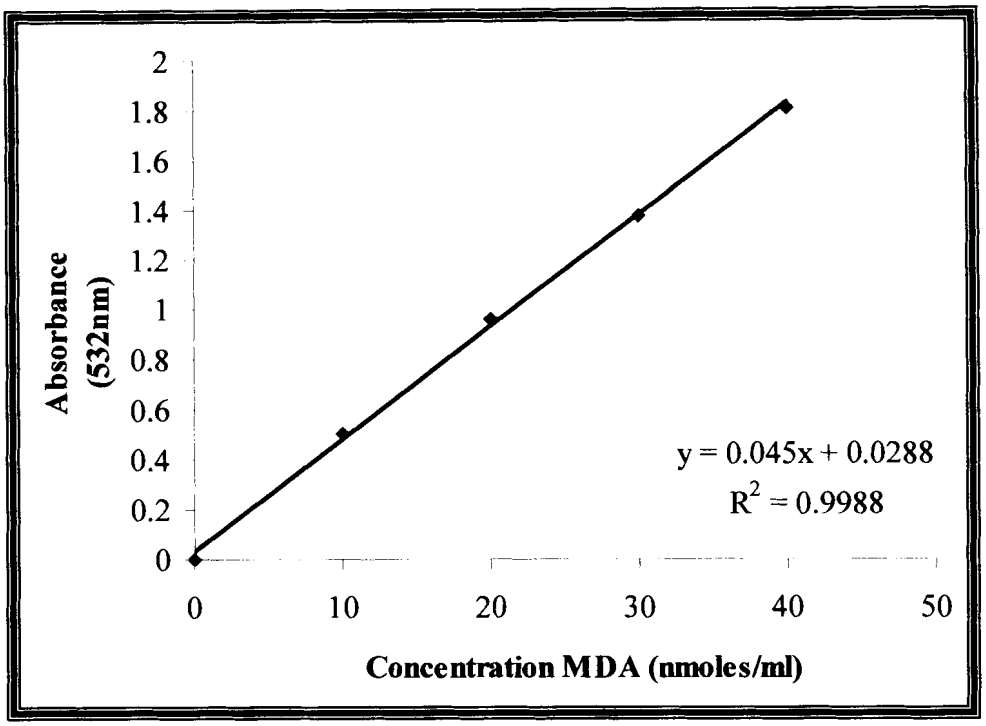


Figure 7.2 Malondialdehyde standard curve generated from 1,1,3,3-tetraethoxypropane.

7.2.3 Tissue preparation

7.2.3.1 Whole rat brain homogenate for *in vitro* studies:

Rat brain homogenate is a useful model for determining the efficacy of agents to reduce or potentiate lipid peroxidation, and is widely used as a rich source of membrane lipids to assess general lipid peroxidation. Each brain was homogenized according to the method described in section 6.2.3.

7.2.3.2 Preparation of the striatum for the *in vivo* assay:

(a) Dosing of animals

Adult male Wistar rats were randomly separated into eight groups of five animals each and were housed in separate cages as described in section 6.2.1.2. The animals were subjected to the same dosing regime described in section 6.2.3.2.*a.*

(b) Surgical procedures

Rats were anaesthetized with diethyl ether and immobilized in a stereotaxic apparatus as described in Chapter 6. The animals were each subjected to the unilateral intrastriatal injection of MPP^+. Sham lesioned rats were subjected to the same surgical procedures. On the fourth day following the intrastriatal infusion of MPP^+, when maximum toxicity has been reached, the rats were sacrificed by cervical dislocation, the brains were removed and the striata were dissected. All procedures followed were identical to those described in Chapter 6 section 6.2.3.2.*b*.

The brains were either used immediately or frozen in liquid nitrogen and stored at -70°C until use.

7.2.4 Instrumentation

The absorbance values for the TBA/MDA complex formed during the reaction were measured at 532 nm, using a Shimadzu UV-160A-visible recording spectrophotometer.

7.2.5 TBARS assay

7.2.5.1 *In vitro* exposure of rat brain to potassium cyanide

The effective concentration of KCN, inducing significant lipid peroxidation was determined by incubating 1 ml rat brain homogenate containing varying concentrations of KCN (0; 0.25; 0.5 and 1mM) in a oscillating water bath for 1 hour at 37 ● 2°C. Termination of the incubation periods was followed by the addition of 0.5 ml methanolic BHT (0.5mg/ml) and 1 ml of TCA (10%) to the mixture. The tubes were sealed and the mixture was heated at 95°C in a boiling water bath for 15 minutes, to release the protein-bound malondialdehyde. Following the 15 minute incubation, the samples were cooled on crushed ice until it reached room temperature and centrifuged at 2000 x g for 20 minutes at 4°C to remove insoluble proteins. The protein free supernatant (2 ml) was removed from each tube and a 0.5 ml aliquot of 0.33% TBA was added to this fraction. All the tubes were sealed and incubated at 95°C in a water bath for 1 hour. After cooling,

the TBA-MDA complexes were extracted with 2 ml of buthanol, of which the absorbance was read at 532 nm.

7.2.5.2 *In vitro* exposure of rat brain to 4-hydroxyquinolines

To determine the effect of the proposed 4-hydroxyquinolines *in vitro*, 1 ml whole brain homogenate containing 1mM KCN in combination with varying concentrations (0, 0.25, 0.5 and 1mM) of the respective 4-hydroxyquinoline was incubated in a oscillating water bath for 1 hour at 37 ± 2°C, in order to induce lipid peroxidation. Termination of the incubation periods was executed and followed by the TBARS assay as described for KCN (section 7.2.5.1)

7.2.5.3 In vivo exposure to 4-hydroxyquinolines

Because lipid peroxidation was induced *in vivo*, the 1 hour incubation at 37°C was excluded, and the homogenate of the striatum for each treated group was heated for 15 min with BHT and TCA in a boiling water bath to release protein bound malondialdehyde. The assay described in section 7.2.5.1 was followed.

7.2.6 Data Collection

The absorbance values obtained at 532 nm was converted to malondialdehyde levels from a standard curve generated from 1,1,3,3-tetraethoxypropane (Fig 7.2). The extent to which lipid peroxidation has occurred was expressed as nmole MDA equivalents/mg tissue.

7.2.7 Statistical analysis

All *in vitro* experiments were done in triplicate and were repeated 5 times. Each group in the *in vivo* study contained five animals. Graphpad instat was used for the statistical analysis of data. Final results are given as the mean ± S.E.M. of 3 replicates per run *in vitro*, and 5 animals per group *in vivo*. Data were analyzed by one way analysis of variance (ANOVA) followed by the Student-Newman Keuls test for multiple comparisons. Differences between groups were considered to be significant when $p<0.05$ (Zar, 1974).

7.3 Results

The influence of the 4-hydroxyquinolines on the pro-oxidant induced lipid peroxidation *in vitro* and *in vivo,* is depicted in tables 7.1 and 7.2 and figures 7.3; 7.4 and 7.5. *In vitro* results for individual compounds are presented in Appendix D.

7.3.1 *In vitro* results

The levels of lipid peroxidation indices in brain homogenate are shown in table 7.1 and figures 7.3 and 7.4. In favour of simplicity, all statistical differences regarding *in vitro* results are not shown graphically, but differences between the tested concentrations of the individual compounds are shown in Appendix D.

Table 7.1 The *in vitro* effect of the proposed 4-hydroxyquinolines on KCN induced lipid peroxidation in rat brain homogenate.

Test Compound	Concentration (mM)	Lipid peroxidation (nmole MDA/mg tissue)	±	S.E.M
Control		0.061	±	0.014
KCN	0.25	0.083	±	0.003
	0.5	0.134	±	0.007
	1	0.178	±	0.002
6-Nitro-4-Hydroxyquinoline **(4)** + 1mM KCN	0.25	0.080	±	0.004
	0.5	0.076	±	0.004
	1	0.070	±	0.007
6-Amino-4-Hydroxyquinoline **(6)** + 1mM KCN	0.25	0.060	±	0.008
	0.5	0.037	±	0.007
	1	0.029	±	0.006
6-Dibuthylamino-4-Hydroxyquinoline **(8)** + 1mM KCN	0.25	0.040	±	0.007
	0.5	0.017	±	0.003
	1	0.013	±	0.002
7-Nitro-4-Hydroxyquinoline **(5)** + 1mM KCN	0.25	0.075	±	0.009
	0.5	0.059	±	0.011
	1	0.076	±	0.013
7-Amino-4-Hydroxyquinoline **(7)** + 1mM KCN	0.25	0.077	±	0.017
	0.5	0.030	±	0.009
	1	0.019	±	0.006
7-Dibuthylamino-4-Hydroxyquinoline **(9)** + 1mM KCN	0.25	0.137	±	0.010
	0.5	0.121	±	0.011
	1	0.086	±	0.010

As evident from figure 7.3, *in vitro* exposure of whole rat brain homogenate to increasing concentrations of KCN caused a significant concentration-dependent increase in lipid peroxidation products in comparison to the control. Furthermore, 1mM KCN showed to have induced the most rigorous lipid peroxidation. Because 1mM KCN induced significantly more lipid peroxidation than 0.25 ($p<0.001$) and 0.5mM ($p<0.001$), this concentration was used in the subsequent experiments.

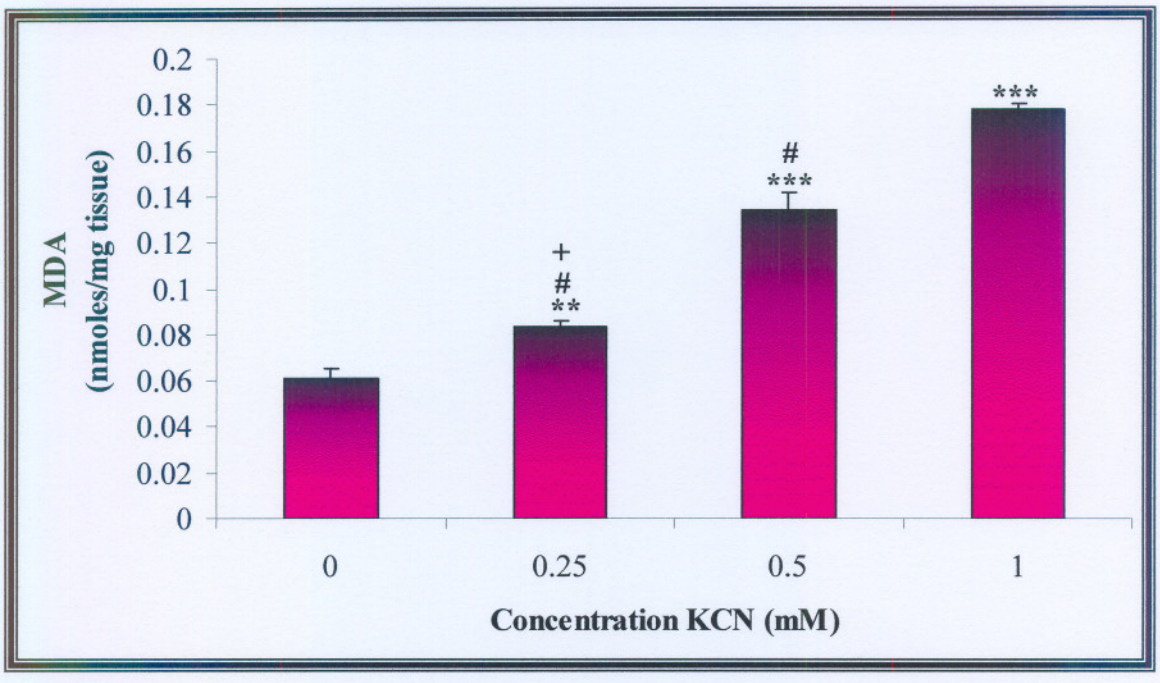


Figure 7.3 The concentration-dependent increase of malondialdehyde induced by KCN. Each bar represents the mean ± S.E.M.; n=3;***$p<0.001$ vs. control; **$p<0.01$ vs. control, #$p<0.001$ vs. 1mM; +$p<0.001$ vs. 0.5mM.

In comparison to the control, KCN induced a significant increase in the extent of lipid peroxidation in rat brain homogenate ($p<0.001$). This increase in the concentration of malondialdehyde equivalents in the rat brain homogenate, was significantly attenuated in the presence of the 4-hydroxyquinolines, as all the tested compounds significantly reduced the level of malondialdehyde equivalents induced by KCN at all concentrations tested ($p<0.001$). Except for the 7-nitro-compound **(5)**, this inhibition of lipid peroxidation *in vitro* appeared to follow a concentration-dependent manner.

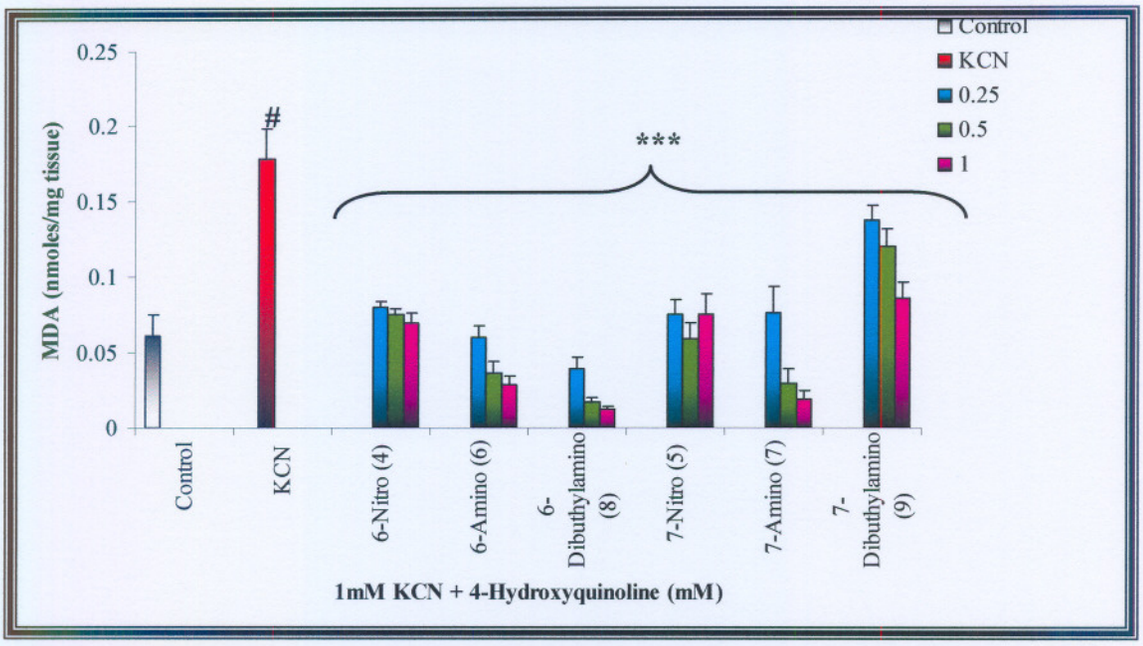


Figure 7.4 The *in vitro* effect of the proposed 4-hydroxyquinolines on lipid peroxidation induced by 1mM KCN in rat brain homogenate. Each bar represents the mean ± S.E.M.; n=3; #p<0.001 vs. control, ***p<0.001 vs. KCN.

7.3.2 *In vivo* results

Inhibition of MPP^+-induced lipid peroxidation by 4-hydroxyquinolines, *in vivo*, is presented in table 7.2 and figure 7.4.

Table 7.2 The effect of the *in vivo* administration of the selected 4-hydroxyquinolines on intrastriatal induction of lipid peroxidation with MPP^+.

Compounds Administered	MDA (nmoles/mg tissue)	±	S.E.M.
Control	0.0037	±	0.0010
MPP^+	0.0124	±	0.0003
6-Nitro-4-Hydroxyquinoline **(4)** + MPP^+	0.0092	±	0.0002
6-Amino-4-Hydroxyquinoline **(6)** + MPP^+	0.0020	±	0.0006
6-Dibuthylamino-4-Hydroxyquinoline **(8)** + MPP^+	0.0052	±	0.0012
7-Nitro-4-Hydroxyquinoline **(5)** + MPP^+	0.0192	±	0.0036
7-Amino-4-Hydroxyquinoline **(7)** + MPP^+	0.0116	±	0.0016
7-Dibuthylamino-4-Hydroxyquinoline **(9)** + MPP^+	0.0076	±	0.0010

An intrastriatal injection of MPP^+, significantly induced a rise in lipid peroxidation *in vivo* ($p<0.01$). However, only 6-amino- **(6)** ($p<0.001$ vs. MPP^+) and 6-dibuthylamino-4-hydroxyquinolines **(8)** ($p<0.05$ vs. MPP^+) were able to significantly curb this rise in lipid peroxidation, and reduced production of malondialdehyde to the level of the basal control.

Overall, the amino- **(6;7)** and dibuthylamino-compounds **(8;9)** showed more activity than the nitro compounds **(4;5)**, and again, with the substituent in the 6-position the hydroxyquinolines were significantly more effective (6-amino-**(6)** vs. 7-amino-4-hydroxyquinoline **(7)** $p<0.01$; 6-nitro-**(4)** vs. 7-nitro-4-hydroxyquinoline **(5)** $p<0.001$).

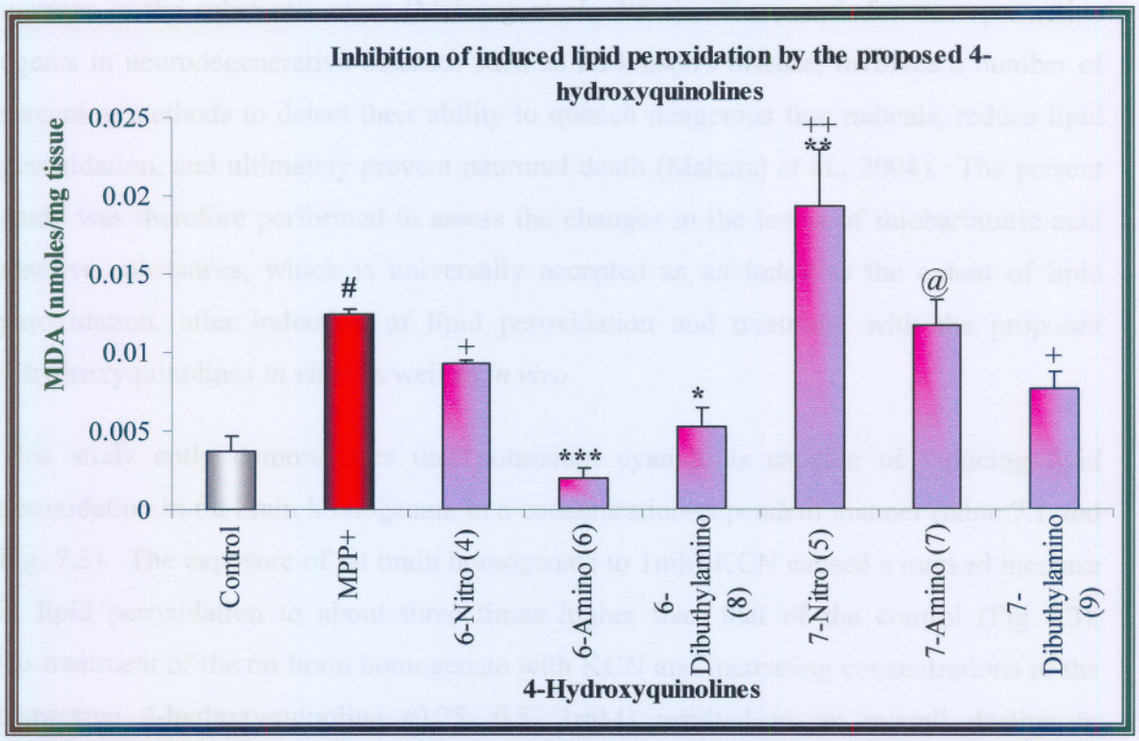


Figure 7.5 The *in vivo* effect of 4-hydroxyquinolines on lipid peroxidation intrastriatally induced with MPP+. Each bar represents the mean ± S.E.M.; n=5. #$p<0.01$ vs. control; ***$p<0.001$ vs. MPP+; *$p<0.05$ vs. MPP+; **$p<0.001$ vs. 6-amino **(6)**; @$p<0.01$ vs. 6-amino **(6)**; +$p<0.05$ vs. 6-amino **(6)**; ++$p<0.001$ vs. 6-dibuthylamino-4-hydroxyquinoline **(8)**.

7.4 Discussion

Ageing may be defined as a gradual, progressive change in an organism that increases the probability of death, and these alterations compromising an organism's ability to meet both internal and external challenges, is worsened by the phenomenon of oxidative stress (Arivazhagan *et al.,* 2002). In recent years a great body of evidence showed that it is oxygen free radicals which ultimately bring about neuronal death of the dopaminergic neurons in the substantia nigra (Maharaj *et al.*, 2004). The search for neuroprotective agents in neurodegenerative diseases such as Parkinson's disease, involves a number of screening methods to detect their ability to quench dangerous free radicals, reduce lipid peroxidation, and ultimately prevent neuronal death (Maharaj *et al.*, 2004). The present study was therefore performed to assess the changes in the levels of thiobarbituric acid reactive substances, which is universally accepted as an index to the extent of lipid peroxidation, after induction of lipid peroxidation and treatment with the proposed 4-hydroxyquinolines *in vitro* as well as *in vivo*.

This study aptly demonstrates that potassium cyanide is capable of inducing lipid peroxidation in rat brain homogenate in a concentration-dependent manner (table 7.1 and Fig. 7.3). The exposure of rat brain homogenate to 1mM KCN caused a marked increase in lipid peroxidation to about three times higher than that of the control (Fig 7.3). Co-treatment of the rat brain homogenate with KCN and increasing concentrations of the respective 4-hydroxyquinoline (0.25, 0.5, 1mM) resulted in an overall decline in malondialdehyde production, as all the compounds significantly curbed the rise in malondialdehyde, induced *in vitro,* at all concentrations tested. Except for the 7-nitro-4-hydroxyquinoline **(5)**, the 1mM concentration of the tested 4-hydroxyquinolines was the most effective concentration in providing protection against the KCN-induced lipid peroxidation, and therefore, these compounds act in a concentration-dependent manner. However, most of the 4-hydroxyquinolines **(4;5;7;9)** had a negligible effect on the basal production of thiobarbituric acid reactive substances (Fig 7.3).

At all the concentrations tested, the 6-amino-4-hydroxyquinoline **(6)** reduced the extent of lipid peroxidation to a level lower than that of the control (Appendix D), and 1mM of

this compound had significantly more scavenging potential than all the concentrations tested for 7-dibuthylamino-4-hydroxyquinoline **(9)** and 6-nitro-4-hydroxyquinoline **(4)**. 0.5mM of the 7-amino-4-hydroxyquinoline **(7)** showed significantly more inhibition of lipid peroxidation, than did all the tested concentrations of 7-dibuthylamino **(9)** and both the nitro-compounds **(4;5)** ($p<0.001$). Because the concentration of MDA obtained, from the 6-amino-4-hydroxyquinoline **(6)** treated homogenate, is below basal control values for all concentrations tested, it indicates that this compound may be effective in preventing induced lipid peroxidation in the rat brain homogenate *in vitro.*

6-Dibuthylamino-4-hydroxyquinoline **(8)** attenuated the level of malondialdehyde to the same level of that of the basal control. This may suggest complete protection from cyanide induced lipid peroxidation. 0.5mM 6-Dibuthylamino-4-hydroxyquinoline **(8)** was significantly more protective than all the concentrations tested for its equivalent 7-dibuthylamino-compound **(9)** and both the nitro-compounds **(8;9)** ($p<0.001$ vs. 0.25; 0.5 and 1mM).

The results presented in figure 7.4 clearly illustrate that intrastriatal infusion of the potent parkinsonian, neurotoxin MPP^+ induced a significant rise of lipid peroxidation in rat striatum when compared to the control group ($p<0.01$). Figure 7.4 also demonstrates the effectiveness of amino-quinolines at attenuating the lipid peroxidation when compared to the toxin group. Administration of 6-amino **(6)** and 6-dibuthylamino-4-hydroxyquinolines **(8)** respectively, after intrastriatal infusion of MPP^+, blocked the lipid peroxidation effect of the toxin in rat striata as there was no significant difference in the malondialdehyde concentration obtained for these two treatment groups and the control group (Fig 7.4). The rise in the concentration of malondialdehyde, induced by MPP^+, was significantly reduced by 83% for 1mM of 6-amino-4-hydroxyquinoline **(6)** and by 58% for 6-dibuthylamino-compound **(8)**. These results are in agreement with results obtained in the *in vitro* study, confirming the effect of 6-amino- and 6-dibuthylamino-4-hydroxyquinolines **(6;8)**.

In addition, results observed for the 7-substituted compounds show that the activity against lipid peroxidation may increase according to the chain length of the alkylamine function, as dibuthylamino-4-hydroxyquinoline **(9)** showed more inhibition than did the amino-compound. However, the same trend wasn't observed for the 4-hydroxyquinolines substituted in the 6 position (Fig 7.4).

The neuroprotective effects of these agents against MPP^+-induced lipid peroxidation are independent of MAO inhibitory activity since MPP^+ was administered directly into the rat striatum.

The present study clearly indicates that 4-hydroxyquinolines significantly reduce KCN induced lipid peroxidation *in vitro.* The amino-4-hydroxyquinolines **(6)** and 6-dibuthylamino-4-hydroxyquinoles **(8)** possessed the most potent antioxidant activities as it inhibited KCN- and MPP^+-induced lipid peroxidation in brain homogenate, *in vitro* and rat striatum *in vivo.* In addition, because these compounds were administered intraperitoneally, and inhibited intrastriatal lipid peroxidation as induced by MPP^+, it can be assumed that these compounds crossed the blood brain barrier, a characteristic essential for neuroprotection. The neuroprotection offered against MPP^+ *in vivo*, by these agents have not been previously reported and is therefore novel.

The important observations made from this study are the antioxidant activity of the proposed amino- and dibuthylamino-4-hydroxyquinolines in attenuating the lipid peroxidation *in vitro* as well as *in vivo.* Since radicals are widely implicated in neurodegeneration of Parkinson's disease, these compounds may potentially have an important role to play at inhibiting the progression of the pathogenesis of Parkinson's disease.

CHAPTER 8. CONCLUSION

Although oxygen is vital for human survival, within the biological system it is the main source of deleterious free radicals and reactive oxygen species, which can cause severe damage to all cellular components (Akyol *et al.*, 2002; Girotti *et al.*, 2002). Eukaryotes have evolved a specific antioxidant defence system in order to survive the obligatory oxidative burden, an un-avoidable by-product of aerobic respiration (Kidd, 2000). However, if an imbalance between the antioxidant defence system and the production of reactive oxygen species occurs, an extremely toxic condition referred to as oxidative stress arises (Jones *et al.*, 2002).

Due to a number of reasons including an exceptionally high utilization of oxygen, high lipid content and remarkably low concentration of antioxidants, the brain is particularly susceptible to damage that occurs during oxidative stress (Driver *et al.*, 2000). In addition, a number of neurological diseases are associated with locally enhanced oxidative stress, affecting functionality of neuronal cells (Schwemmer *et al.*, 2000).

Parkinson's disease has an immense effect on the ageing society. Parkinson's disease, a highly debilitating disorder, is characterized by tremor, rigidity and immobility. The biochemical base of this disorder is a significant deficiency in dopamine in the nigro-striatal pathway, due to destruction of dopaminergic neurons (Maharaj *et al.*, 2004). A persistent increase in free radical generation is believed to be the cause of neuronal death, which reaches more than 80% by middle age (Maharaj *et al.*, 2004). Parkinson's disease is currently managed by replenishing dopamine in the brain or mimicking its actions at dopamine receptors (Kidd, 2000). Although this management improves the clinical symptoms, the benefits rarely persist, as the palliative effects wears of, leading to "on-off" effects which can be as debilitating as Parkinson's disease itself (Przedborski and Vila, 2001). This treatment also does not address the underlying degeneration of dopaminergic neurons nor does it address the closely associated oxidative stress. Logically any agent that could provide antioxidative fortification and hence restoration of striatal dopamine levels, could be neuroprotective, and therefore be useful as a therapeutic tool, curtailing the progression of this disease (Maharaj *et al.*, 2004).

Efforts to counteract the damage caused by reactive oxygen species are gaining acceptance as a basis for novel therapeutic approaches and the field of preventive medicine is experiencing an upsurge of interest in medically useful antioxidants (De Pinedo *et al.,* 2006). The search for neuroprotective antioxidants in neurodegenerative diseases like Parkinson's disease involves a number of screening methods to detect their ability to quench dangerous free radicals, reduce lipid peroxidation and ultimately prevent neuronal death (Maharaj *et al.*, 2004).

Several researchers have shown that compounds containing a quinoline moiety, such as clioquinol **(1)**, 8-hydroxyquinolines **(2)** and fluoro-4-hydroxyquinolines **(3)**, have the potential to protect biological systems against induced oxidative damage, due to their antioxidant activities (Chapter 3).

Therefore, the aim of this study was to investigate the possible neuroprotective ability of a series of 4-hydroxyquinolines, with a nitro-, amino- or dibuthylamino group in the 6 or 7 position respectively. The current investigation was focused on the described models of neurodegeneration and how it could be prevented by hydroxyquinolines. If the proposed 4-hydroxyquinolines are able to scavenge free radicals it might have significant effects in reducing the progression of Parkinson's disease. A series of 4-hydroxyquinolines was synthesized and assayed *in vitro* and *in vivo* for various antioxidative properties.

Chapter 3 describes the selected synthesis route, based on the Gould-Jacobs reaction, which was suited for the dialkylamino-4-hydroxyquinolines as could be seen from the collected physical data, which compared to that reported by Van Dyk, (2001) (section 3.6). The total synthesis route executed is depicted in Scheme 3.9.

Using the ORAC assay (Chapter 4), all the tested hydroxyquinolines delayed the induced fluorescence decay of fluorescein. Therefore, all the tested hydroxyquinolines to some extent have the ability to scavenge peroxyl radicals. Although there were no significant differences between the compounds tested, except for 6-amino-4-hydroyxquinoline, which exerted the best scavenging ability towards the induced peroxyl radicals, hydroxyquinolines with the substituent in the 6 position appeared to have more absorbing

capacity than when in the 7 position. In addition, irrespective of the position of the functional group, amino-compounds had more activity than the corresponding dibuthylamino-compounds which in turn had more activity than the nitro-compounds. 6-Amino-4-hydroxyquinoline **(6)**, was the best scavenger of peroxyl radicals (table 4.1 and Fig 4.4). The damaging effect of the peroxyl radical lies in its ability to induce and sustain the self-perpetuating reaction of lipid peroxidation (Cadenas, 1995). Therefore, scavenging of these radicals will lead to a lower incidence of lipid peroxidation, thus sparing cellular constituents and slowing the progression of oxidative damage related disorders (Bellè *et al.*, 2004).

In the FRAP assay (Chapter 5), 4-hydroxyquinolines with the substituent in the 6 position, rather than the 7 position, had significantly more reducing ability. The amino-4-hydroxyquinolines, irrespective of the position of the amino-group, was the best reducers of iron (table 5.1 and Fig 5.2). In addition, dibuthylamino compounds had more reducing power than the nitro-compounds. The order of activity observed in terms of the substituents thus was amino-> dibuthylamino->nitro-4-hydroxyquinolines. This, in terms of antioxidant properties, is in concordance with results obtained from the ORAC assay. Because these compounds have the ability to reduce iron, it may also act directly as antioxidants, attenuating the initiation or propagation of lipid peroxidation induced by reactive oxygen species resulting from the iron dependent Fenton reaction. In addition, because Fe^{2+} concentrations increase after the administration of MPP^+ in the brain, resulting in the increased production of reactive oxygen species via the Fenton reaction, 4-hydroyxquinolines may prevent MPP^+-induced lipid peroxidation by negating the increase in Fe^{2+} levels induced by MPP^+, and consequent production of reactive oxygen species.

Potassium cyanide was used to induce neurotoxic effects, in order to determine the effect of the synthesized 4-hydroxyquinolines against lipid peroxidation and superoxide anions, *in vitro*. Cyanide neurotoxicity produces a Parkinson-like condition and dopaminergic toxicity. Reactive oxygen species are generated as a result of increased calcium, culminating in a cascade of events ultimately leading to lipid peroxidation (Section 2.3.5.5). Similarly, MPP^+ used for the *in vivo* Parkinson model, leads to damage in

dopaminergic neurons and blockage of the electron transport chain, resulting in energy failure and a consequent increase in the formation of free radicals. Severe energy impairment leads to intra neuronal calcium overload, with the same consequences as seen in cyanide toxicity (Section 2.3.5.3) (Schmidt & Ferger, 2001). Both these agents cause dopaminergic toxicity, inhibit mitochondrial respiration, inhibit several antioxidative enzymes and promote the release of cytochrome *c* into the cytosol which activates a caspase cascade, consequently resulting in severe energy depletion and production of reactive oxygen species. Although different toxins were used to study the *in vitro* and *in vivo* effects, it is evident that their neurotoxicity has the same result: dopaminergic toxicity and free radical formation, ultimately leading to a Parkinsonian like condition.

The extent of protection offered by the proposed 4-hydroxyquinolines against oxidative stress as scavengers of superoxide anions was investigated in Chapter 6. *In vitro* experimental results show that the cyanide acts as a potent concentration-dependent generator of superoxide anions in rat brain homogenate (table 6.2 and Fig. 6.8 and 6.9). It was demonstrated that all the proposed 4-hydroxyquinolines significantly attenuated the cyanide induced superoxide generation *in vitro.* Overall, the nitro-compounds, especially in the 7 position showed more activity in scavenging superoxide anions than the dibuthylamino compounds, which in turn was more effective than the amino-compounds. Also, the hydroxyquinolines with the substituent in the 7 position had more activity than the 6 compounds. In particular, 7-nitro-4-hydroxyquinoline was observed to be the best scavenger of superoxide anions. 7-Nitro-4-hydroxyquinoline afforded significant and complete protection against cyanide-induced generation of superoxide, in a dose-dependent manner, and reduced the level of induced superoxide anions to a level lower than that of the basal control.

The promising superoxide anion scavenging properties of these agents prompted an investigation into the *in vivo* effects of these agents in protecting against MPP^+-induced superoxide generation in the striatum. MPP^+ injected intrastriatally significantly increased the level of superoxide generation *in vivo*, and this increase was significantly curbed by the intraperitoneal injection of the various 4-hydroxyquinolines. However, in contrast to the *in vitro* study, the dibuthylamino-compounds appeared to be superior in

attenuating the MPP^+-induced generation of superoxide anions, though not significantly (table 6.3 and Fig 6.10). These results indicate the antioxidant properties of the 4-hydroxyquinolines, *in vivo*, to be independent of MAO activity. In addition, the dibuthylamino compounds may be more lipophilic and may therefore cross the blood brain barrier more easily than the other tested compounds, explaining the difference between the *in vitro* and *in vivo* results.

In vitro, 7-nitro-4-hydroxyquinoline was the best scavenger of superoxide anions. This test is the only test assayed during the course of this study in which the nitro-compound, as well as the compounds substituted in the 7 position was superior to the rest of the test compounds. This may suggest that the nitro-compounds scavenge superoxide anions and thus prevent the initiation of lipid peroxidation, rather than scavenging peroxyl radicals to shorten the propagation of the lipid peroxidation chain.

Considering the fact that respiratory inhibition of mitochondria by MPP^+ is the basis for its generation of superoxide anions, protection offered by the dibuthylamino-4-hydroxyquinolines may not be due solely to its antioxidative properties, but also to direct inhibition of the MPP^+ insult and complex I activation within the electron transport chain (Shachar *et al.*, 2004). This may probably explain the difference between the *in vitro* and *in vivo* study, as cyanide is known to be an inhibitor of the mitochondrial complex IV, which may be attenuated by the nitro-hydroxyquinolines, counteracting the insult induced by cyanide, *in vitro*.

In chapter 7, the neuroprotective effects of the 4-hydroxyquinolines against cyanide and MPP^+-induced lipid peroxidation were investigated. It was demonstrated that cyanide induced a marked concentration-dependent increase in lipid peroxidation in rat brain homogenates *in vitro*, which was significantly curbed by all the tested 4-hydroxyquinolines. In addition, except in the case of 7-nitro-4-hydroxyquinoline, the protection afforded by these quinolines seemed to follow a concentration-dependent pattern. Overall, the 6-subtituted hydroxyquinolines showed more activity than the 7 substituted compounds. Hydroxyquinolines with the substituent in the 6 position showed that the dibuthylamino compounds had more activity than the amino-compounds,

which in turn had more activity than the nitro-compounds. The same trend, however, was not observed for the 7-substituted hydroxyquinolines where the amino-compounds had more activity than the nitro-compounds, which again had more activity than the dibuthylamino-quinolines. Except for 6-amino-4-hydroxyquinoline, the 4-hydroxyquinolines had a negligible effect on the basal production of lipid peroxidation products. The 6-amino-4-hydroxyquinoline reduced the level of thiobarbituric acid reactive substances to a level lower than that of the control. In this study 6-amino and 6-dibuthylamino-4-hydroxyquinolines were the best inhibitors of lipid peroxidation of all the compounds tested.

MPP^+, administered intrastriatally, caused a significant rise in lipid peroxidation. However, only the 6-amino and 6-dibuthylamino-4-hydroxyquinolines could significantly curb this rise in lipid peroxidation, and because these compounds reduced lipid peroxidation to the level of the basal control, these compounds may offer complete protection against MPP^+ toxicity. This is in accordance with the *in vitro* study where the 6-amino and 6-dibuthylamino-4-hydroxyquinolins also showed the best inhibition of lipid peroxidation.

7-Dibuthylamino-hydroxyquinolines also showed better inhibition of lipid peroxidation *in vivo*, than the other 7-substituted compounds tested. This may be related to its lipophilicity, causing it to cross the blood brain more easily.

The significance of the data obtained from the inhibition of lipid peroxidation is obvious considering that damage to any plasma membrane, albeit the cell or mitochondrial membrane, results in a disruption of membrane fluidity, damaged proteins and affected ATP production - all events detrimental to the survival of the cell.

The only test in which the nitro-compounds had more activity than the rest of the tested 4-hydroxyquinolines was the NBT assay, which may suggest that these compounds scavenge superoxide anions and may thus prevent the formation of the toxic hydroxyl radical to prevent the initiation of lipid peroxidation. According to the ORAC, FRAP and TBARS assays, the amino and dibuthylamino-compounds showed better activity. The amino-4-hydroxyquinolines consistently showed the most promising potential for

antioxidant activity, especially the 6-substituted compounds. 6-Amino-4-hydroxyquinolines were the best scavengers of peroxyl radicals (ORAC), the best reducers of iron (FRAP) as well as the best inhibitor of lipid peroxidation *in vitro* as well as *in vivo* (TBARS).

The phenolic moiety of these compounds is often regarded to be the key structural feature responsible for radical scavenging ability, as it is able to neutralize lipid radicals by donating hydrogen atoms to the radical, forming resonance – stabilized, poorly reactive phenoxyl radicals and obstructing the propagation of lipid peroxidation chain reactions (Zheng *et al.*, 2005) (section 3.1). Probably due to this reason, all the tested hydroxyquinolines have been shown to exert radical absorbing activity.

However, the amino-4-hydroxyquinolines have been shown to have the most promising potential as antioxidants. Quinolines bearing an amino group allow the introduction of a positive charge into the molecule by protonating to a NH_3^+ unit (Zwaans and Thompson, 1996) (Section 3.1). Amino-4-hydroxyquinolines may, via single electron transfer, reduce iron and thereby introduce a positive charge into the molecule. Furthermore, this protonated compound may in turn donate a hydrogen atom to a peroxyl radical more rapidly than the peroxyl radical react with the substrate, forming stable radicals unable to continue the oxidation chain reaction - thereby terminating the chain reaction of lipid peroxidation. The molecule may also receive an electron from a radical with the formation of stable products (Harman, 1998; Young and Woodside, 2001). The charge associated with the presence of the unpaired electron becomes dissociated over the scavenger, resulting in a product that will not readily accept an electron from or donate an electron to another molecule, preventing propagation of the chain reaction (Young and Woodside, 2001).

Amino-4-hydroxyquinolines may thus not prevent the initial formation of carbon centered radicals in a lipid rich environment, but minimizes the formation of secondary radicals, such as malondialdehyde. In this way it shortens the propagation phase of lipid peroxidation, and may thus be considered to be chain breaking antioxidants.

Because these compounds have also been shown to reduce free chelatable iron, it will reduce the production of the highly toxic hydroxyl radical, by blocking the Fenton reaction. In this regard it may also curb the initiation of lipid peroxidation.

These results are in agreement with previous studies that have shown that hydroxyquinolines such as clioquinol and VK-28 are neuroprotective against MPTP toxicity due to antioxidative properties as these compounds are strong iron chelators and inhibitors of reactive oxygen species production (section 3.2).

Results obtained during this study clearly indicate that the position of substitution has an effect on the observed antioxidant activity, as compounds with the substituent in position 6 **(4;6;8)** generally afforded higher antioxidant activity.

It can be concluded that the protection offered by the 4-hydroxyquinolines on pro-oxidant induced toxicity is attributable to the antioxidant properties, in scavenging peroxyl and superoxide anions and due to the reduction of iron, which is accompanied by a commensurate reduction in lipid peroxidation. Because these 4-hydroxyquinolines indeed have antioxidative activity and the potential to limit undesirable effects associated with oxidative stress, it may contribute to increased survival of animals exposed to oxidative stress. In addition, because these 4-hydroxyquinolines, administered intraperitoneally reduced superoxide anion generation and lipid peroxidation induced by intrastriatal infusion of MPP^+, it can be assumed that these compounds crossed the blood brain barrier. These are qualities that are essential for neuroprotective agents. MPP^+ is regarded as the best experimental model for Parkinson's disease (Przedborski and Vila, 2001) and it may therefore be concluded that these agents have the potential to curb the progression of this debilitating disease.

Future recommendations are that histological studies be conducted, determining whether these compounds are capable of maintaining the architectural integrity of the neurons and the arrangement of the neurons in the striatal region and determining if these compounds are able to protect neurons from induced programmed cell death (apoptosis). If these compounds do have the ability to inhibit apoptosis or necrosis, it turns its antioxidant activity to neuroprotection. Complex I of the respiratory chain has been documented to

be intimately associated with the onset of several neurodegenerative disorders as well as the ageing process, thus compounds that stimulate the activity of this complex, can possibly prevent the age and neurodegenerative disease-related decline of complex I activity. Therefore an investigation into the effect of these compounds on complex I is definitely warranted. The toxins used during this study have been documented to inhibit or reduce the activities of several antioxidant enzymes and an investigation of the effect of 4-hydroxyquinolines on the antioxidant enzymes within the brain may also be mandatory.

In conclusion, data presented in this study contribute to the comprehension of the suspected antioxidative effects of 4-hydroxyquinolines and encourage further studies to investigate their mechanism of action. In addition, this study establishes the potential and novel neuroprotective activity of 4-hydroxyquinoline derivatives as strong antioxidants and provides a rational basis for the use of these compounds in development of prophylactic and therapeutical strategies for multiple mental disorders associated with oxidative stress.

.

LITERATURE CITED

AGBAS, A., CHEN, X., HONG, O., KUMAR, K.N. & MICHAELIS, K. 2002. Superoxide modification and inactivation of a neuronal receptor-like complex. *Free Radical Biology and Medicine*, 32(6):512-524.

AKYOL, Ö., HERKEN, H., UZ, E., FADILLIOĞLU, E., ÜNAL, S., SÖĞÜT, S., ÖZYURT, H. & SAVAŞ, H.A. 2002. The indices of endogenous oxidative and antioxidative processes in plasma from schizophrenic patients. The possible role of oxidant/antioxidant imbalance. *Progress in Neuro-Psychopharmacology and Biological Psychiatry,* 26:995-1005.

ALEXI, T., BORLONGAN, C.V., FAULL, R.L.M., WILLIAMS, C.E., CLARK, R.G., GLUCKMAN, P.D. & HUGHES, P.E. 2000. Neuroprotective strategies for basal ganglia degeneration: Parkinson's and Huntington's diseases. *Progress in Neurobiology*, 60:409-470.

ALLEN, R. G. & TRESINI, M. 2000. Oxidative stress and gene regulation. *Free Radical Biology and Medicine,* 28(3):463-499.

ANOOPKUMAR-DUKIE, S., WALKER, R.B., & DAYA, S. 2001. A sensitive and reliable method for the detection of lipid peroxidation in biological studies. *Journal of Pharmacy and Pharmacology*, 53:263-266.

APPELBAUM, P.C. & HUNTER, P.A. 2000. The fluoroquinolone antibacterials: past, present and future perspectives. *International Journal of Antimicrobial Agents*, 16:5-15.

ARDELT, B.K., BOROWITZ, J.L. & ISOM, G.E. 1989. Brain lipid peroxidation and antioxidant protectant mechanisms following acute cyanide intoxication. *Toxicology*, 56:147-154.

ARIVAZHAGAN, P., SHILA, S., KUMARAN, S. & PANNEERSELVAM, C. 2002. Effect of DL-α-lipoic acid on the status of lipid peroxidation and antioxidant enzymes in various brain regions of aged rats. *Experimental Gerontology,* 37:803-811.

ARRIAGA-ALBA, M., RIVERA-SÁNCHEZ, R., PARRA-CERVANTES, G., BARRO-MORENO, F., FLORES-PAZ, R. & GARCÍA-JIMÉNEZ, E. 2000. Antimutagenesis of β-carotene to mutations induced by quinolone on *Salmonella typhimurium. Archives of Medical Research,* 31:156-161.

ARUOMA, O.I. 2003. Methodological considerations for characterizing potential antioxidant actions of bioactive components in plant foods. *Mutation Research,* 523-524:9-20.

ASHOK, B. T. & ALI, R. 1999. The aging paradox: free radical theory of aging. *Experimental Gerontology,* 34:293-303.

BAKER, B.R. & BRAMHALL, R.R. 1972. Irreversible enzyme inhibitors. 189. Inhibition of some dehydrogenase by derivatives of 4-hydroxyquinoline-2- and -3-carboxylic acids. *Journal of Medicinal Chemistry*, 15(3):230-233.

BANAN, A., FITZPATRICK, L., ZHANG, Y. & KESHAVARZIAN, A. 2001. OPC-compounds prevent oxidant-induced carbonylation and depolymerization of the F-actin cytoskeleton and intestinal barrier hyperpermeability. 2001. *Free Radical Biology and Medicine,* 30(3):287-298.

BARC, S., INGRAND, S.S., FAUCONNEAU, B., PAGE, G., PIRIOU, A. & BARRIER, L. 2004. Effect of ischemia on TBARS and lactate production in several cerebral regions of anaesthetized and awake rats. *Life Sciences,* 74:3103-3113.

BEAL, M.F. 2001. Experimental models of Parkinson's disease. *Nature Reviews Neuroscience*, 2:235-334.

BELLÉ, N.A.V. DALMOLIN, G.D., FONINI, G., RUBIN, M.A. & ROCHA, J.B.T. 2004. Polyamines reduces lipid peroxidation induced by different pro-oxidant agents. *Brain Research,* 1008:245-251.

BENZIE, I.F.F. & STRAIN, J.J. 1996. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": The FRAP assay. *Analytical Biochemistry*, 239:70-76.

BENZIE, I.F.F. & STRAIN, J.J. 1999. Ferric reducing/antioxidant power assay: Direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. *Methods in Enzymology,* 299:15-27.

BERNHEIM, F., BERNHEIM, M.L.C. & WILBUR, K.M. 1948. The reaction between thiobarbituric acid and the oxidation products of certain lipids. *Journal of Biological Chemistry*, 174:257-264.

BRAINS, J.S. & SHAW, C.A. 1997. Neurodegenerative disorders in humans: the role of glutathione in oxidative stress-mediated neuronal death. *Brain Research Review,* 25:335-358.

BROWNE, S.E., ROBERTS II, L.J., DENNERY, P.A., DOCTROW, S.R., BEAL, M.F., BARLOW, C. & LEVINE, R.L. 2004. Treatment with a catalytic antioxidant corrects the neurobehavioral defect in ataxia-telengiectasia mice. *Free Radical Biology and Medicine,* 36(7):938-942.

BUSH, A.I. 2002. Metal complexing agents as therapies for Alzheimer's disease. *Neurobiology of Aging*, 23:1031-1038.

BUSH, A. 2002a Response:'... and C is for Clioquinol' – the AβCs of Alzheimer's disease. *Trends in Neuroscience,* 25(3):123.

CADENAS, E. 1995. Mechanisms of oxygen activation and reactive oxygen species detoxification. (*In* Ahmad, S., *ed.* Oxidative stress and antioxidant defences in Biology. New York:Chapman & Hall. p.25-42.).

CAI, Q., RAHN, R. & ZHANG, R. 1997. Dietary flavonoids, quercetin, luteolin and genistein, reduce oxidative DNA damage and lipid peroxidation and quench free radicals. *Cancer Letters*, 119:99-107.

CAMPBELL, N. 1976. Bicyclic compounds containing a pyridine ring: quinoline and its derivatives. (*In* Coffey, S., *ed* Rodd's chemistry of carbon compounds. Vol. 4, part F. 2nd ed. Amsterdam: Elsevier Scientific. pp. 231-356).

CAO, G, GIOVANONI, M. & PRIOR, R.L. 1996. Antioxidant capacity decreases during growth but not aging in rat serum and brain. *Archives of Gerontology and Geriatrics,* 22:27-37.

CAO, G. & PRIOR , R.L. 1999. Measurement of oxygen radical absorbance capacity in biological samples. *Methods in Enzymology,* 299:50-62.

CARNEVALI, S., PETRUZZELI, S., LONGONI, B., VANACORE, R., BARALE, R., CIPOLLINI, M., SCATENA, F., PAGGIARO, P., CELI, A. & GIUNTINI, C. 2003. Cigarette smoke extract induces oxidative stress and apoptosis in human lung fibroblasts. *American Journal of Physiology–Lung Celullular and Molecular Physiology,* 284:L955:L963.

CARRASCO, E. & WERNER, P. 2002. Selective destruction of dopaminergic neurons by low concentrations of 6-OHDA and MPP^+: protection by acetylsalicylic acid (aspirin). *Parkinsonism and Related Disorders,* 8:407-411.

CHENG, C-C. & YAN, S-J. 1982. The Friedländer synthesis of quinolines. (*In* Dauben, W.G., *ed.* Organic reactions. Vol. 28. New York : Wiley. pp.37-201).

CLIFFE, I.A., IFILL, A.D., MANSELL, H.L., TODD, R.S. & WHITE, A.C. 1991. 7-Amino-5,6,7,8-tetrahydroquinolines. Preparation from 5,6-dihydroquinoline and nitrogen nucleophiles. *Tetrahedron letters*, 32(46):6789-6792.

COLLINS, A. R., DOBSON, V.L., DUŠINSKÁ, M., KENNEDY, G. & ŠTĚTINA, R. 1997. The comet assay: what can it really tell us? *Mutation Research*, 375:183-193.

CRAGOE, J.E. & ROBB, C.M. 1973. 3-Hydroxyquinoline. (*In* Baumgarten, H.E., *ed.* Organic syntheses. Coll. vol. 5. New York : Wiley. pp.635-641).

CURTIN, J.F., DONOVAN, M. & COTTER, T.G. 2002. Regulation and measurement of oxidative stress in apoptosis. *Journal of Immunological Methods*, 265:49-72.

DAS, U.N., PADMA, M., SAGAR, P.S., RAMESH, G. & KORATKAR, R. 1990. Stimulation of free radical generation in human leukocytes by various agents including tumor necrosis factor is a calmodulin dependent process. *Biochemical and Biophysical Research Communications,* 167(3):1030-1036.

DE PINEDO, A.T., PEŇALVER, P. & MORALES, J.C. 2006. Synthesis and evaluation of new phenolic-based antioxidants: Structure-activity relationship. *Food Chemistry,*doi:10.1016/j.foodchem.2006.07.026.

DELIBAS, N., OZCANKAYA, R. & ALTUNTAS, I. 2002. Clinical importance of erythrocyte malondialdehyde levels as marker for cognitive deterioration in patients with dementia of Alzheimer's type: a repeated study in 5-year interval. *Clinical Biochemistry*, 32:137-141.

DI MONTE, D., JEWELL, S.A., EKSRÖM, G., SANDY, M.S. & SMITH, M.T. 1986. 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 1-methyl-4-phenylpyridine (MPP^+) cause rapid ATP depletion in isolated hepatocytes. *Biochemical and Biophysical Research Communications*, 137(1):310-315.

DRAPER, H.H. & HADLEY, M. 1990. Malondialdehyde determination as an index of lipid peroxidation. In: Packer, L.; Glazer, A.N., eds. *Methods in Enzymology*, 186:421-431. New York: Academic Press.

DRIVER, A. S., KODAVANTI, P. R. S. & MUNDY, W. R. 2000. Age-related changes in reactive oxygen species production in rat brain homogenates. *Neurotoxicology and Teratology,* 22:175-181.

FINKEL, T. 2003. Oxidant signals and oxidative stress. *Current Opinion in Cell Biology*, 15(2):247-254.

GABORIAU, F., DELCROS, J. & MOULINOUX, J. 2002. A simple assay for the measurement of plasma antioxidant status using spontaneous autoxidation of homovanillic acid. *Journal of Pharmacological and Toxicological Methods,* 47:33-43.

GHISELLI, A., SERAFINI, M., NATELLA, F. & SCACCINI, C. 2000. Total antioxidant capacity as a tool to assess redox status: Critical view and experimental data. *Free Radical Biology and Medicine*, 29(11):1106-1114.

GIASSON, B.I., ISCHIROPOULOS, H., LEE, V.M.-Y. & TROJANOWSKI, J.Q. 2002. The relationship between oxidative/nitrative stress and pathological inclusions in Alzheimer's and Parkinson's diseases. *Free Radical Biology and Medicine,* 32(12):1264-1275.

GIROTTI, S., FERRI, E., MACCAGNANI, L., BUDINI, R. & BIANCHI, G. 2002. Plasma antioxidant capacity determination: comparative evaluation of chemiluminescent and spectrophotometric assays. *Talanta*, 56:407-414.

GLAZER, A.N. 1988. Fluorescence-based assay for reactive oxygen species: a protective role for creatinine. *The FASEB Journal,* 2(9):2487:2491.

GLOWINSKI, J. & IVERSEN, L.L. 1966. Regional studies of catecholamines in the rat brain. I. the disposition of [3H]norepinephrine, [3H]dopamine and [3H]dopa in various regions of the brain. *Journal of Neurochemistry*, 13:655-669.

GOLDFARB, A. H. 1999. Nutritional antioxidants as therapeutic and preventative modalities in exercise-induced muscle-damage. *Canadian Journal of Applied Physiology,* 24:249-266. [Abstract]

GOULD, R.G. & JACOBS, W.A. 1939. The synthesis of certain substituted quinolines and 5,6-benzoquinolines. *Journal of the American Chemical Society,* 61(10):2890-2895.

GRANOT, E. & KOHEN, R. 2004. Oxidative stress in childhood – in health and disease states. *Clinical Nutrition.* 23(1):3-11.

GREENAMYRE, J.T., BETARBET, R. & SHERER, T.B. 2003. The rotenone model of Parkinson's disease and mitochondria. *Parkinsonism and Related Disorders* 9:S59-S64.

GRIMM, T., SCHÄFER, A. & HÖGGER, P. 2004. Antioxidant activity and inhibition of matrix metalloproteinases by metabolites of maritime pine bark extract (pycnogenol). *Free Radical Biology and Medicine* 36(6):811-822.

GÜLÇIN, E., OKTAY, M., KÜFREVIOĞLU, Ö. I. & ASLAN, A. 2002. Determination of antioxidant activity of *Cetraria islandica* (L) Ach. *Journal of Ethnopharmacology,* 79:325-329.

GUTTERIDGE, J.M.C. 1987. In B., Halliwell (Ed.), *Oxygen Radicals and Tissue injury: Proceedings of an Upjohn Symposium,* The Upjohn Company: USA p. 9.

HALLIWELL, B. 1995. Antioxidant characterization Methodology and mechanism. *Biochemical Pharmacology,* 49(10):1341-1348.

HALLIWELL, B. & GUTTERIDGE, J.M.C. 1984. Oxygen toxicity, oxygen radicals, transition metals and disease. *Biochemical Journal*, 219:1-14.

HALLIWELL, B. & GUTTERIDGE, J.M.C. 1989. *Free radicals in Biology and Medicine,* 2nd ed. Oxford University Press: Oxford, p. 12.

HALLIWELL, B. & GUTTERIDGE, J.M.C. 1999. *Free Radicals in Biology and Medicine,* 3rd ed. Oxford University Press, New York. p. 210.

HAMAI, D., BONDY, S. 2004. Pro-or anti-oxidant manganese: a suggested mechanism for reconciliation. *Neurochemistry International.* 44(4):223-229.

HARMAN, D., 1956. Aging: a theory based on free radical and radiation chemistry. *Journal of Gerontology*, 11:298-300.

HARMAN, D. 1998. Free radical theory of ageing: applications. *Asia Pacific Heart Journal,* 7(3):169-177.

HEIM, K. E., TAGLIAFERRO, A. R. & BOBILYA, D. J. 2002. Flavonoid antioxidants: chemistry, metabolism and structure activity. *The Journal of Nutritional Biochemisty,* 13:572-584.

HEINECKE, J. W. 2002. Oxidized amino acids: culprits in human atherosclerosis and indicators of oxidative stress. *Free Radical Biology and Medicine,* 32(11):1090-1101.

HO, P. & SAVILLE, D.J. 2001. Inhibition of human CYP3A4 activity by grapefruit flavonoids, furanocoumarins and related compounds. *Journal of Pharmacy and Pharmaceutical Sciences* 4(3):217-227.

HUANG, D., OU, B., HAMPSH-WOODILL, M., FLANAGAN, J.A. & DEEMER, E.K. 2002. Development and validation of oxygen radical absorbance capacity assay for lipophilic antioxidants using randomly methylated β-cyclodextrin as the solubility enhancer. *Journal of Agricultural and Food Chemistry,* 50:1815-1821.

HUDLICKÝ, M. 1996. Reductions in organic chemistry. 2nd ed. Washington, DC : American Chemical Society. 429 p.

INAL, M.E., KANBAK, G. & SUNAL, E. 2001. Antioxidant enzyme activities and malondialdehyde levels related to aging. *Clinica Chimica Acta*, 305:75-80.

ISHIGE, K., SCHUBERT, D. & SAGARA, Y. 2001. Flavonoids protect neuronal cells from oxidative stress by three distinct mechanisms. *Free Radical Biology and Medicine*, 30(4):433-446.

JANISCH, K., HIPPELI, S., DORNISCH, K., KERN, S. & ELSTNER, E.F. 2002. Determination of the antioxidative potential of human plasma after supplementation with pycnogenol and whey. *Food Research International,* 35:257-266.

JIMÉNEZ-JIMÉNEZ, F.J., MOLINA, J.A., DE BUSTOS, F., GARCÍA-REDONDO, A., GÓMEZ-ESCALONILLA, C., MARTÍNEZ-SALIO, A., BERBEL, A., CAMACHO, A., ZURDO, M., BARCENILLA, B., ENRÍQUEZ DE SALAMANCA, R. & ARENAS, J. 2000. Serum levels of coenzyme Q_{10} in patients with Parkinson's disease. *Journal of Neural Transmission*, 107:177-181.

JONES, G. 1977. Synthesis of the quinoline ring system. (*In* Jones, G. *ed.* Quinolines. Part 1. London: Wiley. pp.93-318).

JONES, D.C., PRABHAKARAN, K., LI, L., GUNASEKAR, P.G., SHOU, Y., BOROWITZ, J.L. & ISOM, G.E. 2003. Cyanide enhancement of dopamine-induced apoptosis in mesencephalic cells involves mitochondrial dysfunction and oxidative stress. *NeuroToxicology*, 24:333-432.

JONES, D.P., MODY Jr, V.C., CARLSON, J.L., LYNN, M.J. & STERNBERG Jr, P. 2002. Redox analysis of human plasma allows separation of pro-oxidant events of age from decline in antioxidant defenses. *Free Radical Biology and Medicine,* 33(9):1290-1300.

JUNQUEIRA, V.B.C., BARROS, S.B.M., CHAN, S.S., RODRIQUES, L., GIAVAROTTI, L., ABUD, R.L. & DEUCHER, G.P. 2004. Aging and oxidative stress. *Molecular Aspects of Medicine*, 25:5-16.

KANG, S.S, LEE, J.Y., CHOI, Y.K., KIM, G.S. & HAN, B.H. 2004. Neuroprotective effects of flavones on hydrogen peroxide-induced apoptosis in SH-SY5Y neuroblostoma cells. *Bioorganic and Medicinal Chemistry Letters* 14:2261-2264.

KARLSON, J. 1997. Introduction to nutraology and radical formation. In: Antioxidants and Exercise. Illinois: Human kinetics Press, pp. 1-143.

KAYIRERE, M., MAHAMOUD, A., CHEVALIER, J., SOYFER, J., CRÉMIEUX, A. & BARBE, J. 1998. Synthesis and antibacterial activity of new 4-alkoxy, 4-aminoalkyl and 4-alkylthioquinoline derivatives. *European Journal of Medicinal Chemistry*, 33:55-63.

KHALDY, H., ESCAMES, G., LEÓN, J., BIKJDAOUENE, L. & ACUÑA-CASTROVIEJO, D. 2003. Synergistic effects of melatonin and deprenyl against MPTP-induced mitochondrial damage and DA-depletion. *Neurobiology of Aging,* 24:491-500.

KIDD, P.M. 2000. Parkinson's disease as multifactorial oxidative neurodegeneration: Implications for integrative management. *Alternative Medicine Review,* 5(6): 502-529.

KIM, J.W., NO, J.K., IKENO, Y., YU, B.P., CHOI, J.S., YOKOZAWA, T. & CHUNG, H.Y. 2002. Age-related changes in redox status of rat serum. *Archives of Gerontology and Geriatrics*, 34:9-17.

KLÖHN, P-C., MASSALHA, H. & NEUMANN, H-G. 1995. A metabolite of carcinogenic 2-acetylaminofluorene, 2-nitrosofluorene, induces redox cycling in mitochondria. *Biochemica et Biophysica Acta,* 1229:363-372.

KLOPMAN, G., FERCU, D., LI, L.Y., ROSENKRANZ, H.S. & JACOBS, M.R. 1995. Antimycobacterial quinolones: a comparative analysis of structure–activity and structure-cytotoxicity relationships. *Forum of microbiology 13^{ed},* 86-96.

KOHN, H. I. & LIVERSEDGE, M. 1944. On a new aerobic metabolite whose production by brain is inhibited by apomorphine, emetine, ergotamine, epinephrine and menadione. *Journal of Pharmacology and Experimental Therapeutics*, 83:292-300.

KÖNIG, J.F.R. & KLIPPEL, R.A. 1963. The rat brain. A stereotaxic atlas of the forebrain and lower parts of the brain stem. Baltimore : Williams and Wilkins. pp.108-114.

KONO, Y. & FRIDOVICH, I. 1982. Superoxide radical inhibits catalase. *The Journal of Biological Chemistry*, 257(10):5751-5754.

KOVÁCS, T. 2004. Mechanisms of olfactory dysfunction in aging and neurodegenerative disorders. *Ageing Research Reviews*, 3(2):215-232.

KUTI, J.O. 2004. Antioxidant compounds form four *Opuntia* cactus pear fruit varieties. *Food Chemistry,* 85:527-533.

LANGE, J.H.M., VERVEER, P.C., OSNABRUG, S.J.M. & VISSER, G.M. 2001. Rapid microwave-enhanced synthesis of 4-hydroxyquinolones under solvent-free conditions. *Tetrahedron letters,* 42(7):1367-1369.

LARANJINHA, J., VIEIRA, O., MADEIRA, V. & ALMEIDA, L. 1995. Two related phenolic antioxidants with opposite effects on vitamin E content in low density

lipoproteins oxidized by ferrylmyoglobin: Consumption vs regeneration. *Archives of Biochemistry and Biophysics,* 323(2):373-381.

LEE, C. 2000. Antioxidant ability of caffeine and its metabolites based on the study of oxygen radical absorbing capacity and inhibition of LDL peroxidation. *Clinica Chimica Acta.*, 295(1-2):141-154.

LEE, S.H. 2004. Green tea in the fight of cancer [Web:] http://www.teaforhealth.com [2006-02-24].

LEVINE, R.L. & STADTMAN, E.R. 2001. Oxidative modification of proteins during aging. *Experimental Gerontology,* 36:1495-1502.

LEVINE, R.L. 2002. Serial Review: Oxidatively modified proteins in aging and disease. Carbonyl modified proteins in cellular regulation, aging, and disease. *Free Radical Biology and Medicine,* 32(9):790-796.

LIU, Z., HAN, K., LIN, Y. & LUO, X. 2002. Antioxidative or prooxidative effect of 4-hydroxyquinoline on free-radical-initiated hemolysis of erythrocytes is due to its distributive status. *Biochimica et Biophysica Acta*, 1570:97:103.

LLORENTE, B., LECLERC, F. & CEDERGREN, R. 1996. Using SAR and QSAR analysis to model the activity and structure of the quinolone – DNA complex. *Bioorganic and Medicinal Chemistry,* 4(1):61-71.

LOGHIN, F., CHAGRAOUI, A., ASENCIO, M., COMOY, E., SPEISKY, H., CASSELS, B.K. & PROTAIS, P. 2003. Effects if some antioxidative aporphine derivatives on striatal dopaminergic transmission and on MPTP-induced striatal dopamine depletion in B6CBA mice. *European Journal of Pharmaceutical Sciences,* 18:133-140.

LOWRY, O.H., ROSEBRONGH, N.J., FARA, A.L. & RANDALL, R.J. 1951. Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* 193,265-275.

MAHARAJ, D.S. 2003. An investigation into the physico-chemical and neuroprotective properties of melatonin and 6-hydroxymelatonin. Grahamstown:Rhodes. (Thesis-Ph.D.) p. 400.

MAHARAJ, D. S., SARAVANAN, K. S., MAHARAJ, H., MOHANAKUMAR, K. P. & DAYA, S. 2004. Acetaminophen and aspirin inhibit superoxide anion generation and lipid peroxidation, and protect against 1-methyl-4-phenyl pyridinium-induced dopaminergic neurotoxicity in rats. *Neurochemistry International*, 44:355-360.

MAHARAJ, D.S., WALKER, R.B., GLASS, B.D. & DAYA, S. 2003. 6-Hydroxymelatonin protects against cyanide induced oxidative stress in rat brain homogenates. *Journal of Chemical Neuroanatomy*, 26:103-107.

MAHARAJ, H. 2004. An investigation into the neuroprotective properties of acetylsalicylic acid and acetaminophen. Grahamstown:Rhodes (Thesis – Ph.D.) p. 367.

MAHARAJ, H., MAHARAJ, D.S. & DAYA, S. 2006. Acetylsalicylic acid and acetaminophen protect against MPP^+-induced mitochondrial damage and superoxide anion generation. *Life Sciences,* 78:2438-2443.

MALFROY, B., DOCTROW, S.R., ORR, P.L., TOCCO, G., FEDOSEYEVA, E.V. & BENICHOU, G. 1997. Prevention and suppression of autoimmune encephalomyelitis by EUK-8, a synthetic catalytic scavenger of oxygen-reactive metabolites. *Cellular Immunology,* 177:62-68.

MANSKE, R.H.F. & KULKA, M. 1953. The Skraup synthesis of quinolines. (*In* Adams, R. *ed.* Organic reactions. Vol. VII. New York: Wiley. pp. 59-98).

MARUYAMA, W. & NAOI, M. 1999. Neuroprotection by (-)- deprenyl and related compounds. *Mechanisms of Ageing and Development*, 111(2-3):189-200.

MCCORD, J. M. 2000. The evolution of free radicals and oxidative stress. *The American Journal of Medicine,* 108:652-659.

MELOV, S. 2002. Animal models of oxidative stress, aging and therapeutic antioxidant interventions. *The International Journal of Biochemistry and Cell Biology,* 34:1395-1400.

MELOV, S. 2002a. '...and C is for Clioquinol'-the AβC of Alzheimer's disease. *Trends in Neuroscience*, 25(3):121-122.

MICHAELIS, E.K. 1998. Molecular biology of glutamate receptors in the central nervous system and their role in excitotoxicity, oxidative stress and aging. *Progress in Neurobiology*, 54(4):369-415.

MIOLO,G., VIOLA, G., VADALDI, D., DALL'ACQUA, F., FRAVOLINI, A., TABARRINI, O. & CECCHETTI, V. 2002. In vitro phototoxic properties of new 6-desfluoro and 6-fluoro-8-methylquinolones. *Toxicology in Vitro*, 16:683-693.

MIZUNO, Y., OHTA, S., TANAKA, M., TAKAMIYA, S., SUZUKI, K., SATO, T., OYA, H., OZAWA, T. & KAGAWA, Y. 1989. Deficiencies in complex I subunits of the respiratory chain in Parkinson's disease. *Biochemical and Biophysical Research Communications,* 163:1450-1455.

MOREL, P., FAUCONNEAU, B., PAGE, G., MIRBEAU, T. & HUGUET, F. 1998. Inhibitory effects of ascorbic acid on dopamine uptake by rat striatal synaptosomes: relationship to lipid peroxidation and oxidation of protein sulfhydryl groups. *Neuroscience Research*, 32:171-179.

MUSIOL, R., JAMPILEK, J., BUCHTA, V., SILVA, L., NIEDBALA, H., PODESZWA, B., PALKA, A., MAJERZ-MANIECKA, K., OLEKSYN, B. & POLANSKI, J. 2006. Antifungal properties of new series of quinoline derivatives. *Bioorganic and Medicinal Chemistry,* 14(10):3592-3598.

NAIDU, P.S., SINGH, A & KULKARNI, S.K. 2003. Quercetin, a bioflavonoid, attenuates haloperidol-induced orofacial dyskinesia. *Neuropharmacology,* 44:1100-1106.

NARENDER, P., SRINIVAS, U., RAVINDER, M., RAO, B. A., RAMESH, C, HARAKISHORE, K., GANGADASU, B., MURTHY, U.S.N. & RAO, V.J. 2006. Synthesis of multisubstituted quinolines from Baylis-Hillman adducts obtained from substituted 2-chloronicotinaldehydes and their antimicrobial activity. *Bioorganic and Medicinal Chemistry*, 14(13):4600-4609.

NATHAN, B.R., SCHELD, W.M. 2003. The efficacy of trovafloxacin versus ceftriaxone in the treatment of experimental brain abcess/cerebritis in the rat. *Life Sciences,* 73 (14): 1773-1782.

OKATANI, Y., WAKATSUKI, A., REITER, R.J. & MIYAHARA, Y. 2002. Melatonin reduces oxidative damage of neural lipids and proteins in senescence-accelerated mouse. *Neurobiology of Aging,* 23:639-644.

OTTINO, P. & DUNCAN, J.R. 1997. Effect of α-tocopherol succinate on free radical and lipid peroxidation levels in BL6 melanoma cells. *Free Radical Biology and Medicine,* 22(7):1145-1151.

OU, B., HAMPSCH-WOODILL, M. & PRIOR, R.L. 2001. Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. *Journal of Agriculture and Food Chemistry,* 49:4619-4626.

OU, B., HUANG, D., HAMPSCH-WOODILL, M., FLANAGAN, J.A. & DEEMER, E.K. 2002. Analysis of antioxidant activities of common vegetables employing oxygen radical absorbance capacity (ORAC) and ferric reducing antioxidant power (FRAP) assays: A comparative study. *Journal of Agricultural and Food Chemistry,* 50:3122-3128.

PACKER, L., RIMBACH, G. & VIRGILI, F. 1999. Antioxidant activity and biologic properties of a procyanidin – rich extract from pine (*Pinus maritima*) bark, pycnogenol. *Free Radical Biology and Medicine,* 27;(5-6):704-724.

PAN, T., FEI, J., ZHOU, X., JANKOVIC, J. & LE, W. 2003. Effects of green tea polyphenols on dopamine uptake and on MPP^+ - induced dopamine neuron injury. *Life Sciences,* 72:1073-1083.

PAPUCCI, L., SCHIAVONE, N., WITORT, E., DONNINI, M., LAPUCCI, A., TEMPESTINI, A., FORMIGLI, L., ZECCHI-ORLANDINI, S., ORLANDINI, G., CARELLA, G., BRANCATO, R. & CAPACCIOLI, S. 2003. Coenzyme Q_{10} prevents apoptosis by inhibiting mitochondrial depolarization independently of its free radical scavenging property. *Journal of Biological Chemistry,* 278(30):28220-28228.

PAREJO, I., VILADOMAT, F., BASTIDA, J., ROSAS-ROMERO, A., SAAVEDRA, G., MURCIA, M.A., JIMÉNEZ, A.M. & CODINA, C. 2003. Investigation of Bolivian plant extracts for their radical scavenging activity and antioxidant activity. *Life Sciences,* 73:1667-1681.

PARIKH, V., KHAN, M.M. & MAHADIK, S.P. 2003. Differential effects of antipsychotics on expression of antioxidant enzymes and membrane lipid peroxidation in rat brain. *Journal of Psychiatric Research,* 37:43-51.

PARK, H., KIM, T.H. & BARK, K. 2002. Physicochemical properties of quinolone antibiotics in various environments. *European Journal of Medicinal Chemistry,* 37:443-460.

PAXINOS, G. & WATSON, C. 1998. The rat brain in stereotaxic coordinates. *Academic press,* New York : pp.41-44.

PERSONAL HEALTH LIFESTYLES, INC. 2002. Parkinson's disease facts, disease prevention and treatment strategies. [Web:] http://www.healingwithnutrition.com/pdisease/parkinosons.html [Date of access: 21 Sept. 2005].

POLIDORI, M.C., STAHL, W., EICHLER, O., NIESTROJ, I. & SIES, H. 2001. Profiles of antioxidants in human plasma. *Free Radical Biology and Medicine,* 30(5):456-462.

PRIOR, R.L. & CAO, C. 1999. In vivo total antioxidant capacity: comparison of different analytical methods. *Free Radical Biology and Medicine,* 27(11-12):1173-1181.

PRIOR, R.L., HOANG, H., GU. L., WU, X., BACCHIOCCA, M., HOWARD, L., HAMPSCH-WOODILL, M., HUANG, D., OU, B. & JACOB, R. 2003. Assays for hydrophilic and lipophilic antioxidant capacity (oxygen radical absorbance capacity ($ORAC_{FL}$)) of plasma and other biological and food samples. *Journal of Agricultural and Food Chemistry,* 51:3273-3279.

PRZEDBORSKI, S. & VILA, M. 2001. MPTP: a review of its mechanisms of neurotoxicity. *Clinical Neuroscience Research,* 1:407-418.

PULIDO, R., BRAVO, L. & SAURA-CALIXTO, F. 2000. Antioxidant activity of dietary polyphenols as determined by a modified ferric reducing/antioxidant power assay. *Journal of Agriculture and Food Chemistry,* 48:3396-3402.

REIGEL, B., LAPPIN, G.R., ADELSON, B.H., JACKSON, R.I., ALBISETTI, C.J., DODSON, R.M. & BAKER, R.H. 1946. The synthesis of some 4-quinolinols and 4-chloroquinolines by the ethyoxymethylenemalonic ester method. *Journal of the American Chemical Society,* 68(7):1264-1266.

REIL, E., HÖFLE, G., DRABER, W. & OETTMEIER, W. 1997. Quinolones and their *N*-oxides as inhibitors of mitochondrial complexes I and III. *Biochemica et Biophysica Acta,* 1318:291-298.

REIL, E., HÖFLE, G., DRABER, W. & OETTMEIER, W. 2001. Quinolones and their *N*-oxides as inhibitors of photosystem II and the cytochrome b_6/f-complex. *Biochemica et Biophysica Acta,* 1506:127-132.

ROIG, R., CASCÓN, E., AROLA, L., BLADÉ, C. & SALVADO, M.J. 2002. Procyanidins protect Fao cells against hydrogen peroxide – induced oxidative stress. *Biochimica et Biophysica Acta,* 1572:25-30.

ROSEN, J.E., CHEN, D., PRAHALAD, A.K., SPRATT, T.E., SCHLUTER, G. & WILLIAMS, G.M. 1997. A Fluoroquinolone antibiotic with a methoxy group at the

8-position yields reduced generation of 8-oxo-7,8-dihydro-2'-deoxyguanosine after ultraviolet-A irradiation. *Toxicology and Applied Pharmacology,* 145:381-387.

SAGAR, P.S., DAS, U.N., KORATKAR, R., RAMESH, G., PADMA, M. & SRAVAN KUMAR, G. 1992. Cytotoxic action of cis-unsaturated fatty acids on human cervical carcinoma (HeLa) cells: relationship to free radicals and lipid peroxidation and its modulation by calmodulin antagonists. *Cancer Letters*, 63:189-198.

SAMIEC, P. S., DREWS-BOTSCH, C., FLAGG, E. W., KURTZ, J. C., STERNBERG, P. Jr., REED, R. L. & JONES, D. P. 1998. Glutathione in human plasma: Decline in association with aging, age-related macular degeneration, and diabetes. *Free Radical Biology and Medicine,* 24(5):699-704.

SANTAMARIA, A., FLORES-ESCARTIN, A., MARTINEZ, A. C., OSORIO, L., GALVAN-ARZATE, S., CHAVERRI, J.P., MALDONADO, P.D., MEDINA-CAMPOS, O.N., JIMENEZ-CAPDEVILLE, M.E., MANJARREZ, J. & RIOS, C. 2003. Copper blocks quinolinic acid neurotoxicity in rats: Contribution of antioxidant systems. *Free Radical Biology and Medicine* 35(4):418-427.

SCHAPIRA, A.H.V., COOPER, J.M., DEXTER, D., CLARK, J.P. & MARSDEN, C.D. 1989. Mitochondrial complex I deficiency in Parkinson's disease. *Lancet.*, 1:1269.

SCHMIDT, N. & FERGER, B. 2001. Neurochemical findings in the MPTP model of Parkinson's disease. *Journal of neural transmission*, 108:1263-1282.

SCHWEMMER, M., FINK, B., KÖCKERBAUER, R. & BASSENGE, E. 2000. How urine analysis reflects oxidative stress – nitrotyrosine as a potential marker. *Clinica Chimica Acta,* 297:207-216.

SEELEY, R.R., STEPHENS, T.D. & TATE, P. 2000. Anatomy and Physiology. 5th ed. Boston: Mc Graw Hill. 1106 p.

SHACHAR, D.B., KAHANA, N., KAMPEL, V., WARSHAWSKY, A. & YOUDIM, M.B.H., 2004. Neuroprotection by a novel brain permeable iron chelator, VK-28, against 6-hydroxydopamine lesion in rats. *Neuropharmacology,* 46:254-263.

SHAH, K.J. & COATS, E.A. 1977. Design, synthesis and correlation of 7-substituted 4-hydroxyquinoline-3-carboxylic acids as inhibitors of cellular respiration. *Journal of Medicinal Chemistry,* 20(8):1001-1006.

SHILA, S., KOKILAVANI, V., SUBATHRA, M. & PANNEERSELVLAM, C. 2005. Brain regional responses in antioxidant system α-lipoic acid in arsenic intoxicated rat. *Toxicology,* 210:25-36.

SHOU, Y., GUNASEKAR, P.G., BOROWITZ, J.L. & ISOM, G.E. 2000. Cyanide-induced apoptosis involves oxidative stress-activated NF-κB in cortical neurons. *Toxicology and Applied Pharmacology,* 164:196-205.

SILVERSTEIN, R.M. & WEBSTER, F.X. 1998. Spectrometric identification of organic compounds. 6th ed. New York:Wiley. 482 p.

SKIBSKA, B., JÓZEFOWICZ-OKONKWO, G. & GORĄCA, A. 2006. Protective effects of early administration of alpha-lipoic acid against lipopolysaccharide-induced plasma lipid peroxidation. *Pharmacological Reports,* 58:3999-404.

SMITH, C.J., HANSCH, C., MORTON, M.J. 1997. QSAR treatment of multiple toxicities: the mutagenicity and cytotoxicity of quinolines. *Mutation Research,* 379:167:175.

SOHMIYA, M., TANAKA, M., TAK, N.W., YANAGISAWA, M., TANINO, Y., SUZUKI, Y., OKAMOTO, K. & YAMAMOTO, Y. 2004. Redox status of plasma coenzyme Q10 indicates elevated systemic oxidative stress in Parkinson's disease. *Journal of the Neurological Sciences,* 223:161-166.

SOMAYAJULU, M., MCCARTHY, S., HUNG, M., SIKORSKA, M., BOROWY-BOROWSKI, H. & PANDEY, S. 2005. Role of mitochondria in neuronal cell death induced by oxidative stress; neuroprotection by coenzyme Q_{10}. *Neurobiology of Disease,* 18:618-627.

SOUTHGATE, G. & DAYA, S. 1999. Melatonin reduces quinolinic acid-induced lipid peroxidation in rat brain homogenate. *Metabolic Brain Disease*, 14(3):165-171.

STERN, E., MILLET, R., DEPREUX, P. & HÉNICHART, J-P. 2004. A versatile and efficient synthesis of 3-aroyl-1,4-dihydroquinolin-4-ones. *Tetrahedron Letters,* 45:9257-9259.

TAHARA, S., MATSUO, M. & KANEKO, T. 2001. Age-related changes in oxidative damage to lipids and DNA in rat skin. *Mechanisms of Ageing and Development,* 122:415-426.

TURSKI, L., BRESSLER, K., RETTIG, K.J., LOSCHMAN, P.A. & WACHTEL, H. 1991. Protection of substantia nigra from MPP^+ by N-methyl-D-aspartate antagonists. *Nature*, 349(6308):414-418.

UTTI, R.J., RAJPUT, A.H., ASHENHURST, E.M. & ROZDILSKY, B. 1985. Cyanide-induced parkinsonism: a clinicopathologic report. *Neurology,* 35:921-925

VAN DYK, S. 2001. Synthesis and biological activity of selected 4-hydroxyquinolines. Potchefstroom: PU for CHE. (Thesis – Ph.D.). p. 133.

VIOLA, G., FACCIOLO, L., DALL'ACQUA, S., DI LISA, F., CANTON, M., VEDALDI, D., FRAVOLINI, A., TABARRINI, O. & CECCHETTI, V. 2004. 6-Aminoquinolones: photostability, cellular distribution and photoxicity. *Toxicology in Vitro,* 18(5):581-592.

VIRGILI, F., KOBUCHI, H. & PACKER, L. 1998. Procyanidins extracted form pinus *Maritima* (Pygnogenoc®): scavengers of free radical species and modulators of nitrogen monoxide metabolism in activated murine. *Free Radical Biology and Medicine*, 24(7-8):1120-1129.

WAN, X. S., ZHOU, Z., WARE, J. H., KENNEDY, A.R. 2005. Standardization of a fluorometric assay for measuring oxidative stress in irradiated cells. *Radiation Research,* 163:232-240.

WANG, H.D., PAGANO, P.J., DU, Y., CAYATTE, A.J., QUINN, M.T., BRECHER, P. & COHEN, R. 1998. Superoxide anion from the adventitia of the rat thoracic aorta inactivates nitric oxide. *Circulation Research,* 82:810-818.

WAYPA, G.B. & SCHUMACKER, P.T. 2002. O_2 sensing in hypoxic pulmonary vasoconstriction: the mitochondrial door re-opens. *Respiratory Physiology and Neurobiology,* 132(1):81-91.

WERSINGER, C., CHEN, J. & SIDHU, A. 2004. Bimodel induction of dopamine-mediated striatal neurotoxicity is mediated through both activation of D1 dopamine receptors and autoxidation. *Molecular and Cellular Neuroscience*, 25(1):124-137.

WILBUR, K.M., BERNHEIM, F. & SHAPIRO, O.N. 1949. The thiobarbituric acid reagent as a test for the oxidation of unsaturated fatty acids by various agents. *Archives of Biochemistry*, 24:305-313.

YOUDIM, M. B. H., BEN-SCHACHAR, D. & RIEDERER, P. 1989. Is Parkinson's disease a progressive siderosis of substantia nigra resulting in iron and melanin induced neurodegeneration? *Acta Neurologica Scandinavica,* 126:47-54.

YOUNG, I.S. & WOODSIDE, J.V. 2001. Antioxidants in health and disease. *Journal of Clinical Pathology*, 54:176-186.

ZAIDI, S.M.K.R. & BANU, N. 2004. Antioxidant potential of vitamins A, E and C in modulating oxidative stress in rat brain. *Clinica Chemica Acta*, 340:229-233.

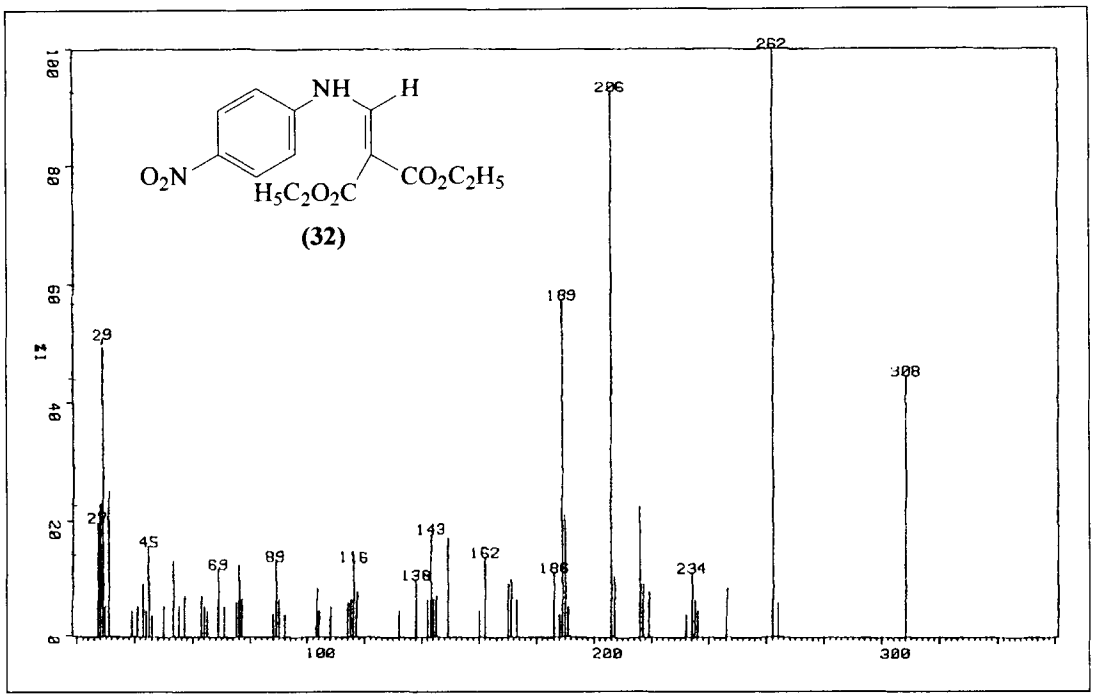
ZAR, J.H. 1974. Biostatistical Analysis, 1st ed. Prentice Hall, Englewood Cliffs, N.J., pp. 151-466.

ZHENG, H., WEINER, L. M. BAR-AM, O., EPSZTEJN, S., CABANTCHIK, Z. I., WARSHAWSKY, A., YOUDIM, M. B. H. & FRIDKIN, M. 2005. Design, synthesis, and evaluation of novel bifunctional iron-chelators as potential agents for neuroprotection in Alzheimer's, Parkinson's, and other neurodegenerative diseases. *Bioorganic and Medicinal Chemistry,* 13:773-783.

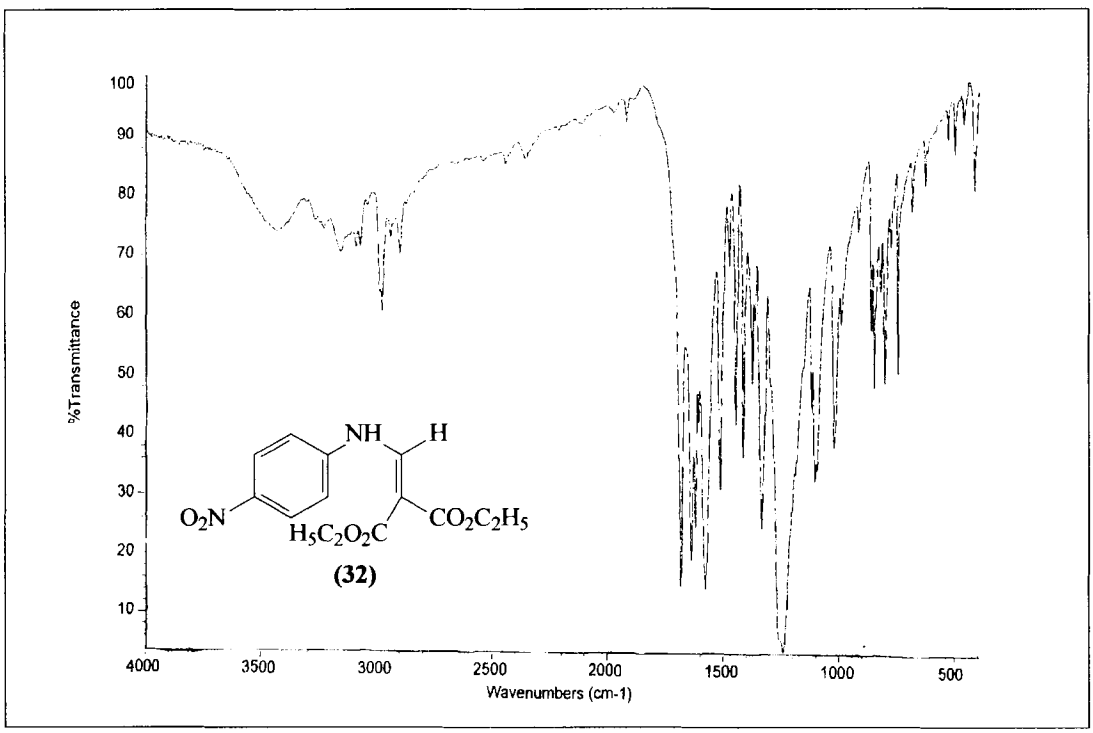
ZWAANS, R. & THOMSON, C. 1996. Ab initio calculations on 2-, 3- and 4- substituted quinolines in relation with their activity as protein tyrosine kinase inhibitors. *Journal of Molecular Structure (Theochem)*, 362:51-68.

APPENDIX A

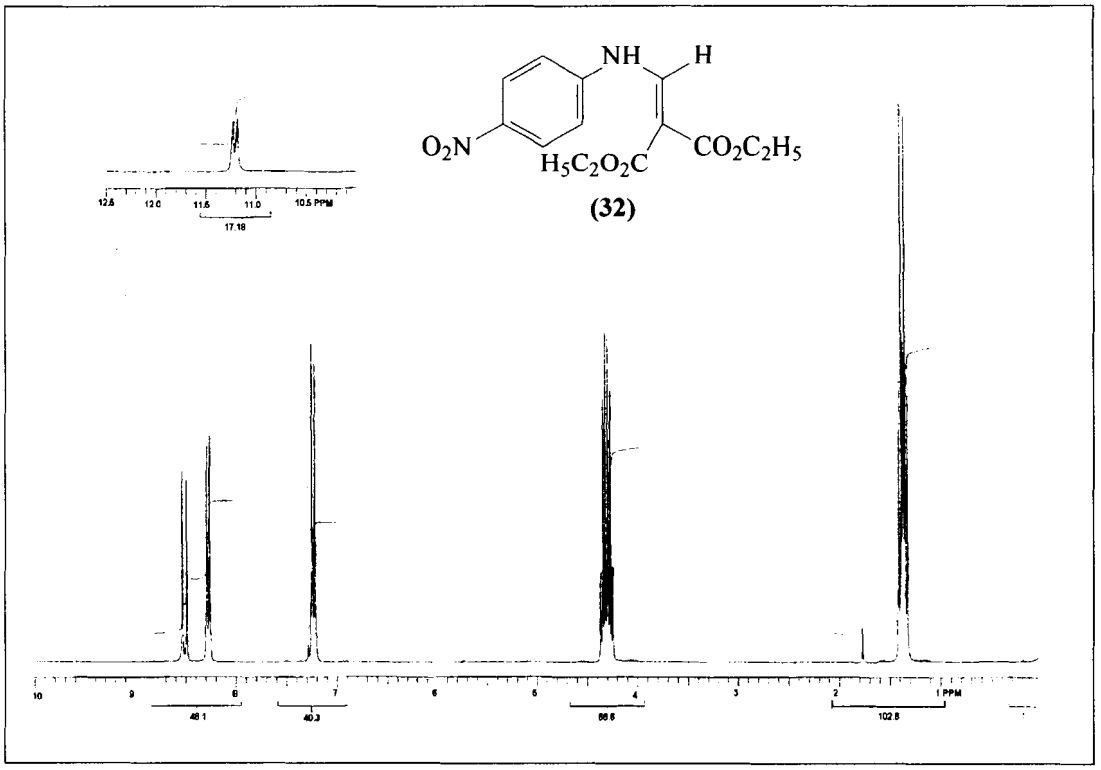
Spectrum 1



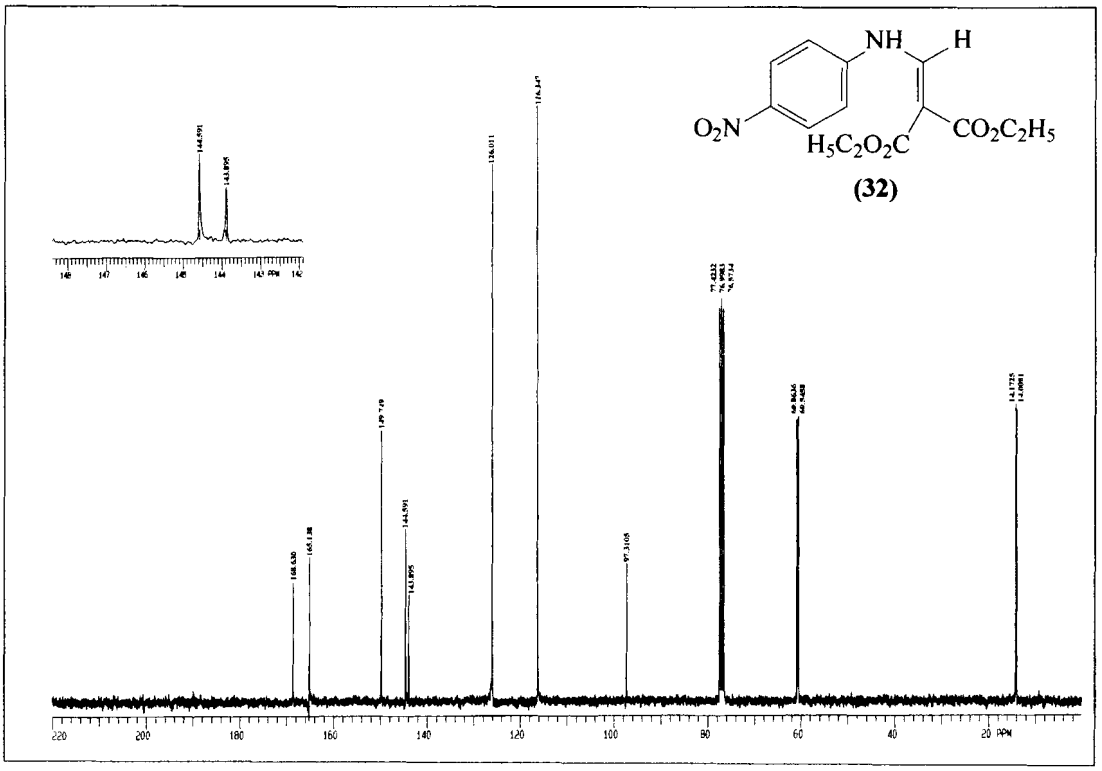
Spectrum 2



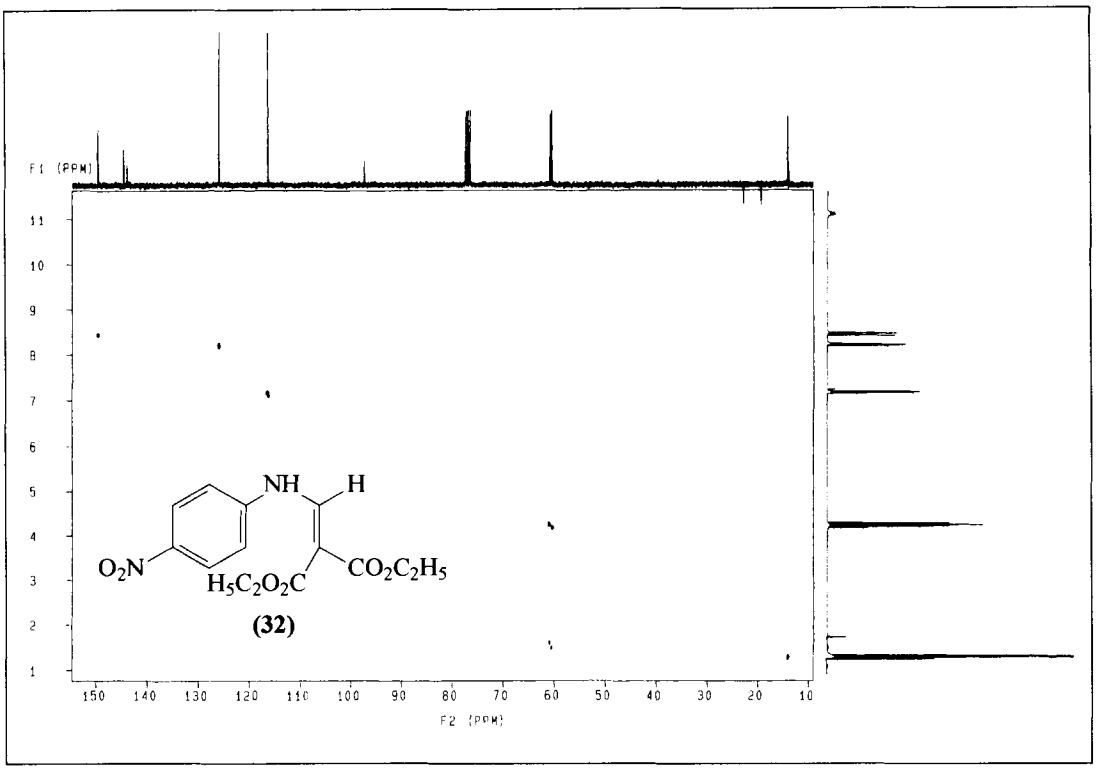
Spectrum 3



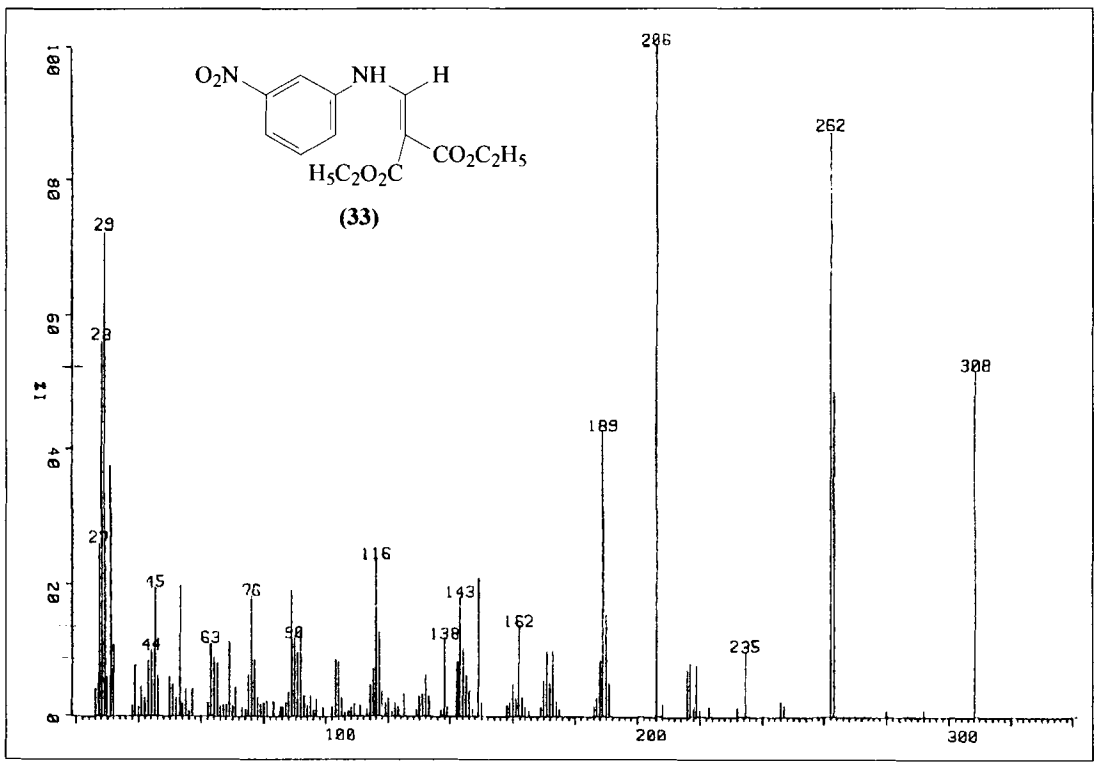
Spectrum 4



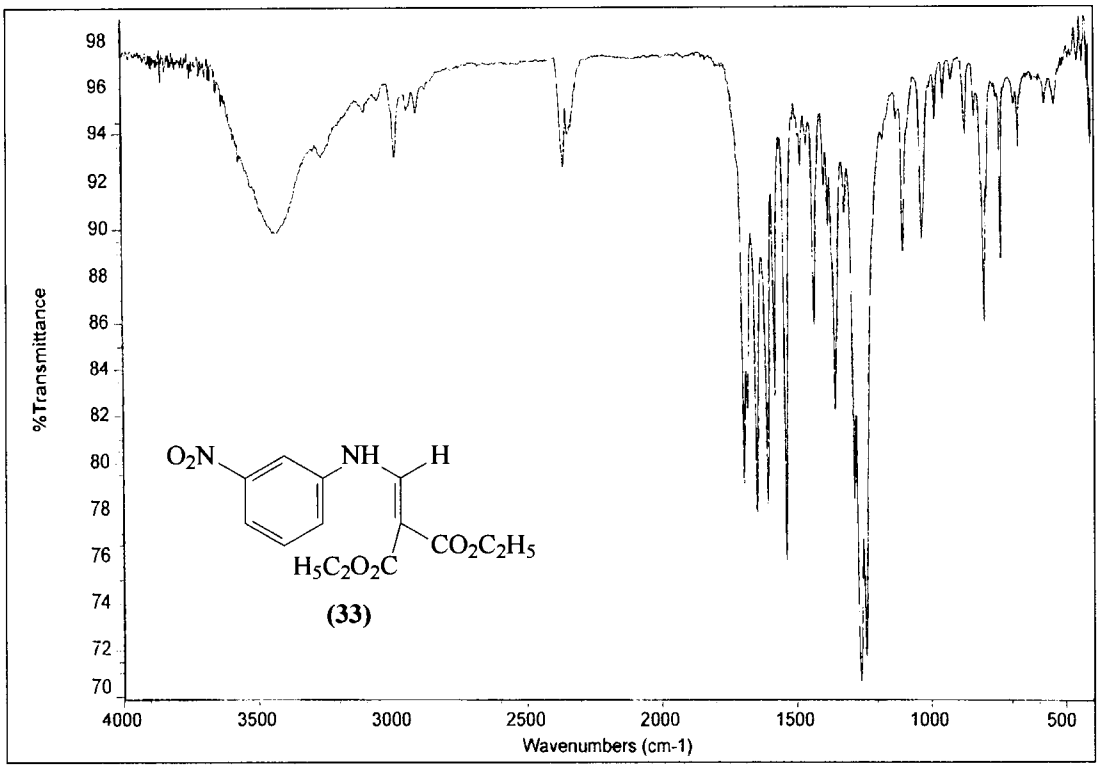
Spectrum 5



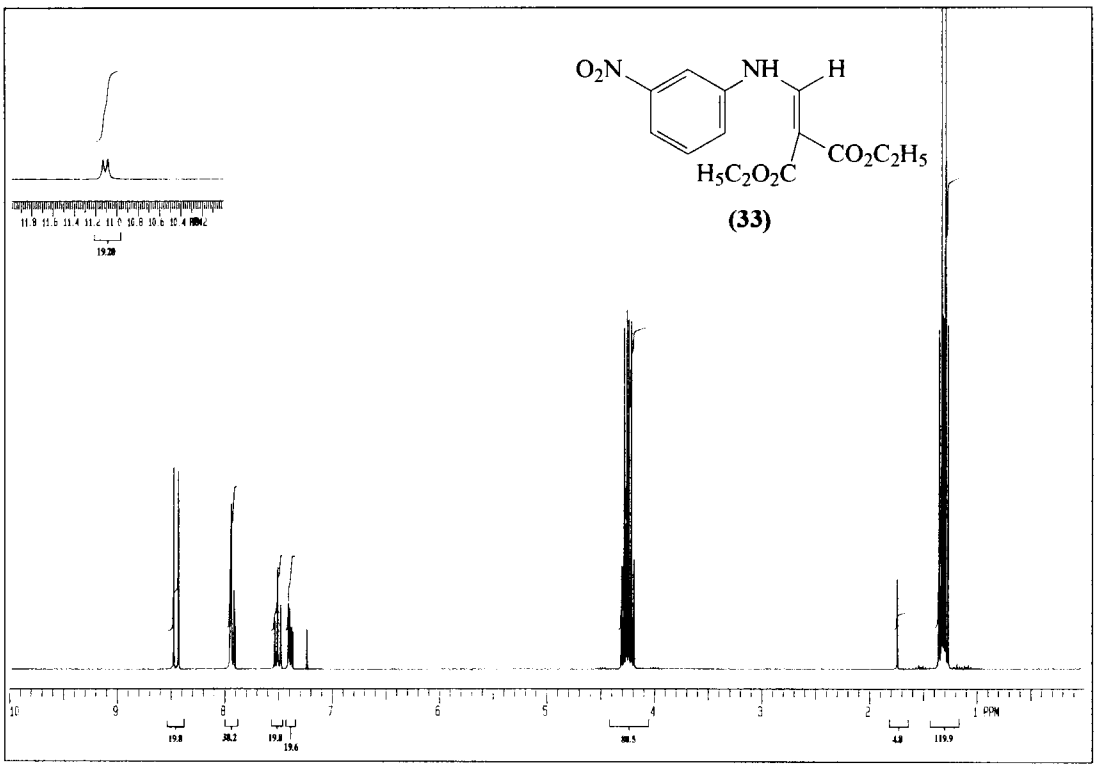
Spectrum 6



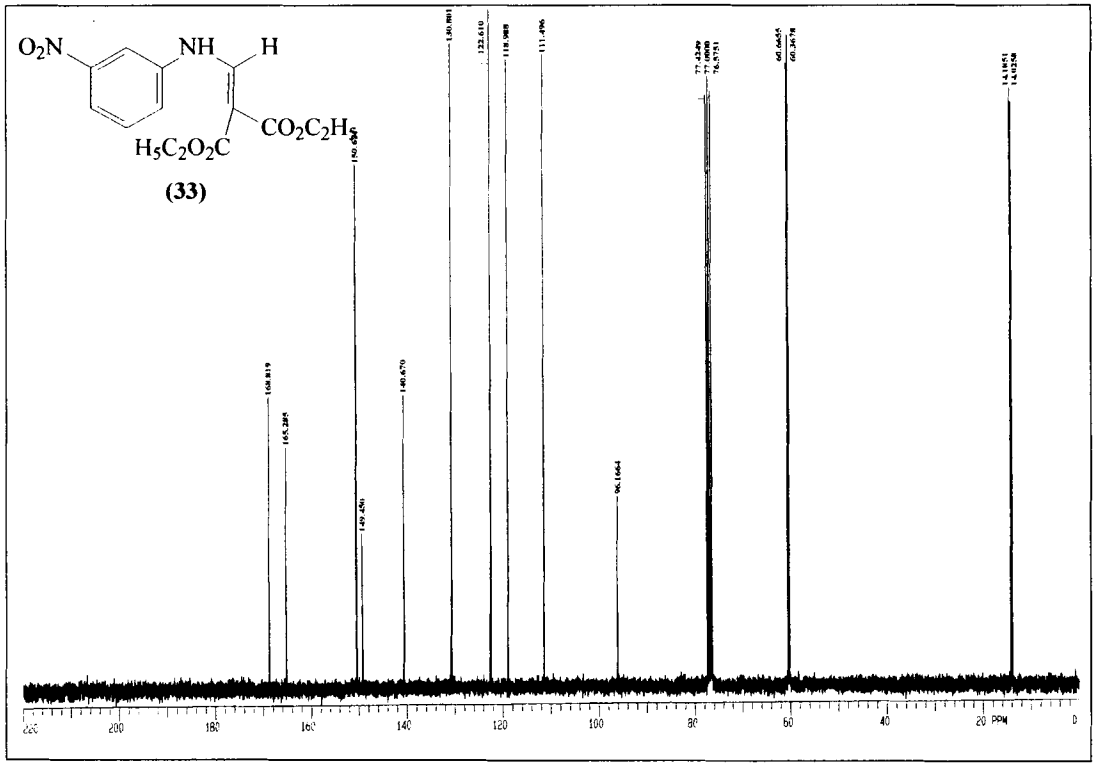
Spectrum 7



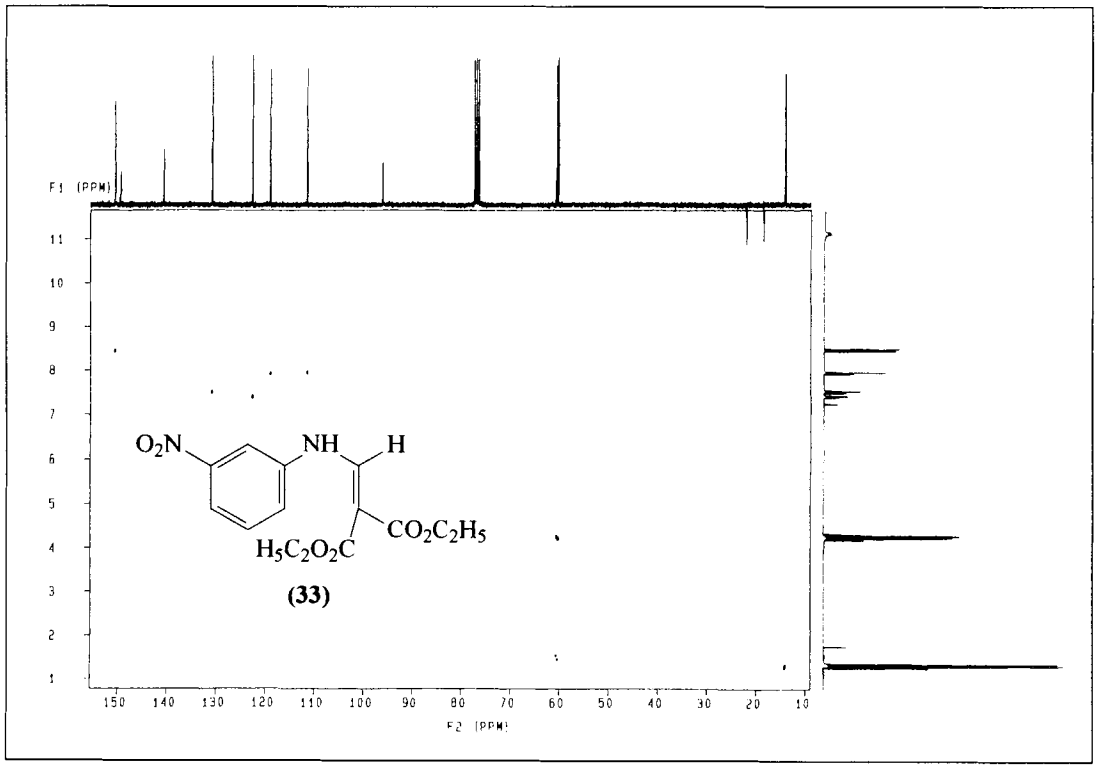
Spectrum 8



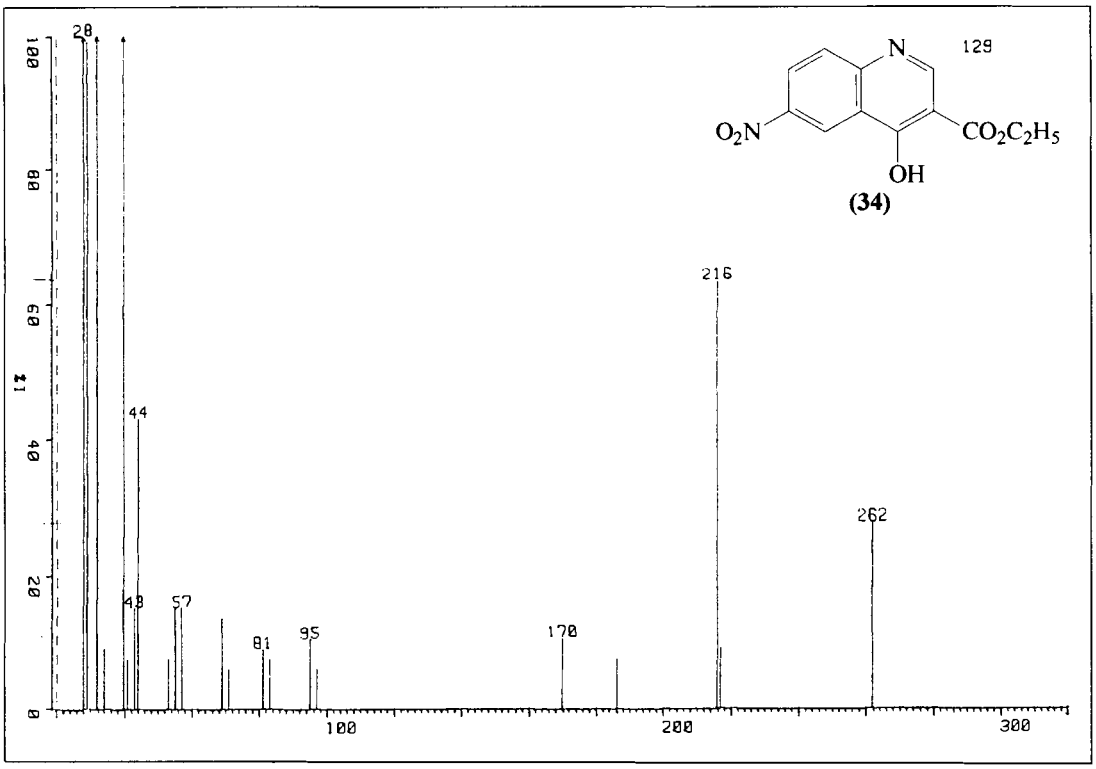
Spectrum 9



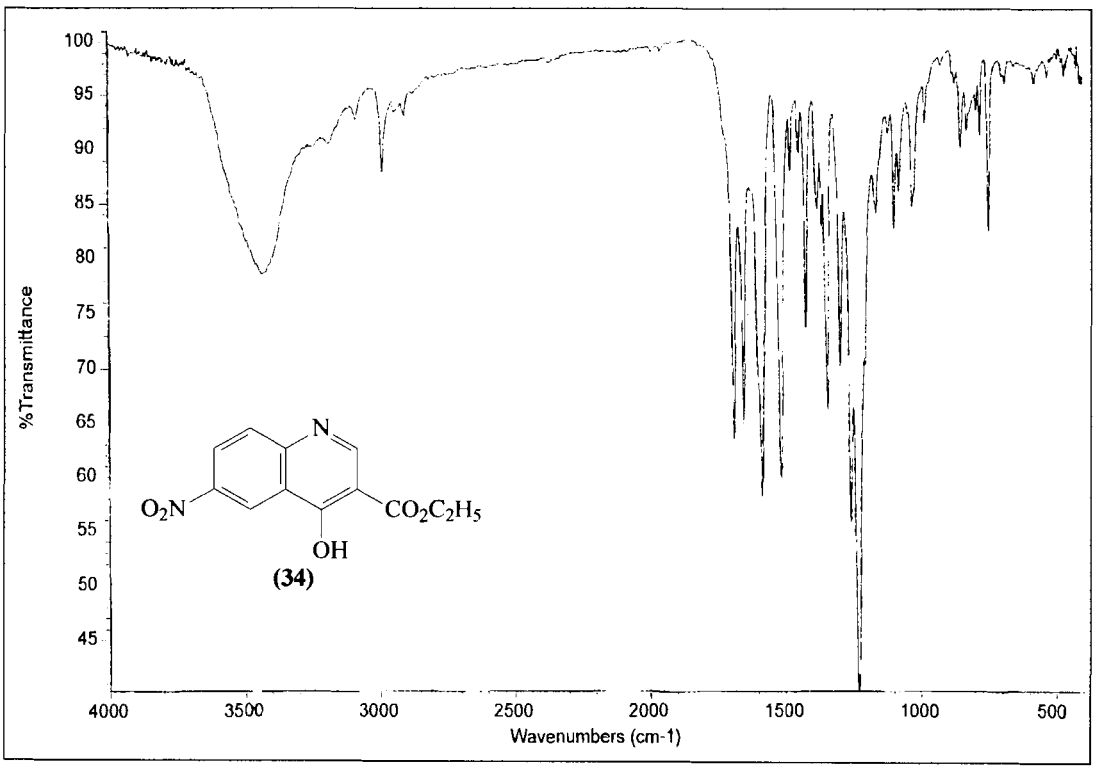
Spectrum 10



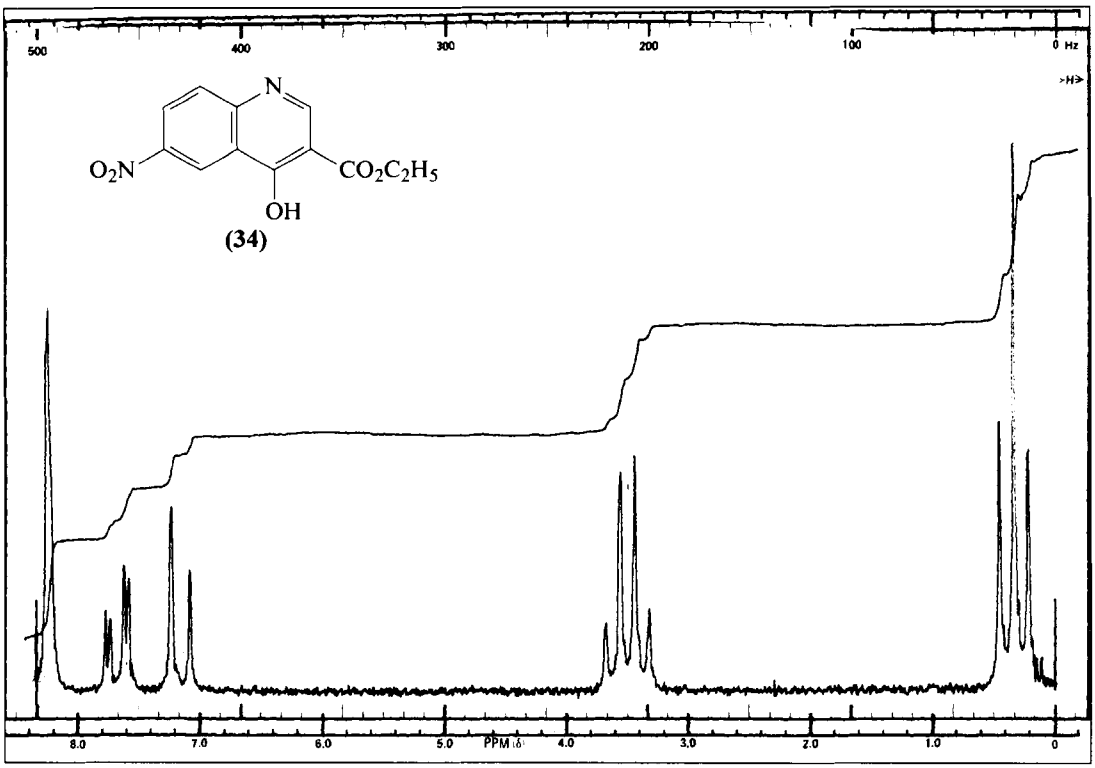
Spectrum 11



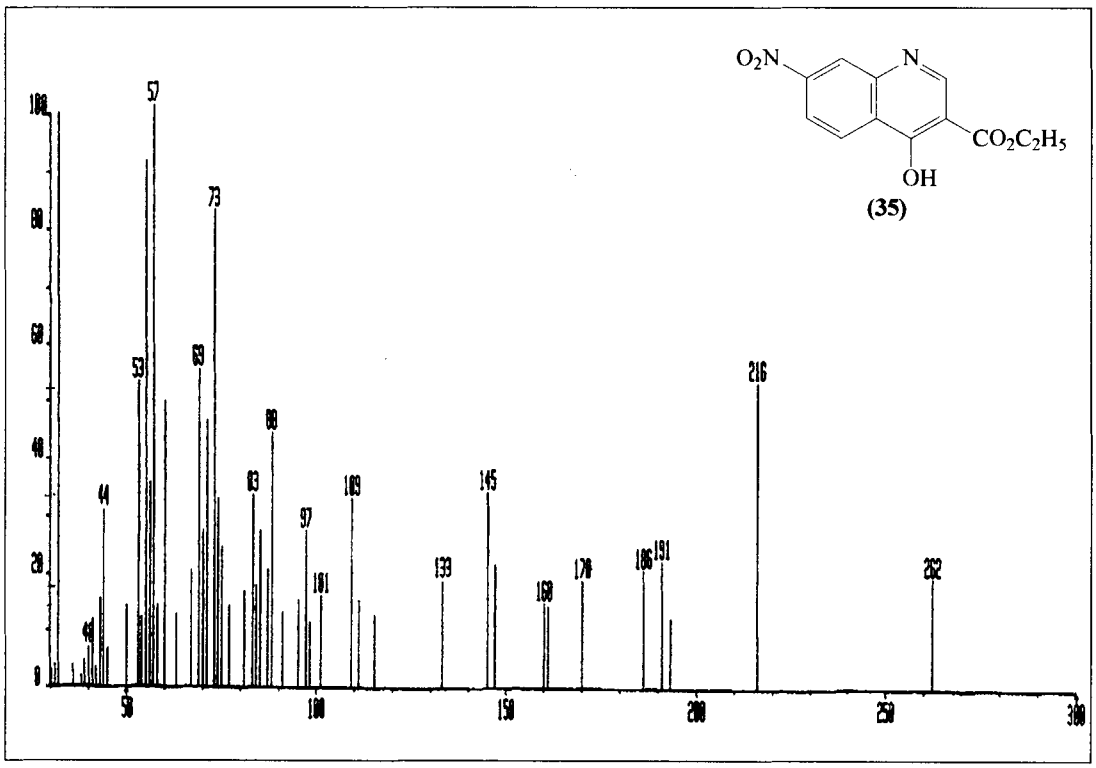
Spectrum 12



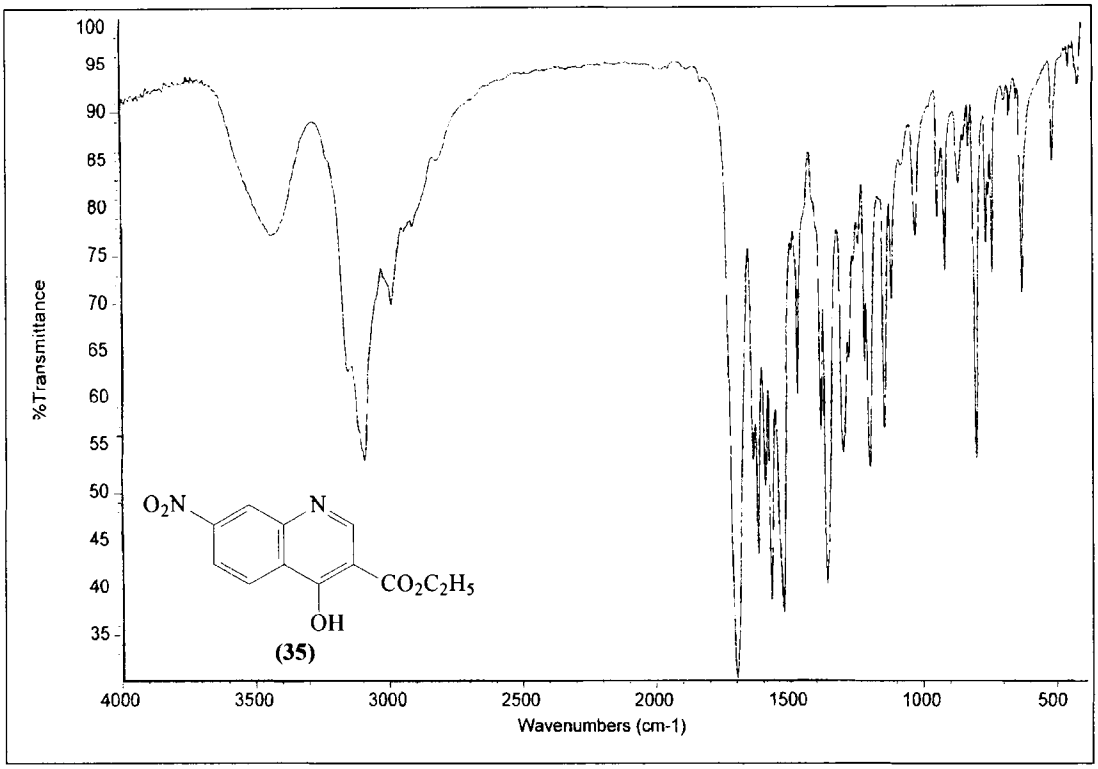
Spectrum 13



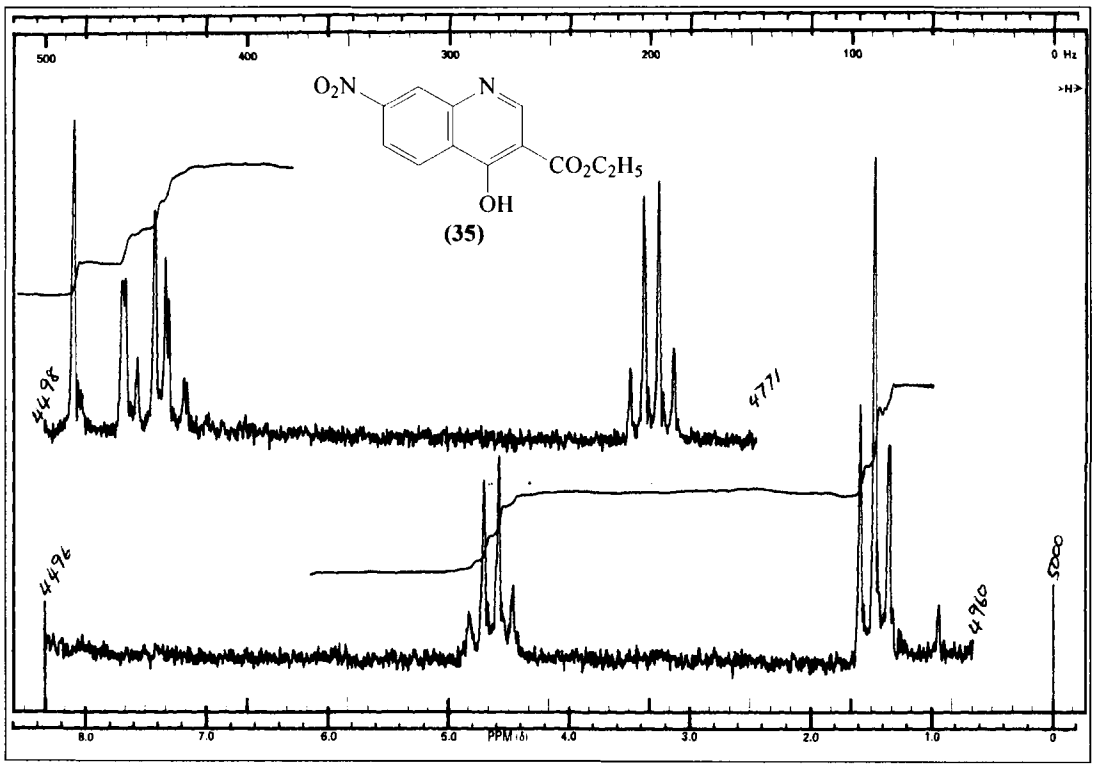
Spectrum 14



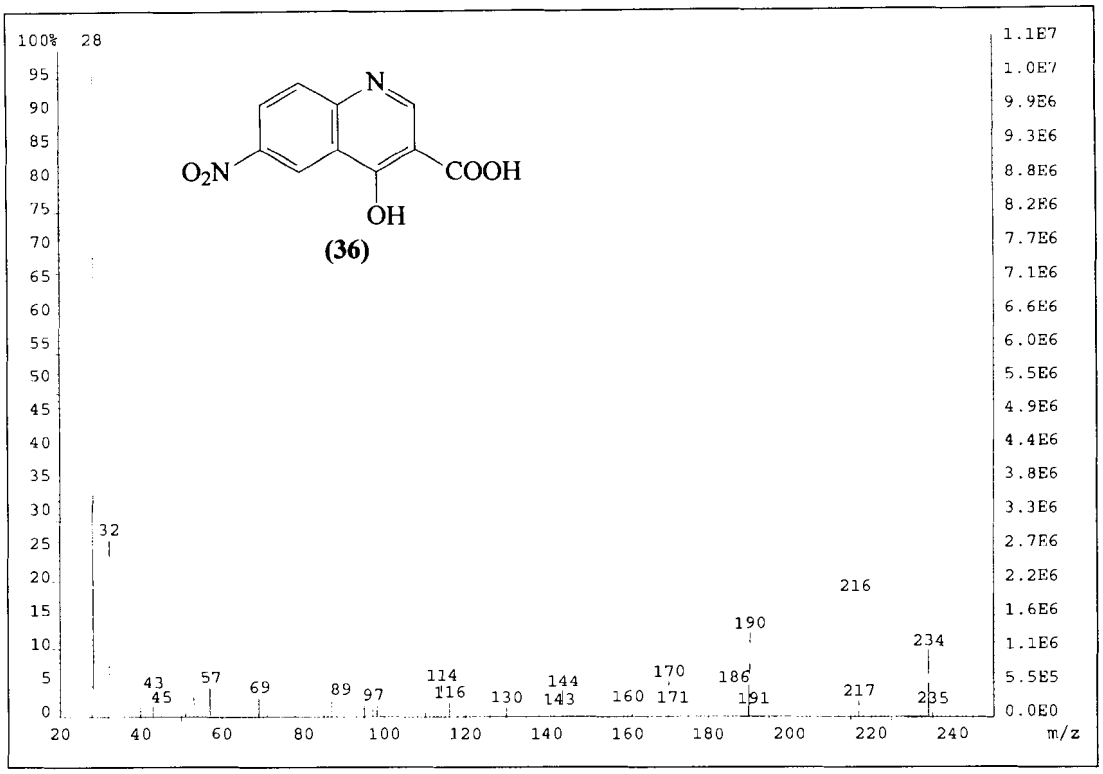
Spectrum 15



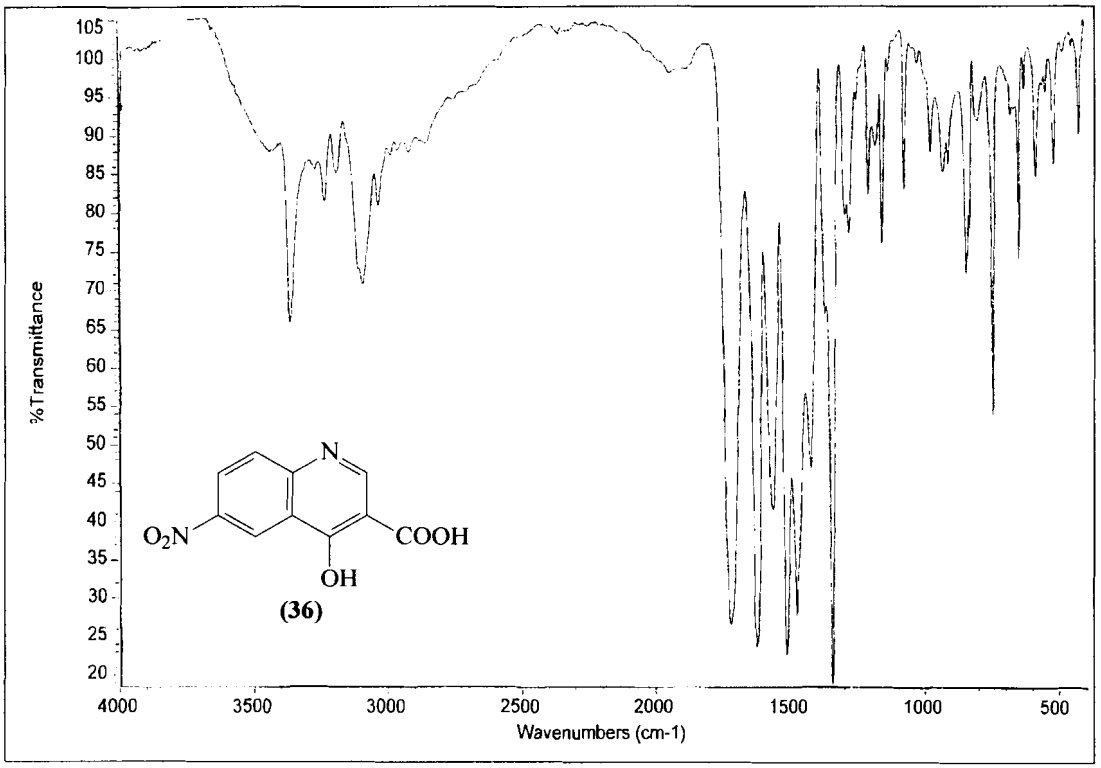
Spectrum 16



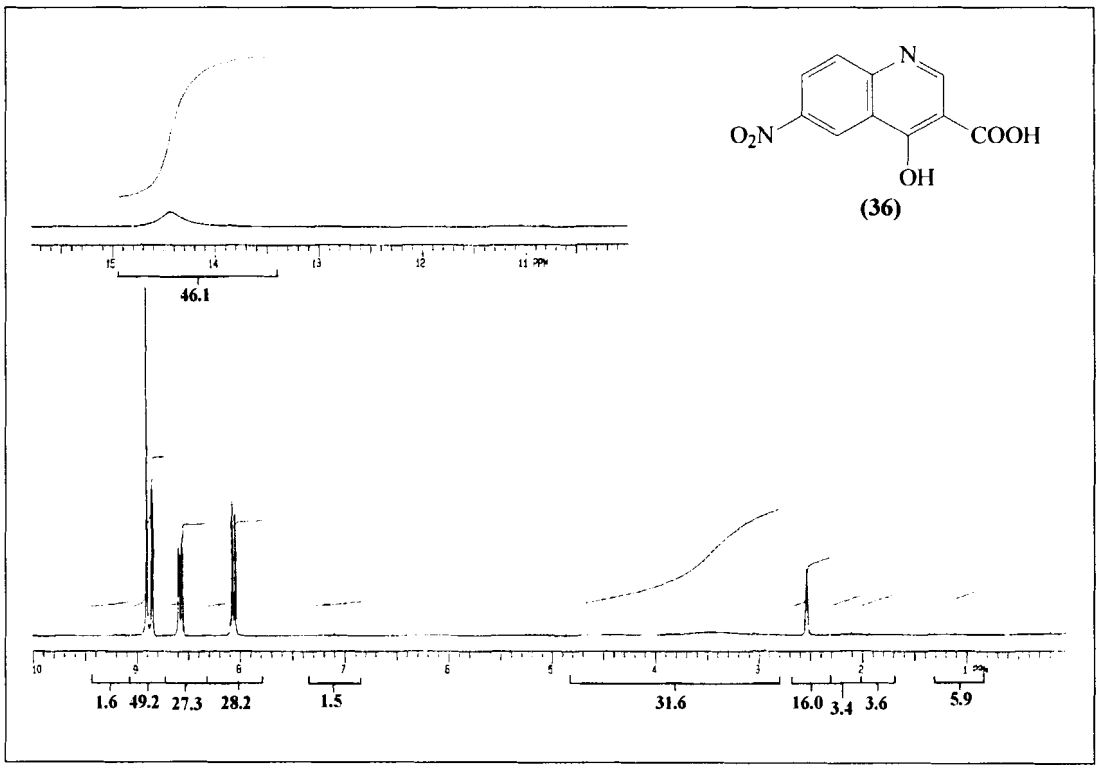
Spectrum 17



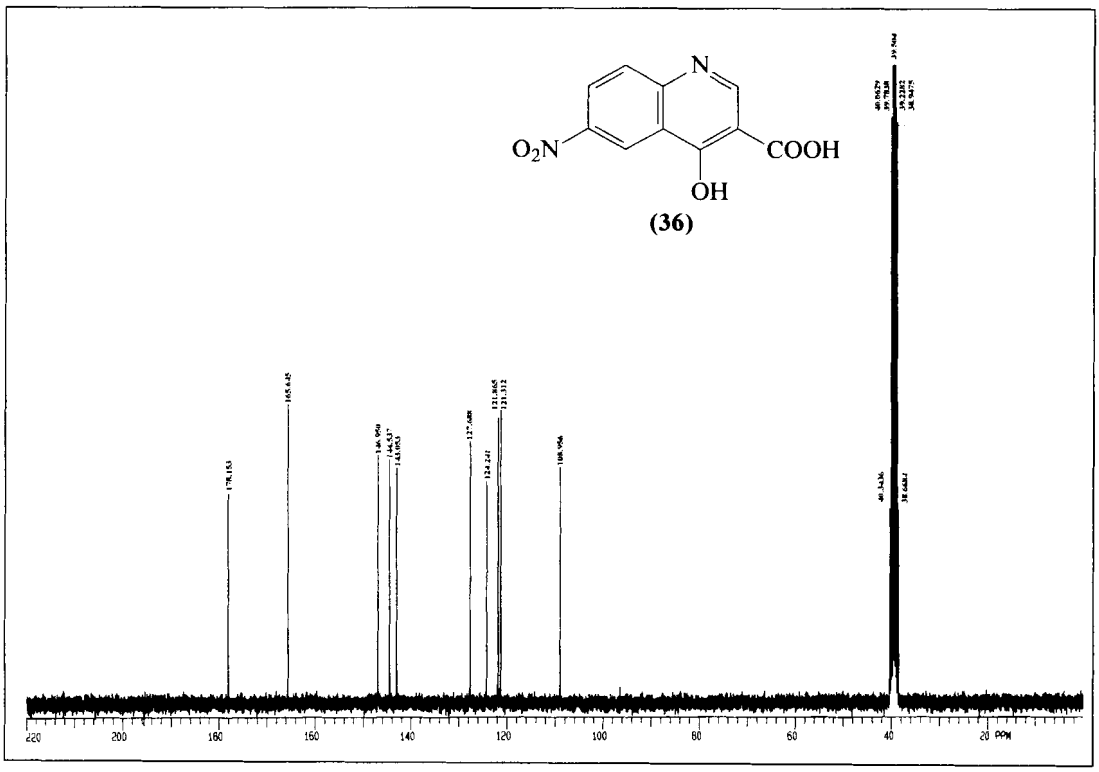
Spectrum 18



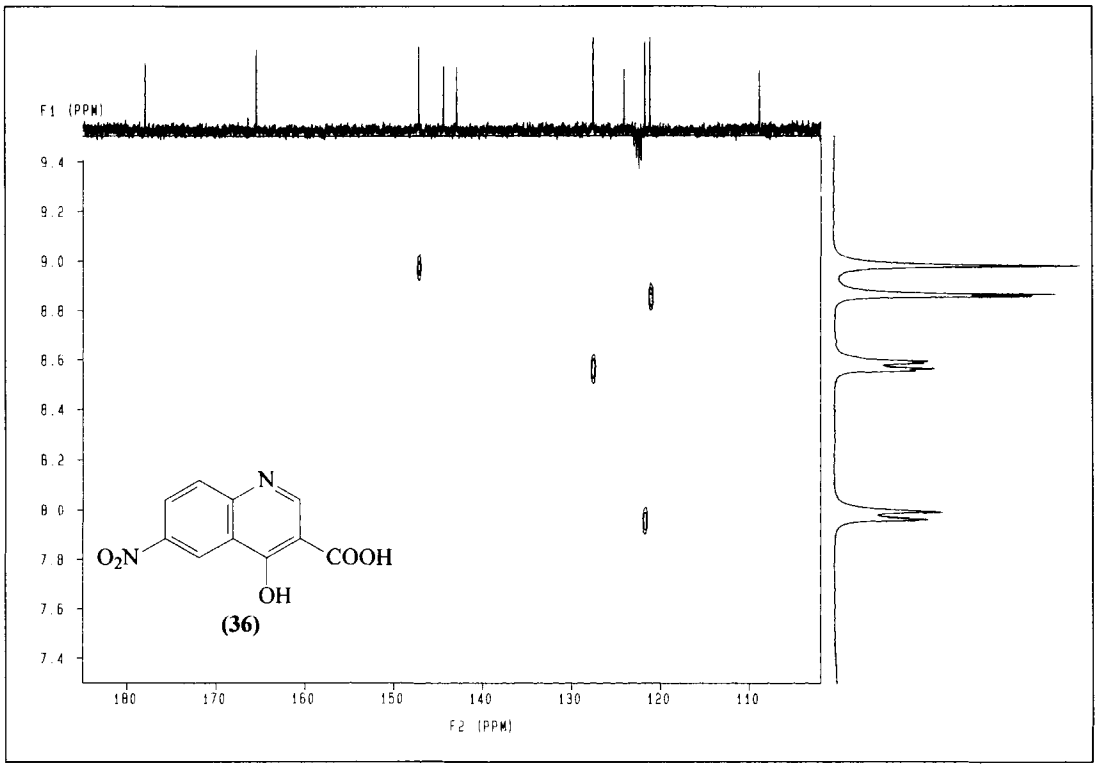
Spectrum 19



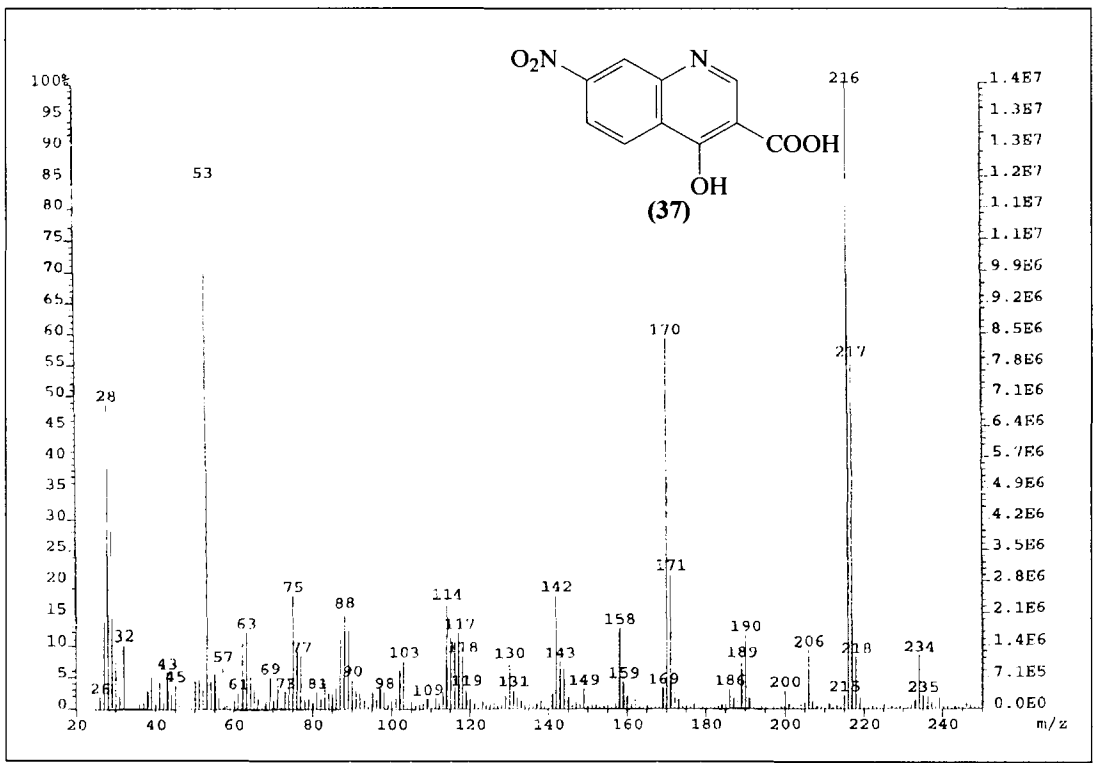
Spectrum 20



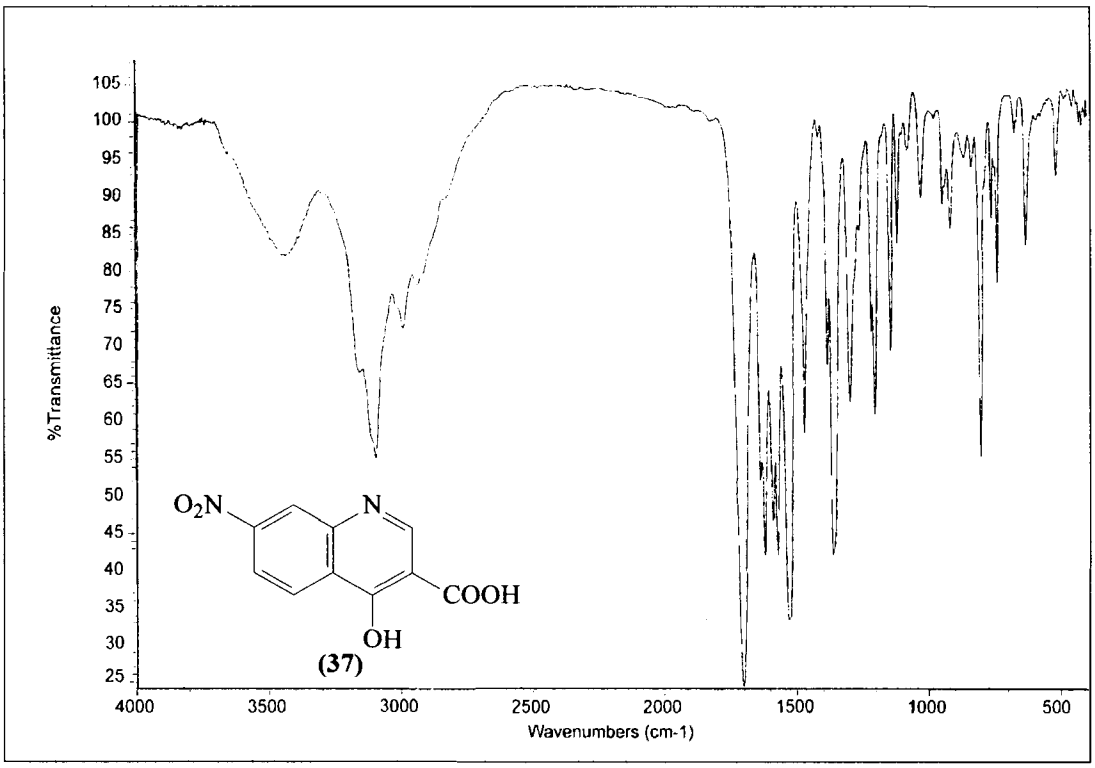
Spectrum 21



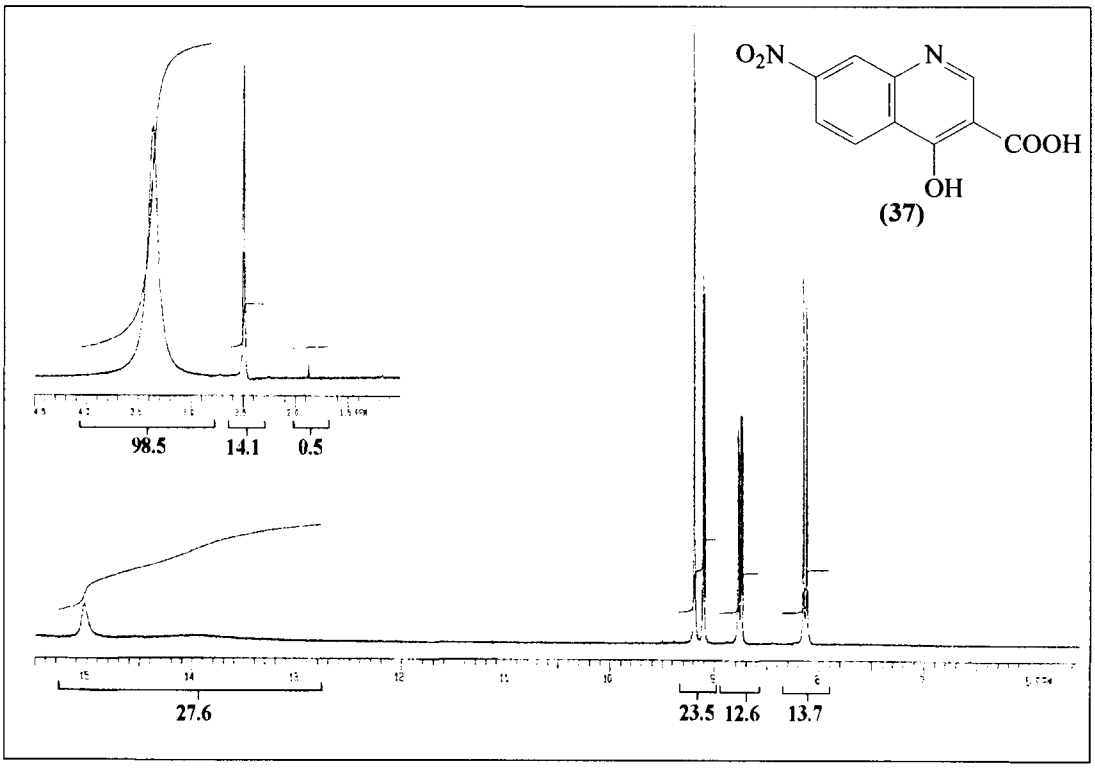
Spectrum 22



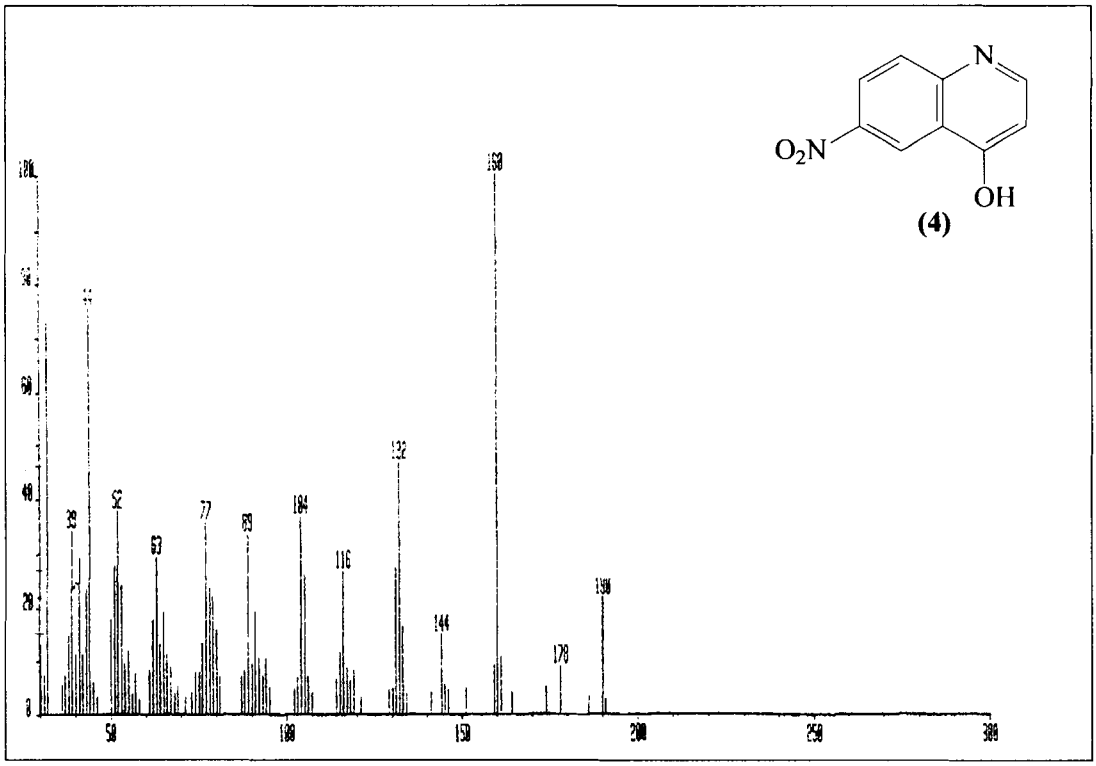
Spectrum 23



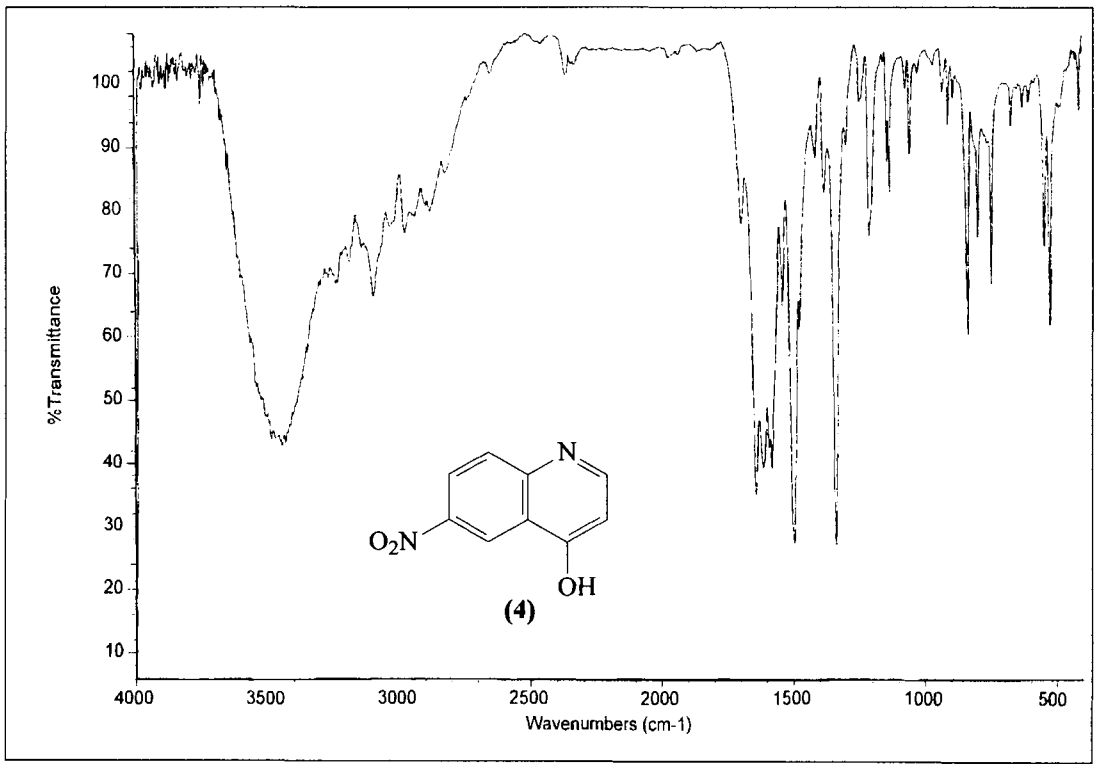
Spectrum 24



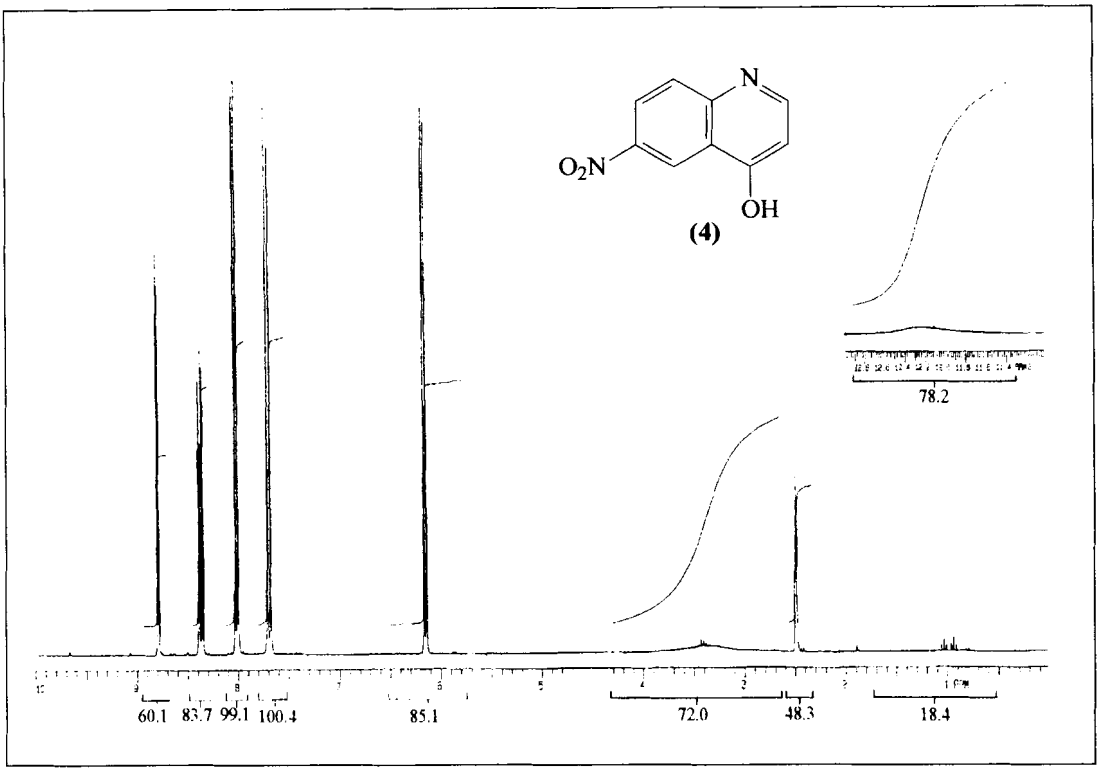
Spectrum 25



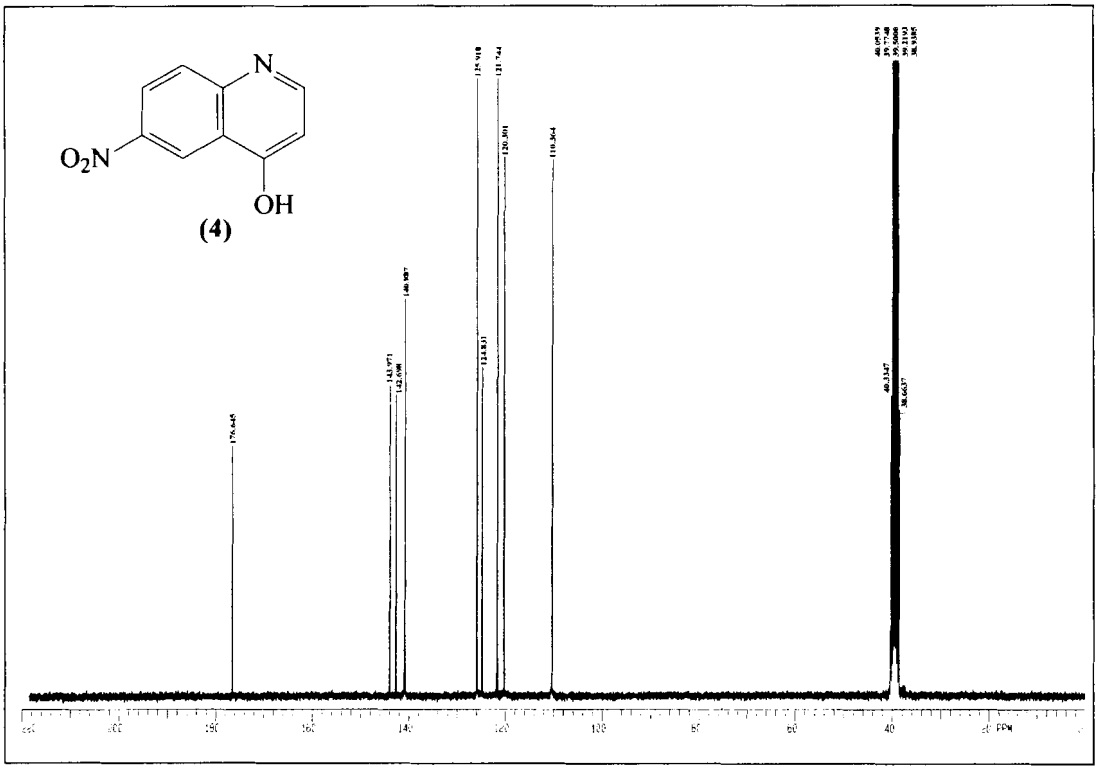
Spectrum 26



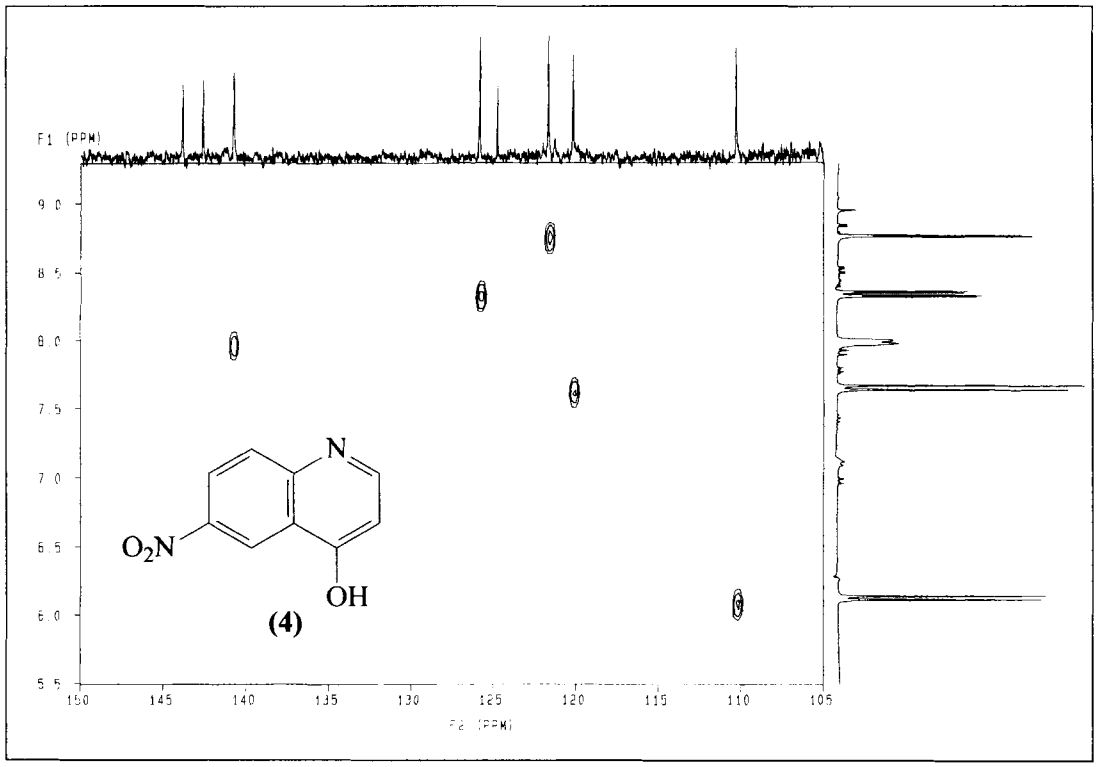
Spectrum 27



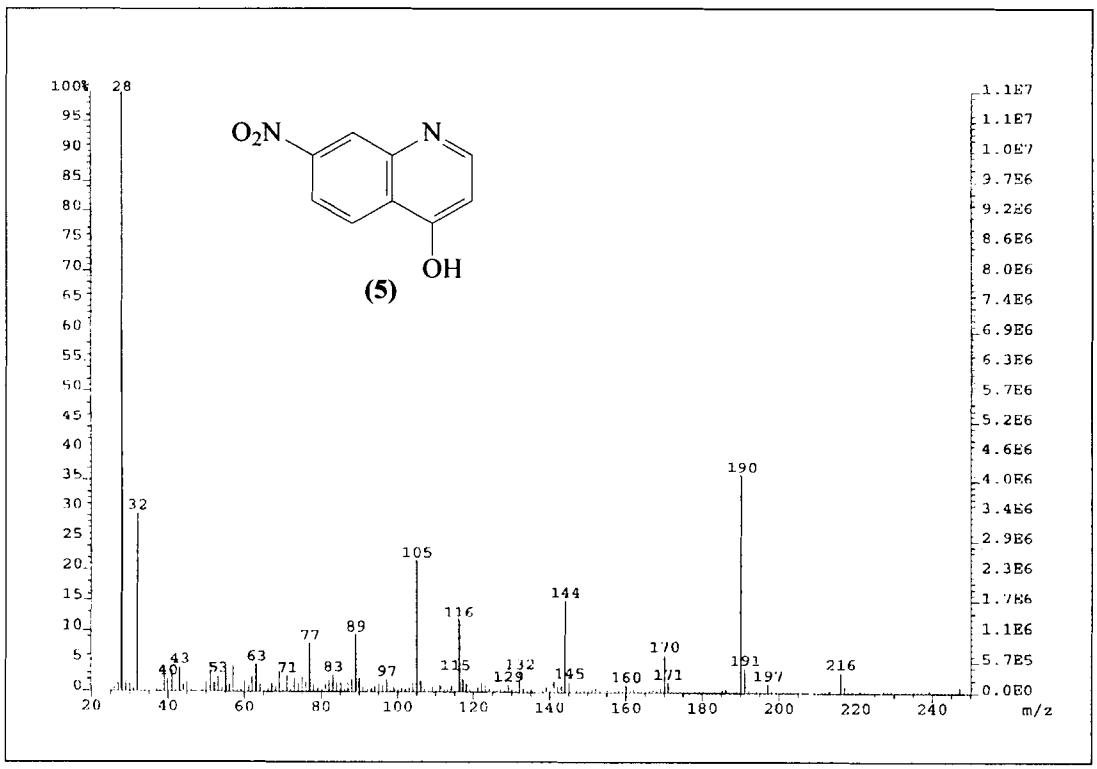
Spectrum 28



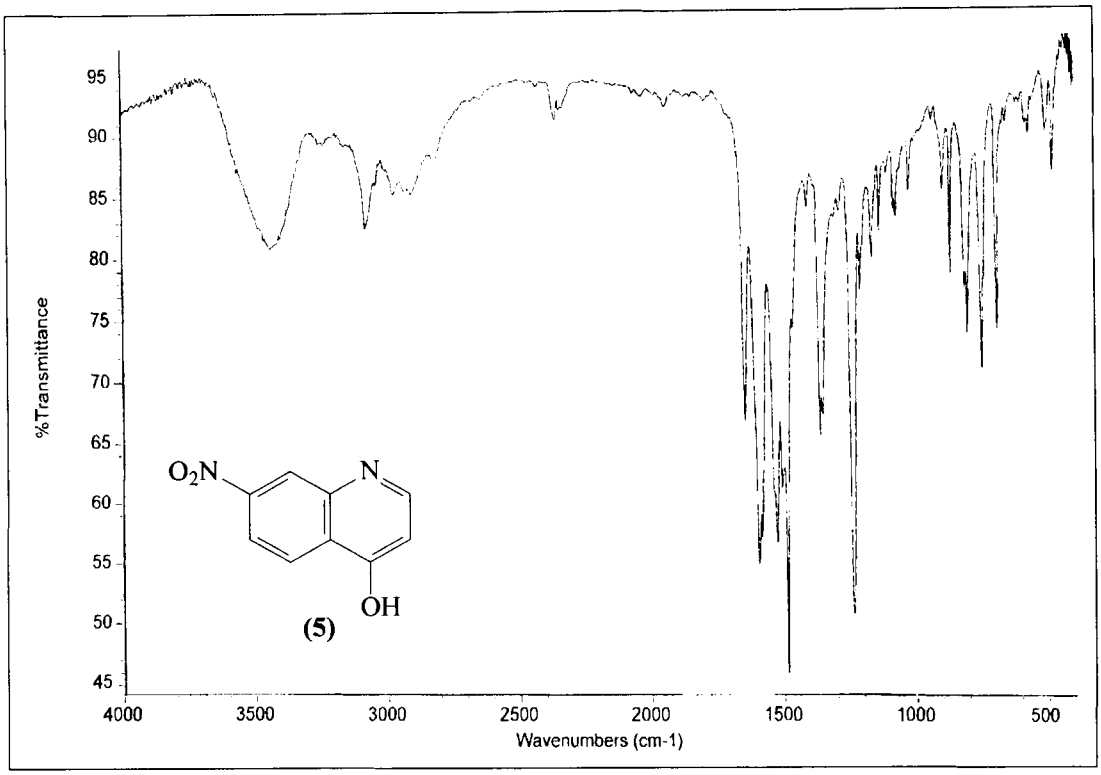
Spectrum 29



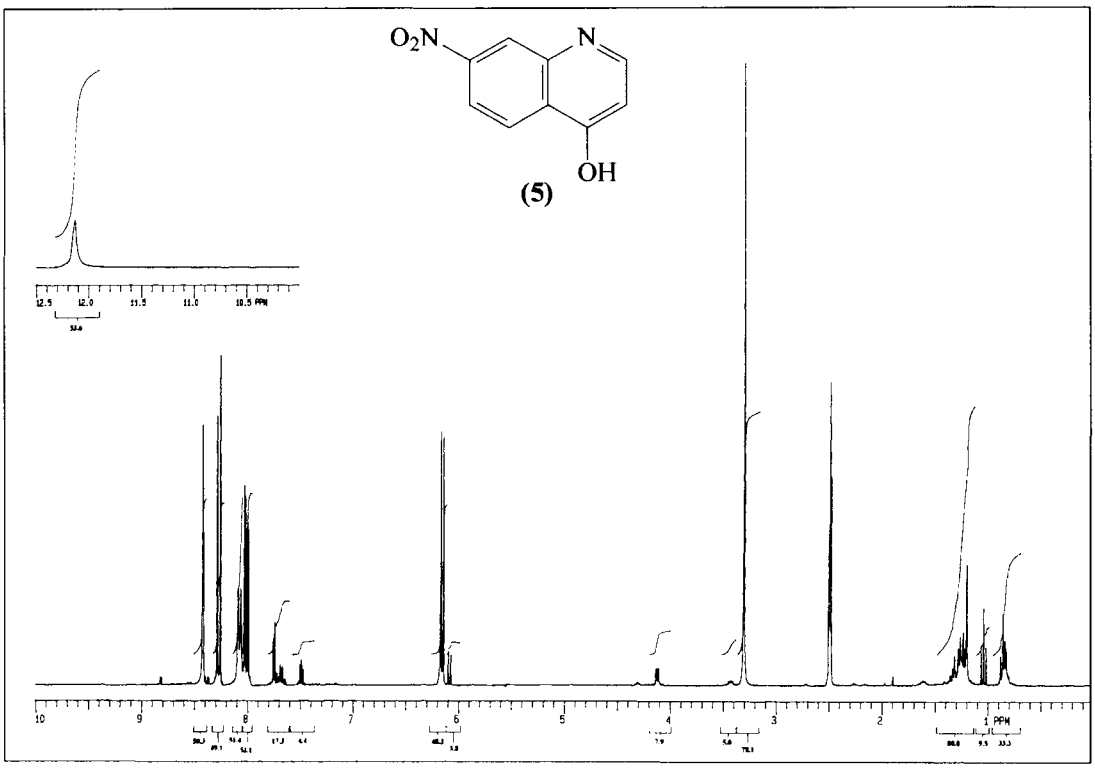
Spectrum 30



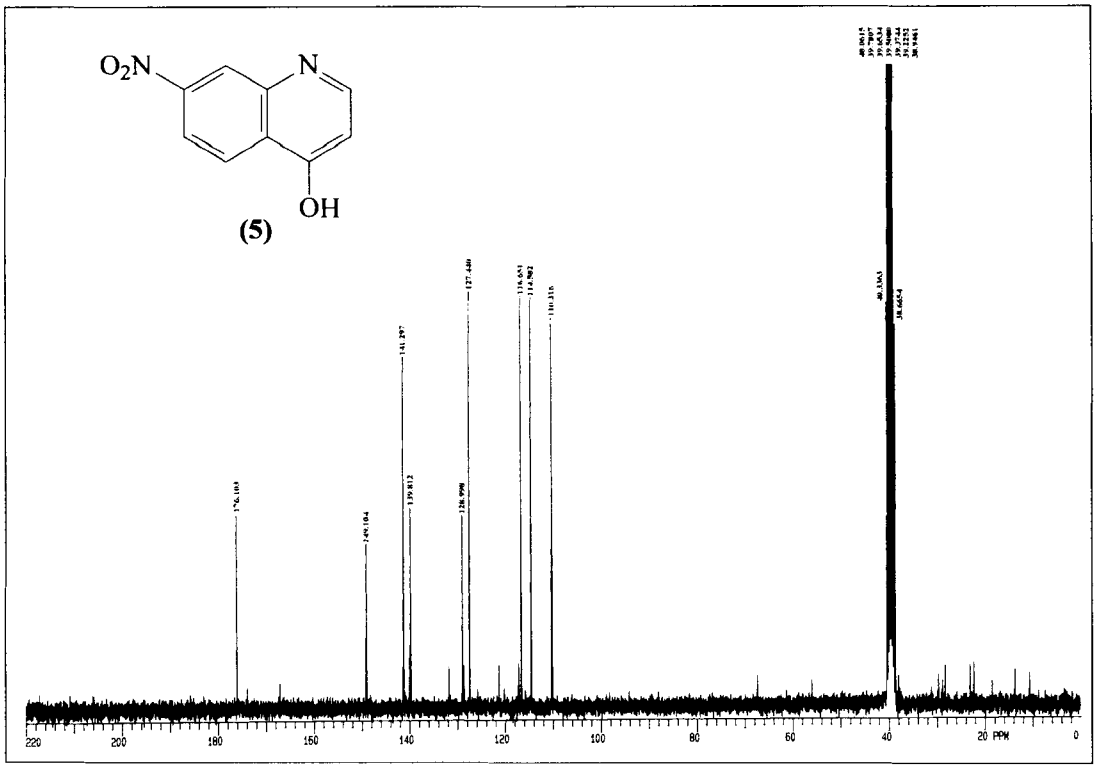
Spectrum 31



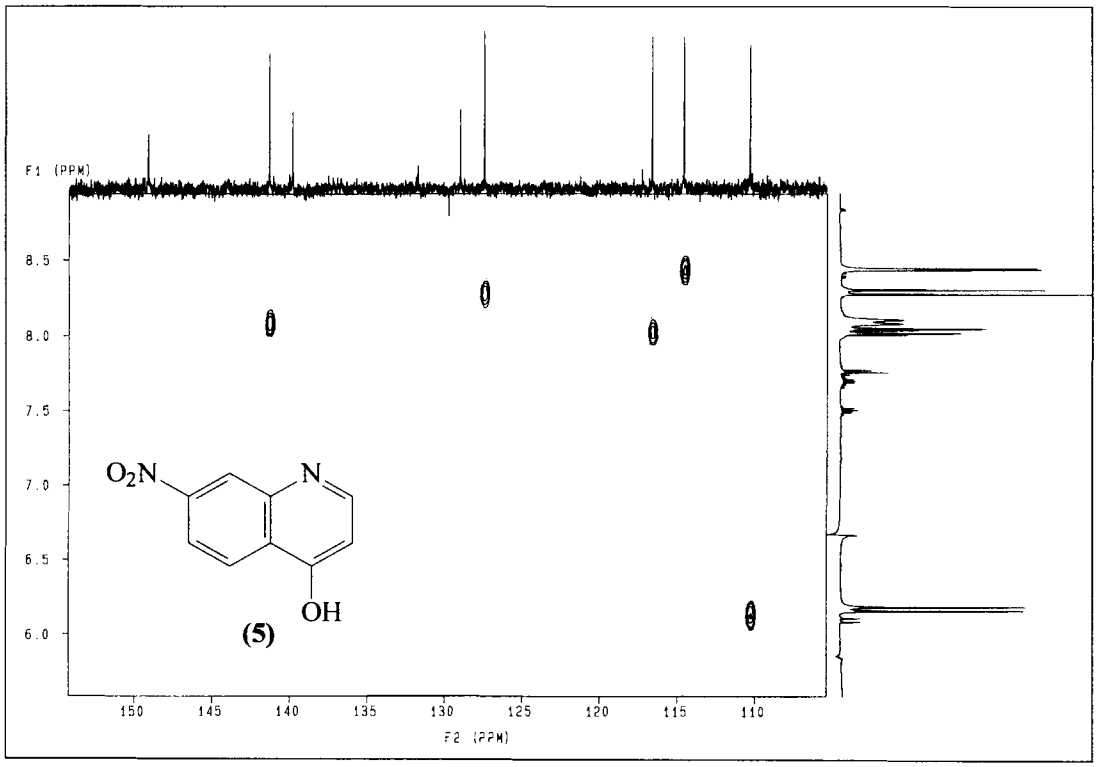
Spectrum 32



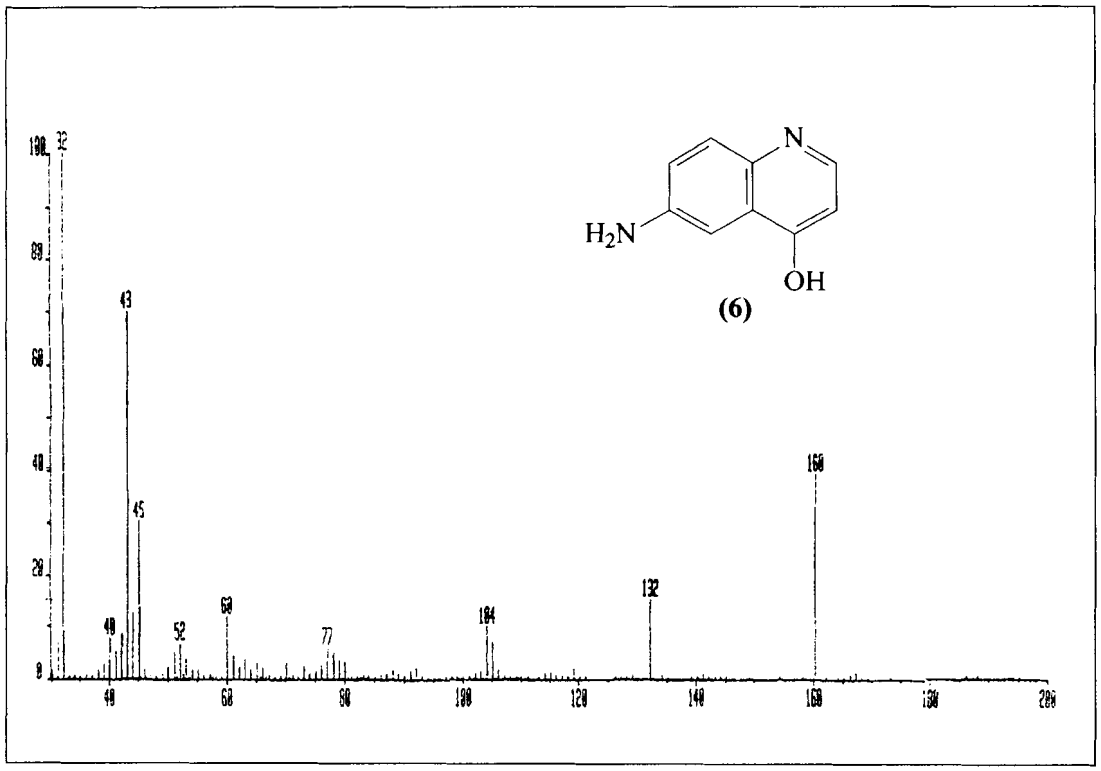
Spectrum 33



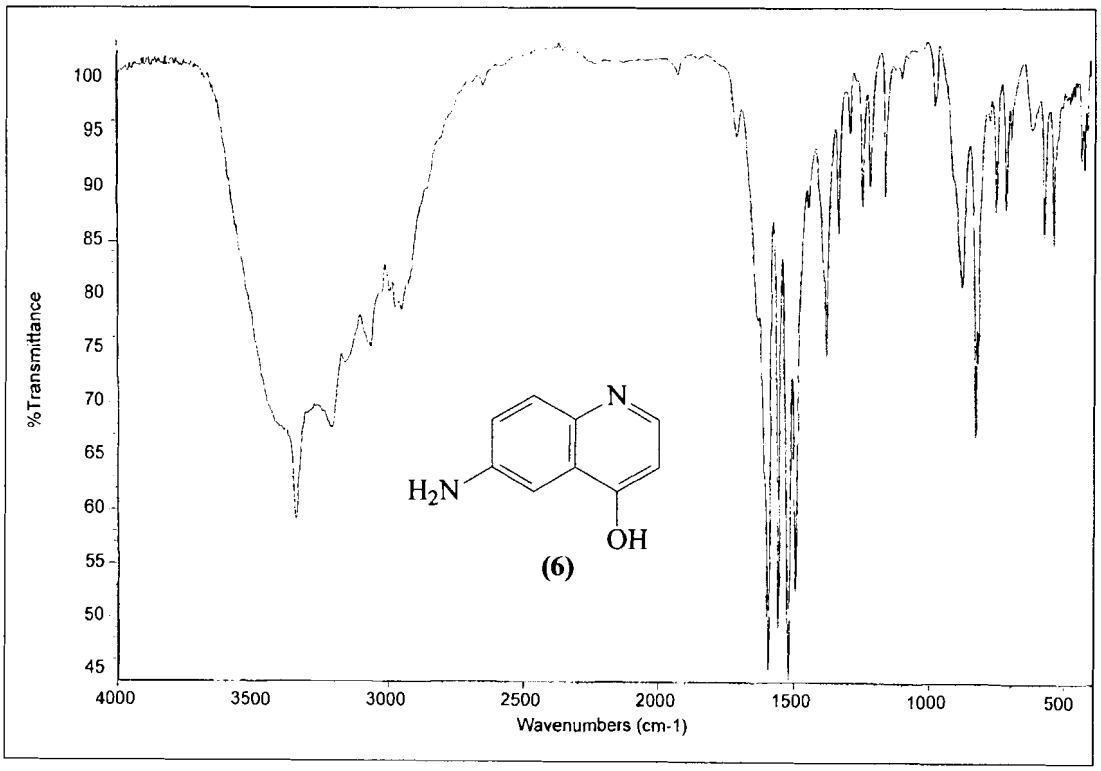
Spectrum 34



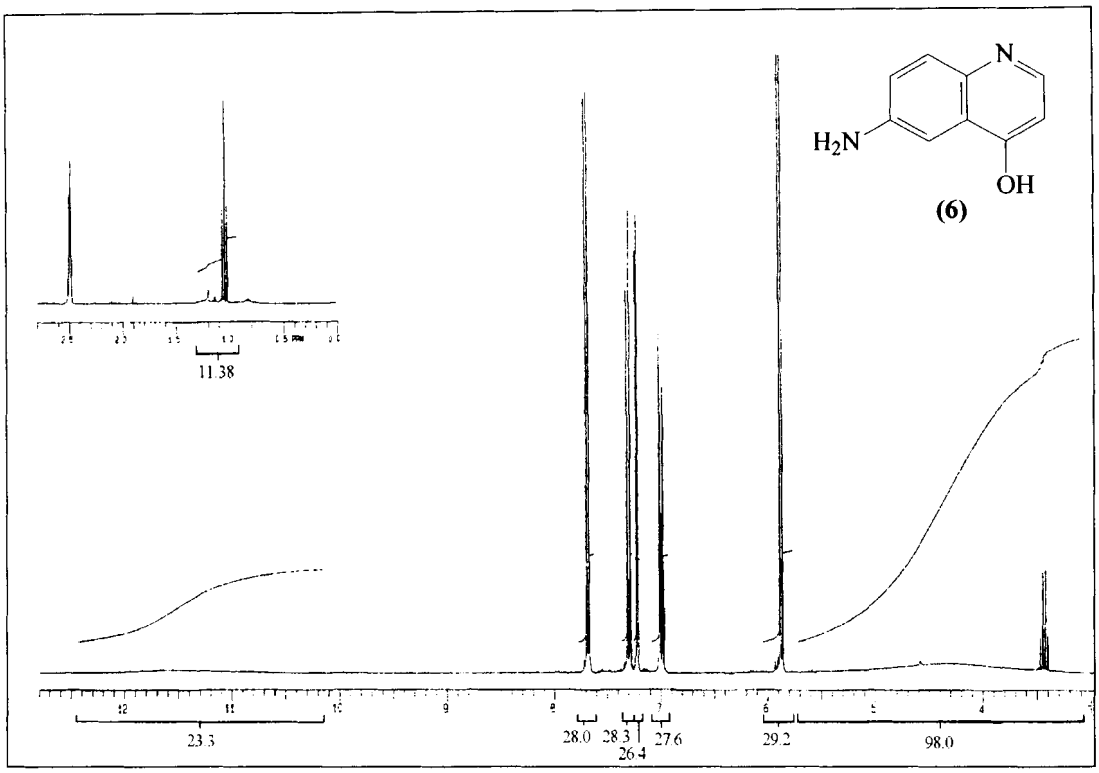
Spectrum 35



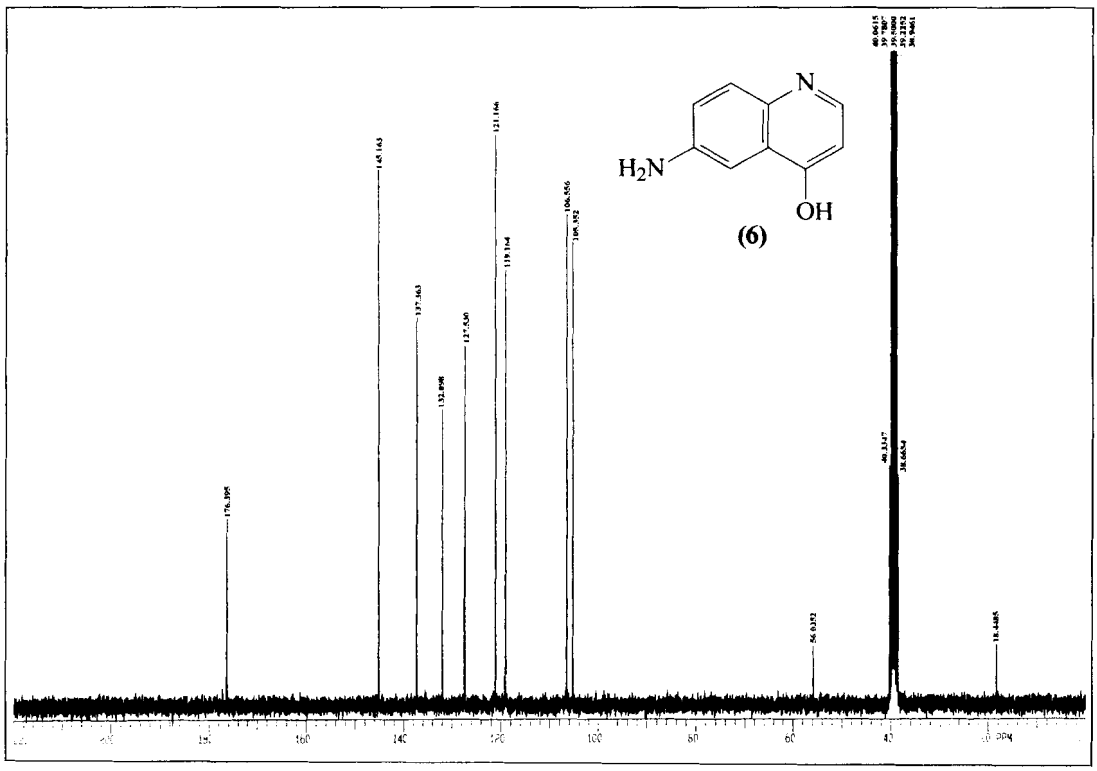
Spectrum 36



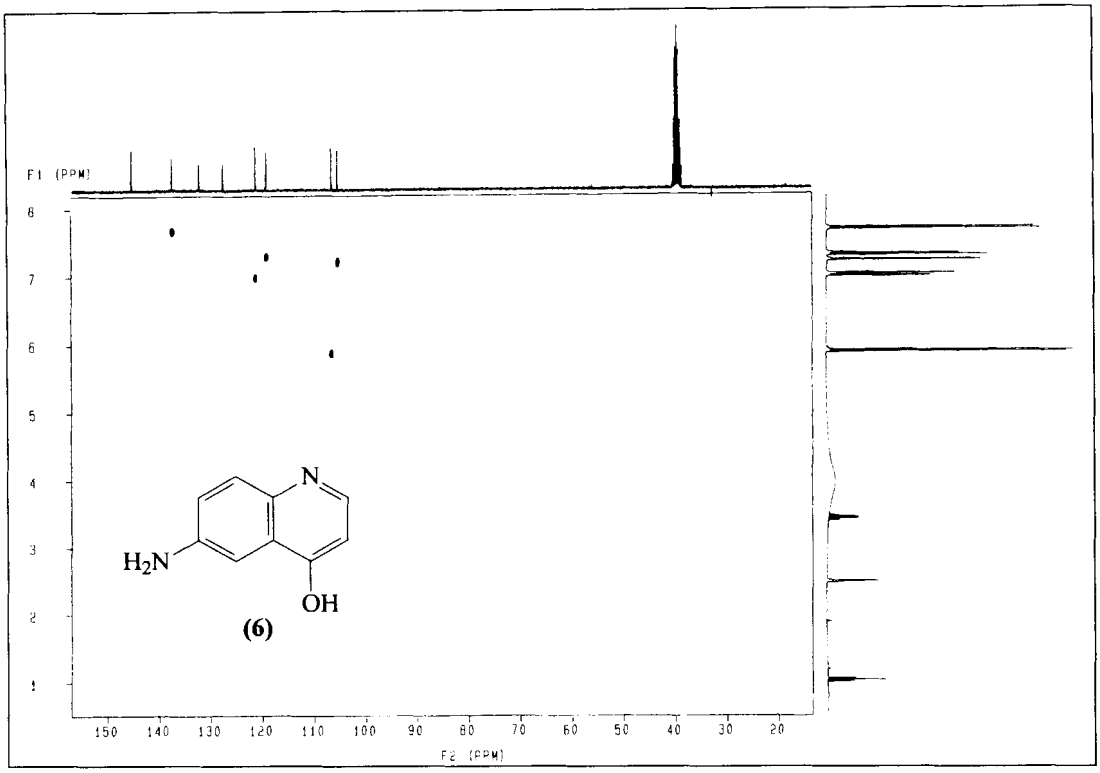
Spectrum 37



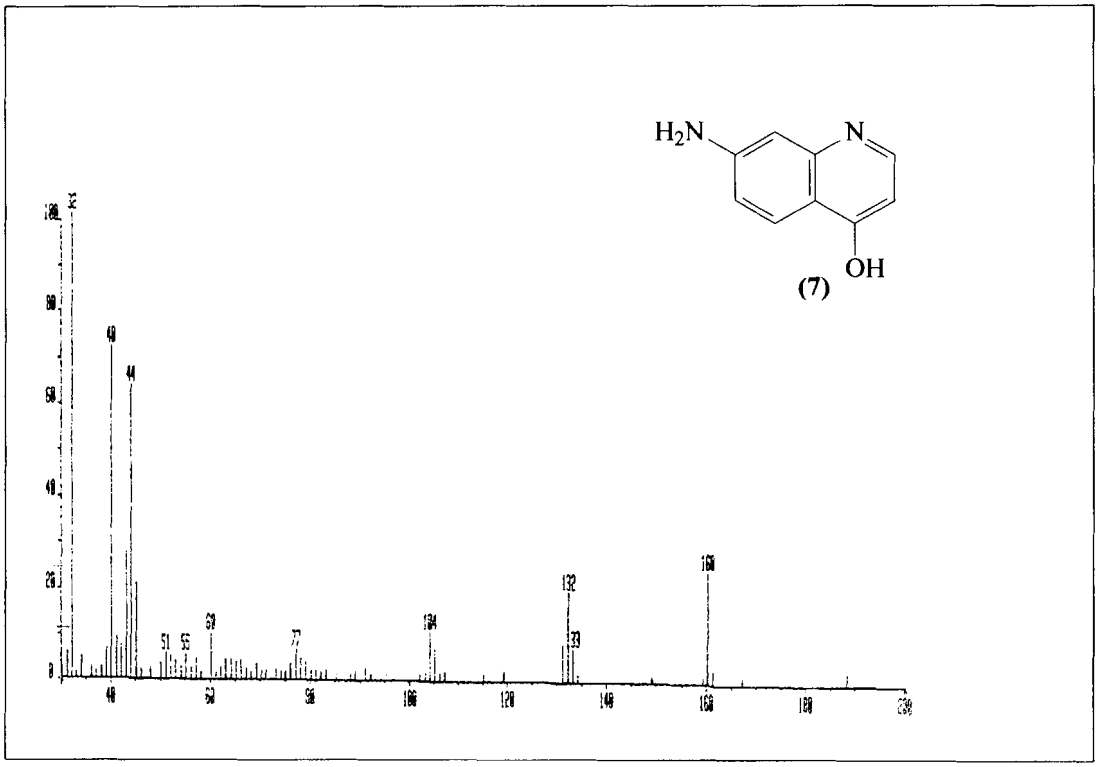
Spectrum 38



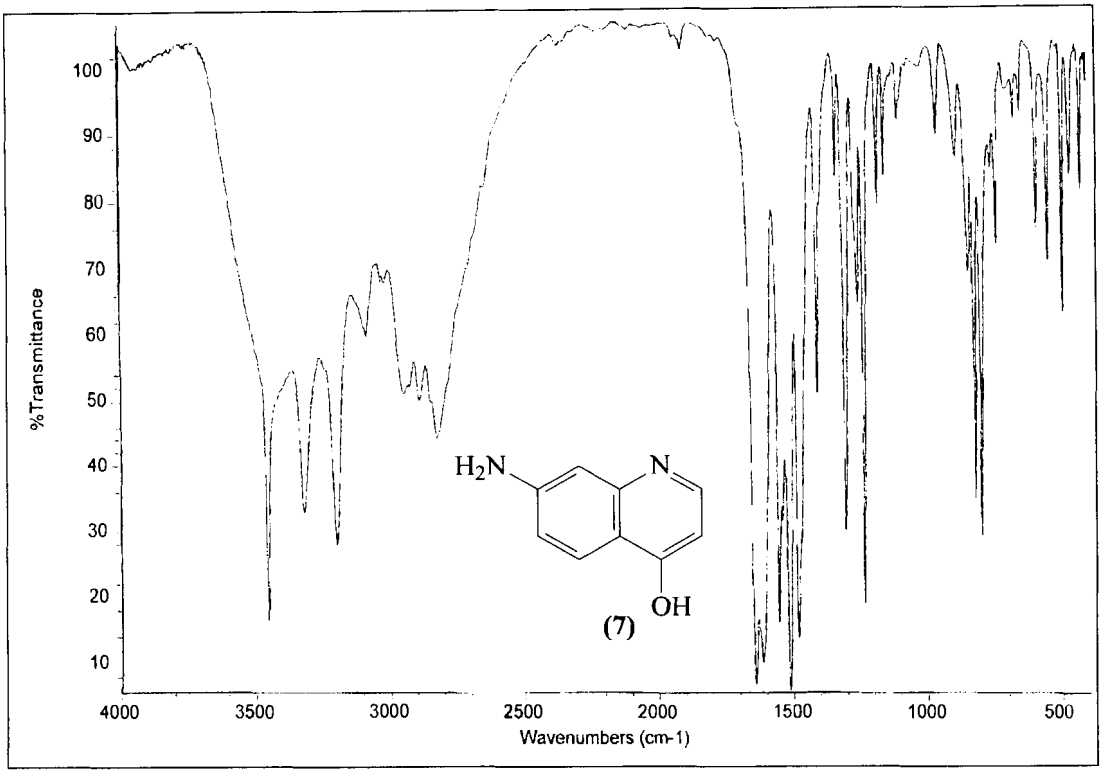
Spectrum 39



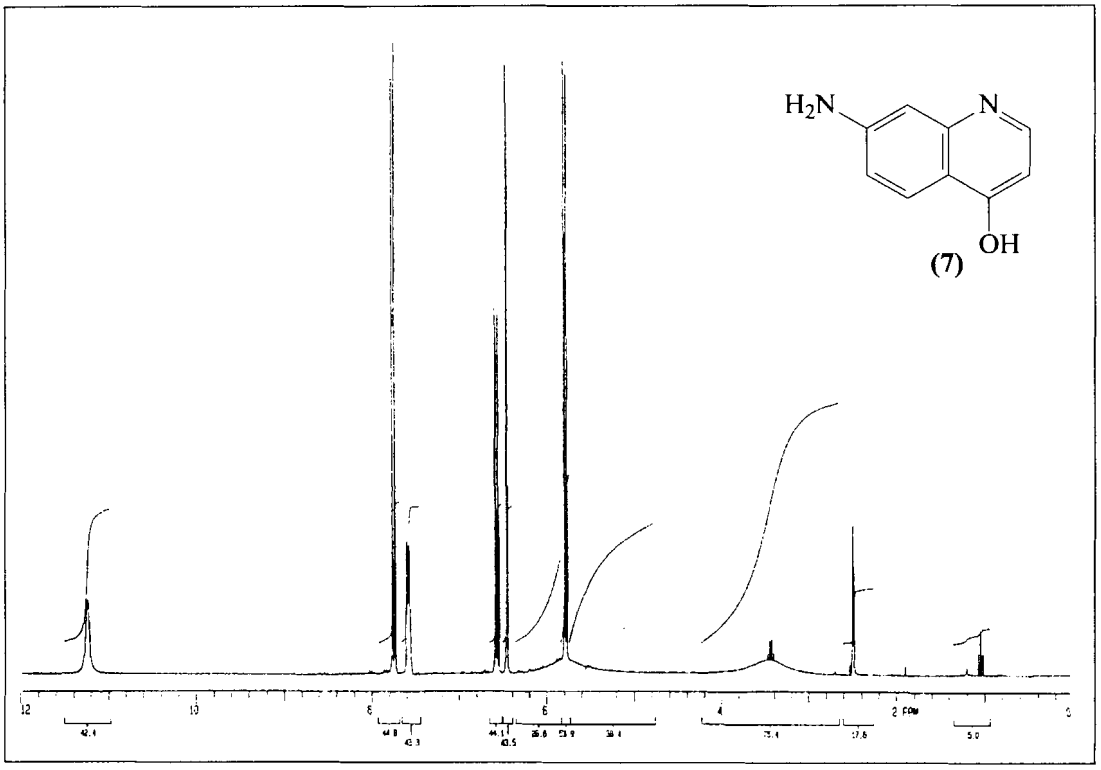
Spectrum 40



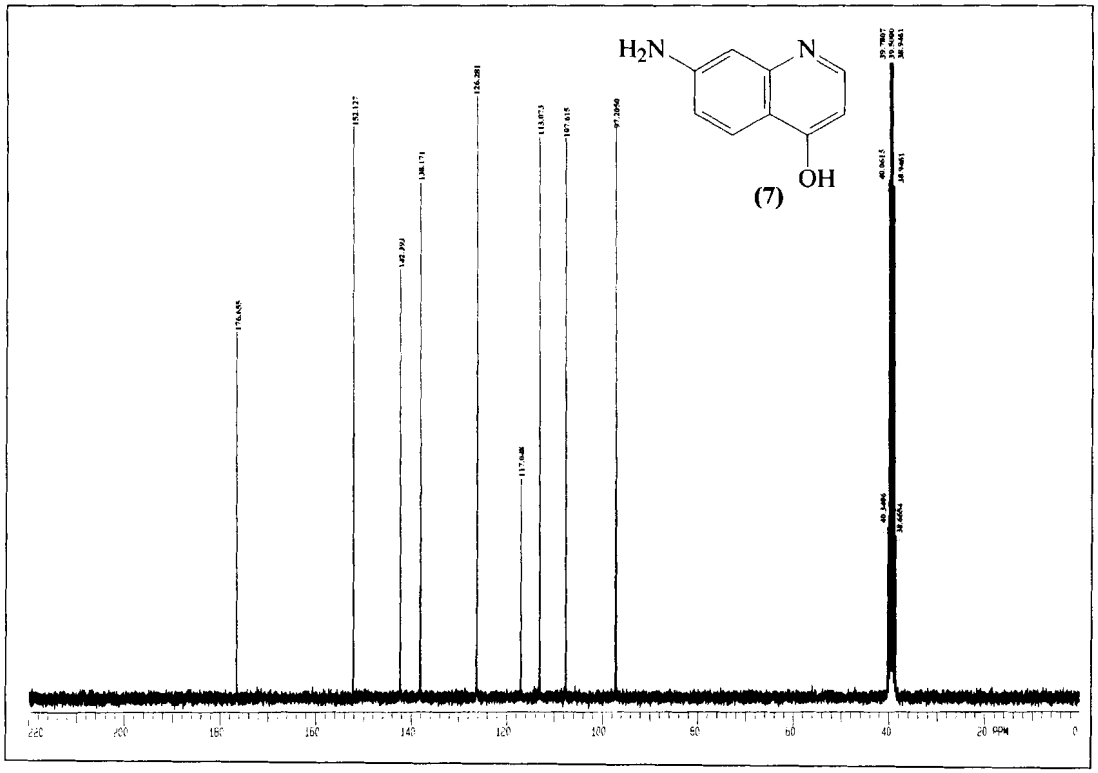
Spectrum 41



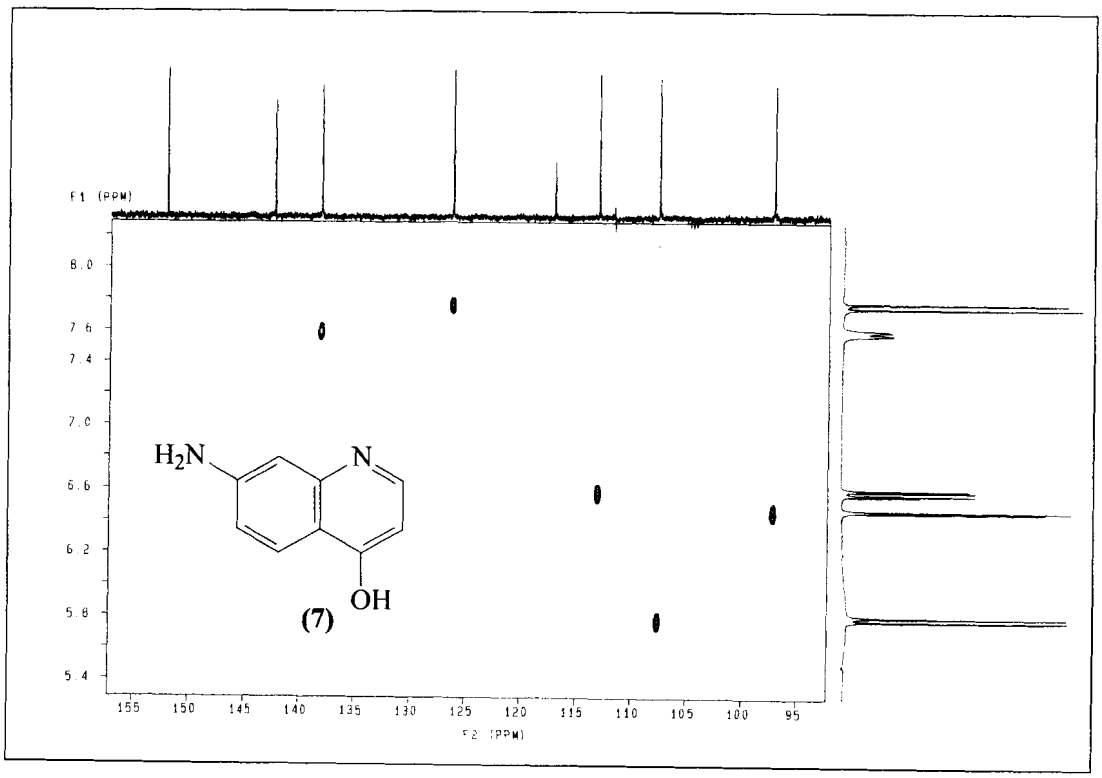
Spectrum 42



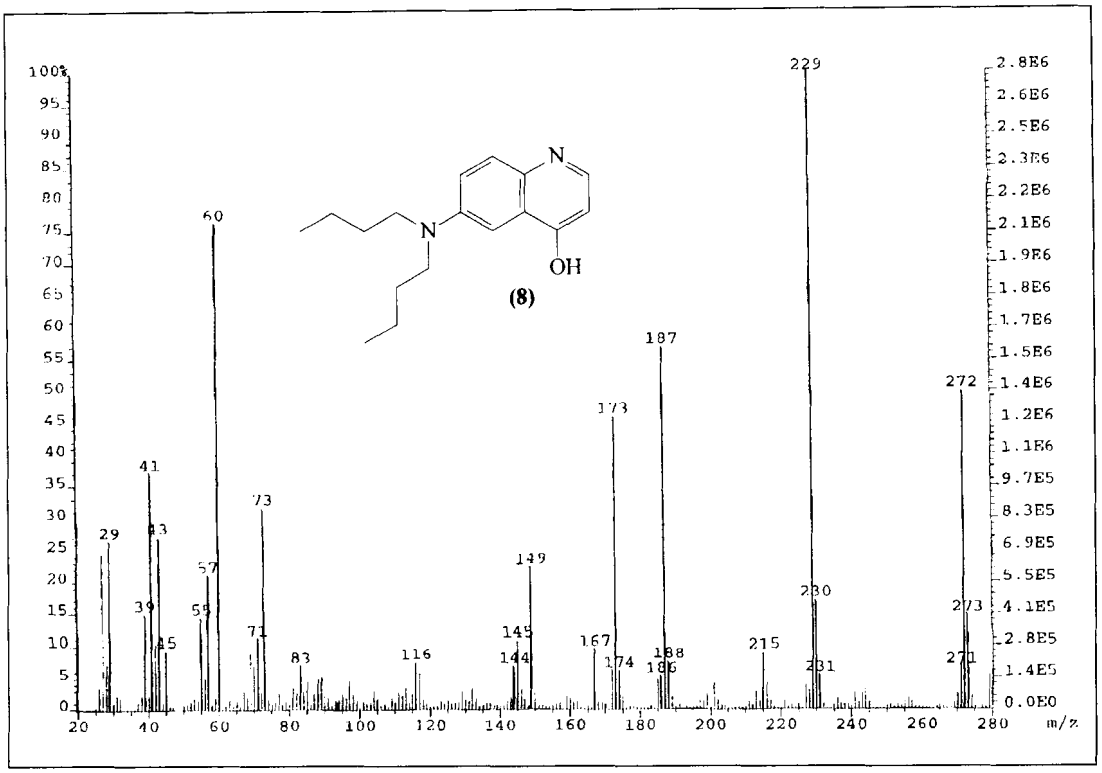
Spectrum 43



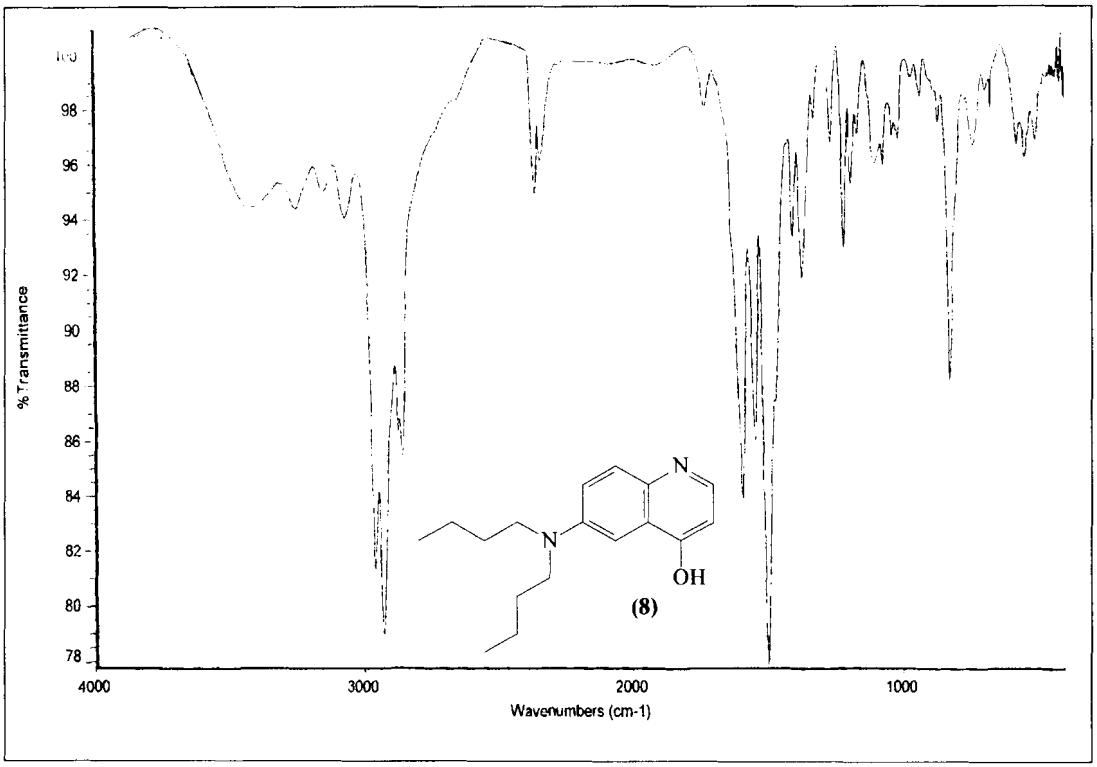
Spectrum 44



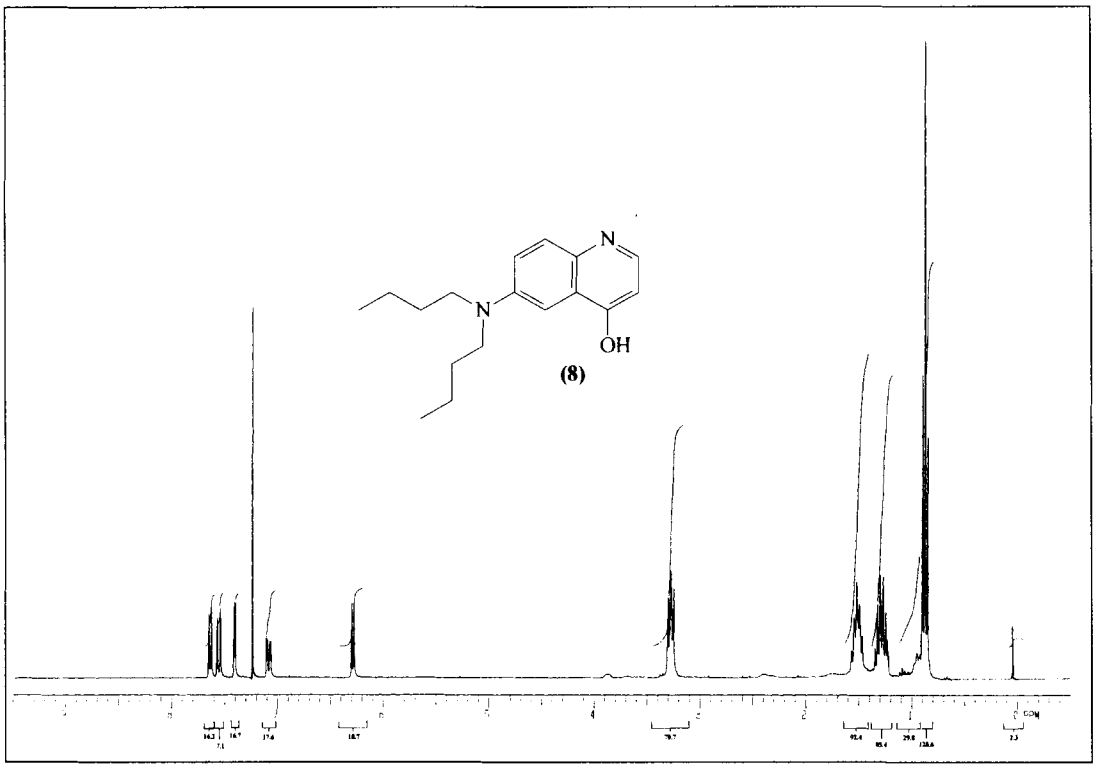
Spectrum 45



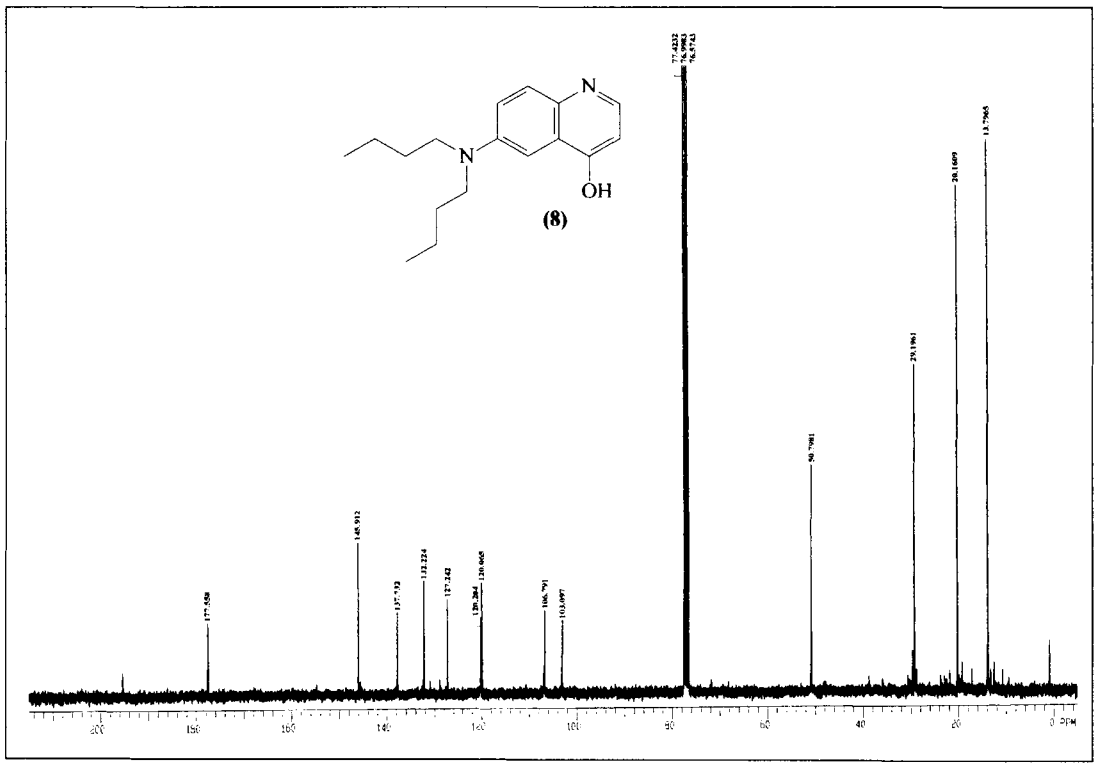
Spectrum 46



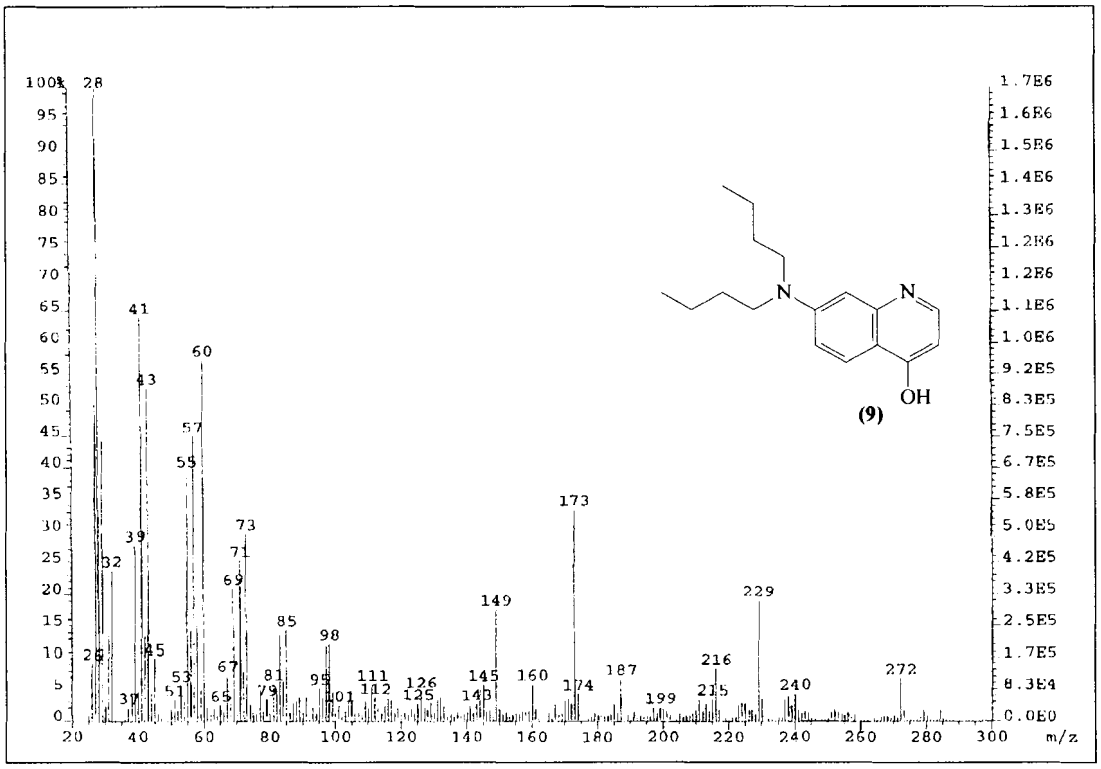
Spectrum 47



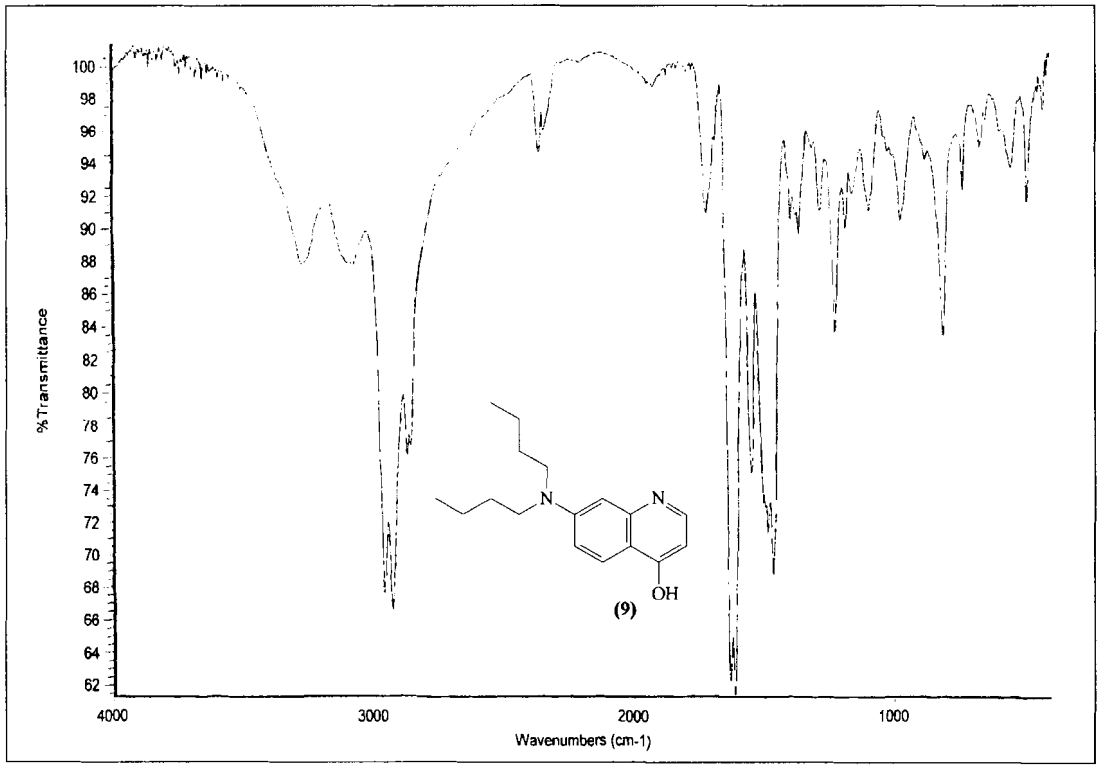
Spectrum 48



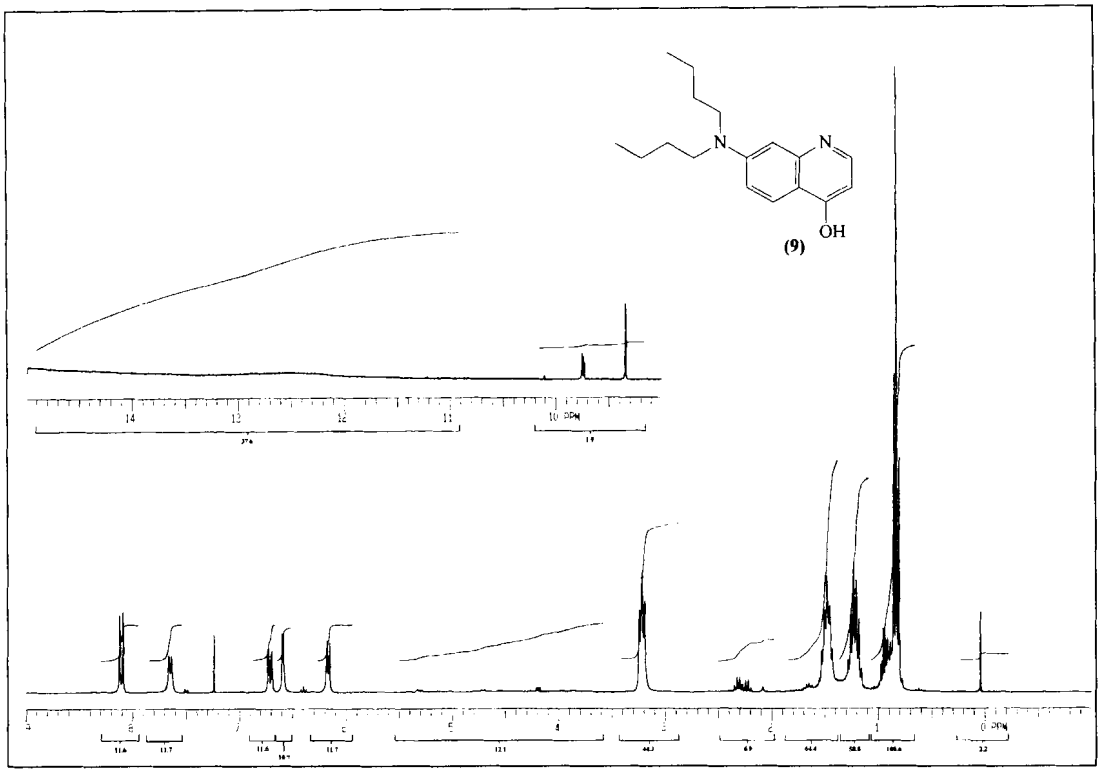
Spectrum 49



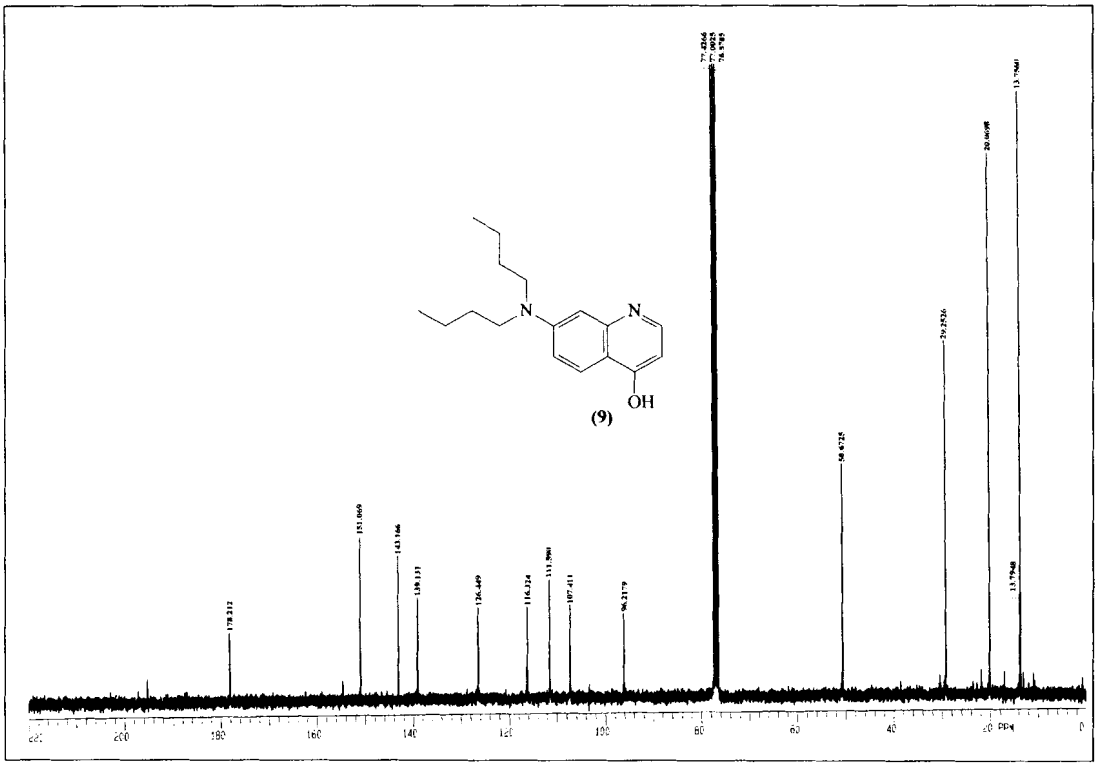
Spectrum 50



Spectrum 51

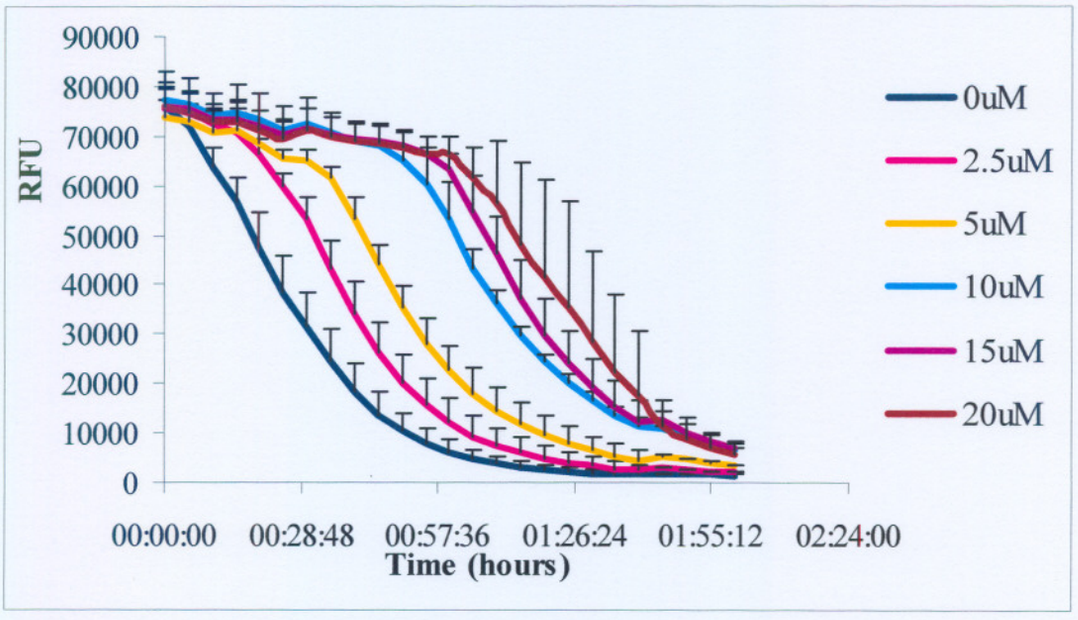


Spectrum 52

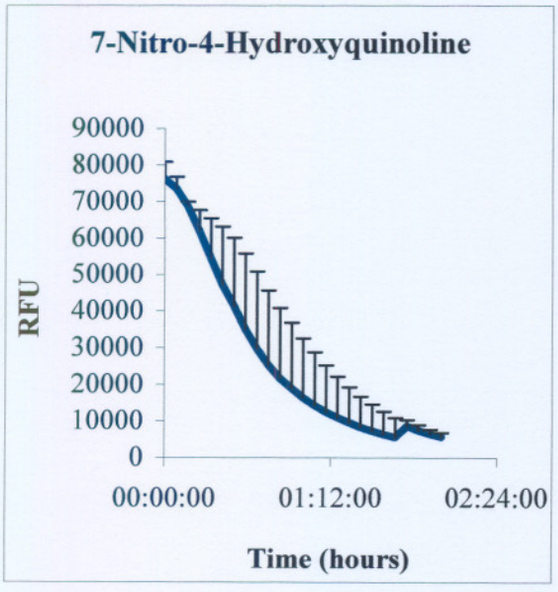


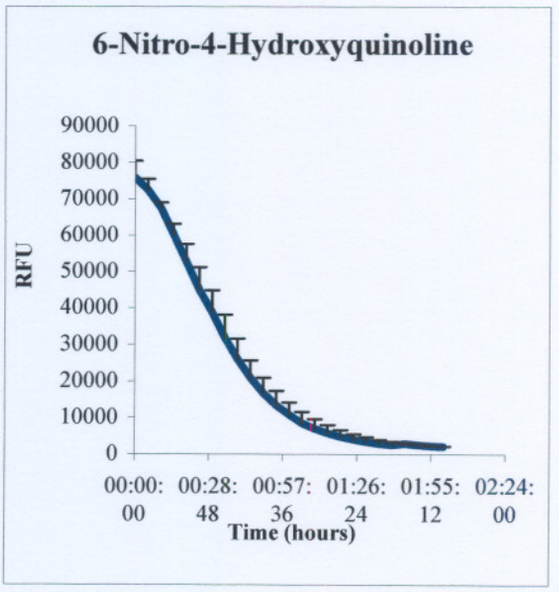
APPENDIX B

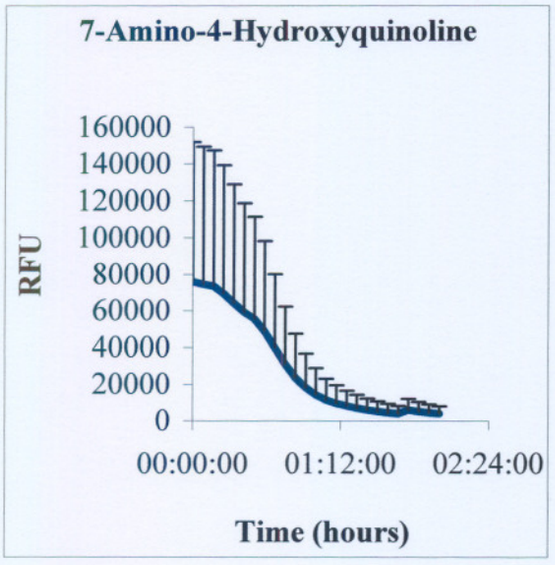
AAPH induced Fluorescence decay curves in the presence of Trolox

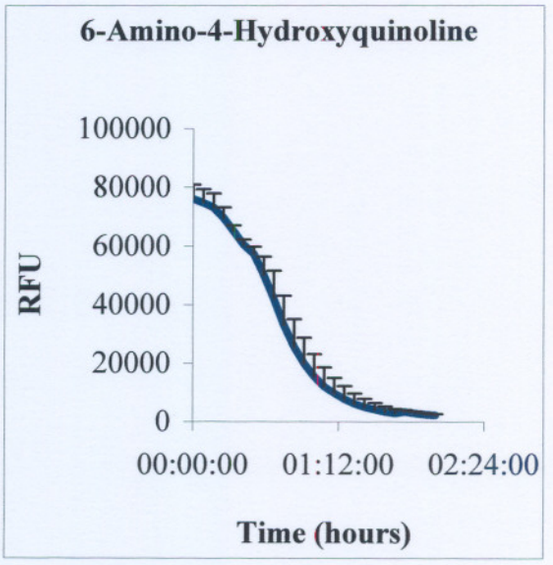


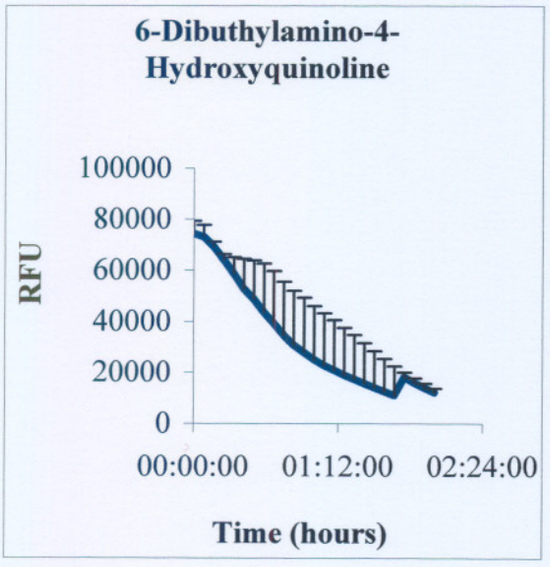
Fluorescence decay curves induced by AAPH in the presence of respective 4-Hydroxyquinolines

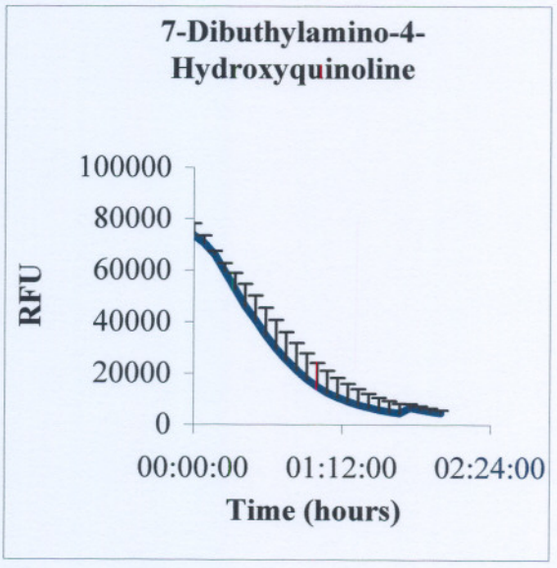





7-Amino-4-Hydroxyquinoline
RFU
160000
140000
120000
100000
80000
60000
40000
20000
0
00:00:00
01:12:00
02:24:00
Time (hours)


6-Amino-4-Hydroxyquinoline
RFU
100000
80000
60000
40000
20000
0
00:00:00
01:12:00
02:24:00
Time (hours)


6-Dibuthylamino-4-
Hydroxyquinoline
RFU
100000
80000
60000
40000
20000
0
00:00:00
01:12:00
02:24:00
Time (hours)


7-Dibuthylamino-4-
Hydroxyquinoline
RFU
100000
80000
60000
40000
20000
0
00:00:00
01:12:00
02:24:00
Time (hours)

APPENDIX C

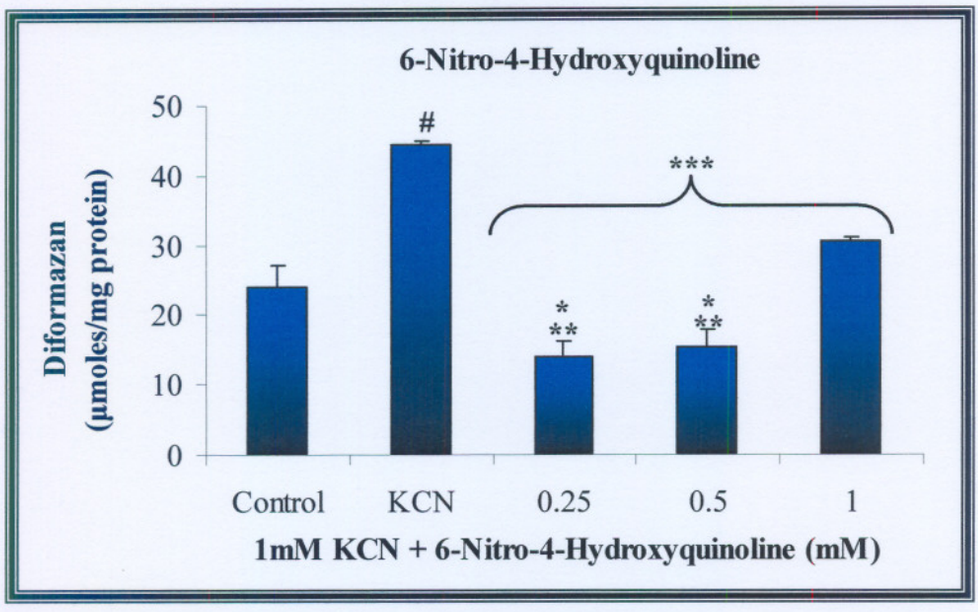


Figure C.1. The superoxide scavenging ability of 6-nitro-4-hydroxyquinoline (4). Each bar represents the mean ± S.E.M. **p<0.01 vs. control; ***p<0.001 vs KCN;*p<0.01 vs. 1mM; #p<0.001 vs. control.

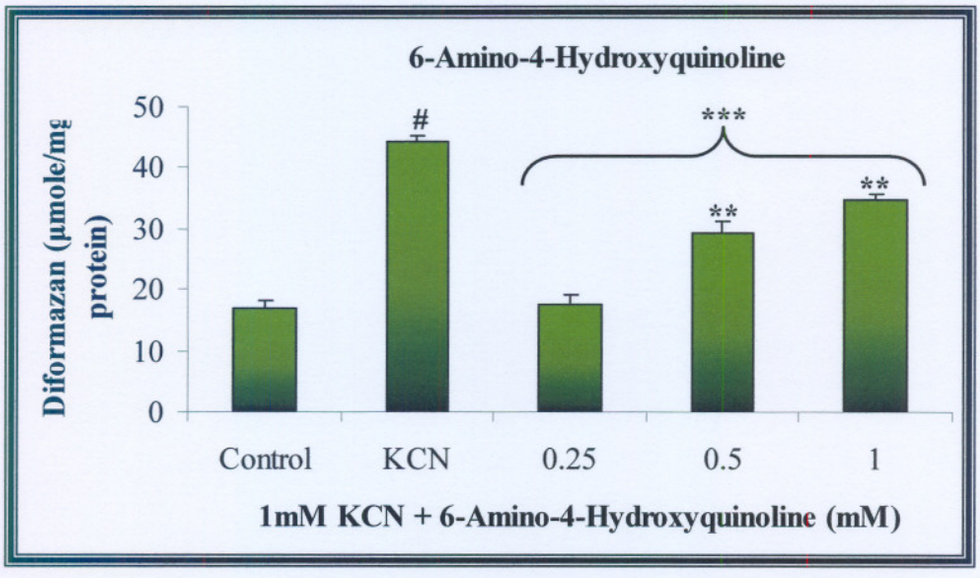


Figure C.2. The superoxide scavenging ability of 6-amino-4-hydroxyquinoline (6). Each bar represents the mean ± S.E.M. ***p<0.001 vs KCN; **p<0.001 vs. 0.25mM; #p<0.001 vs. control.

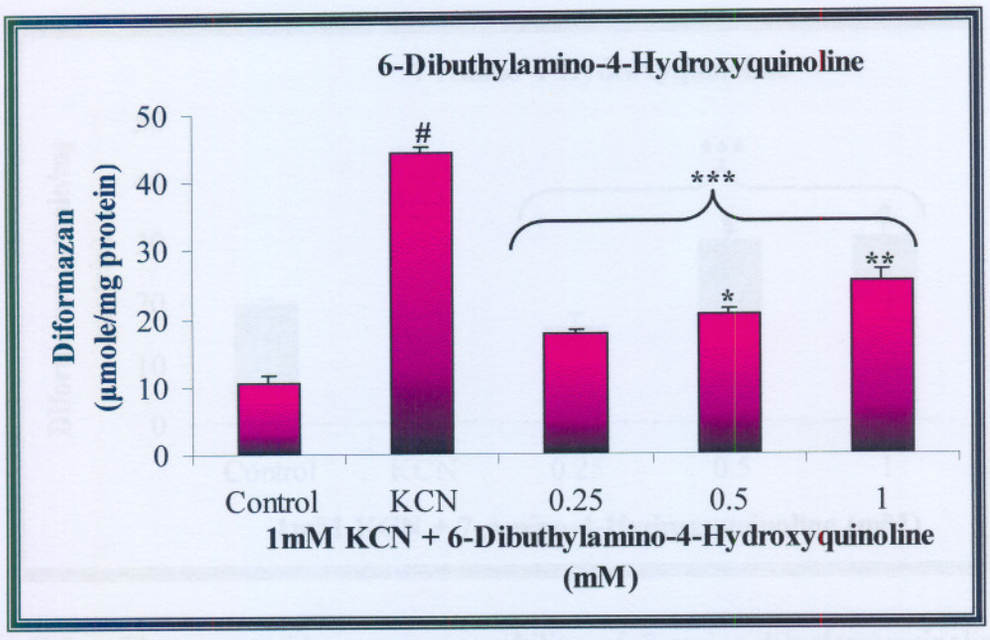


Figure C.3. The superoxide scavenging ability of 6-dibuthylamino-4-hydroxyquinoline (8). Each bar represents the mean ± S.E.M. ***p<0.001 vs KCN; **p<0.001 vs. 1mM;*p<0.01 vs 1mM; #p<0.001 vs. control.

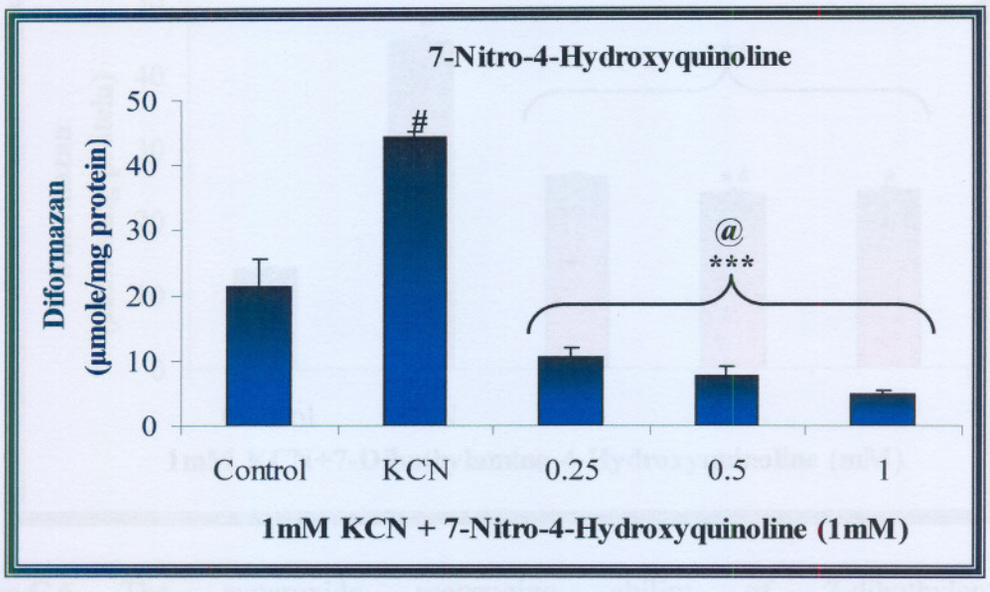


Figure C.4. The superoxide scavenging ability of 7-nitro-4-hydroxyquinoline (5). Each bar represents the mean ± S.E.M. ***p<0.001 vs KCN; @p<0.001 vs control; #p<0.001 vs. control.

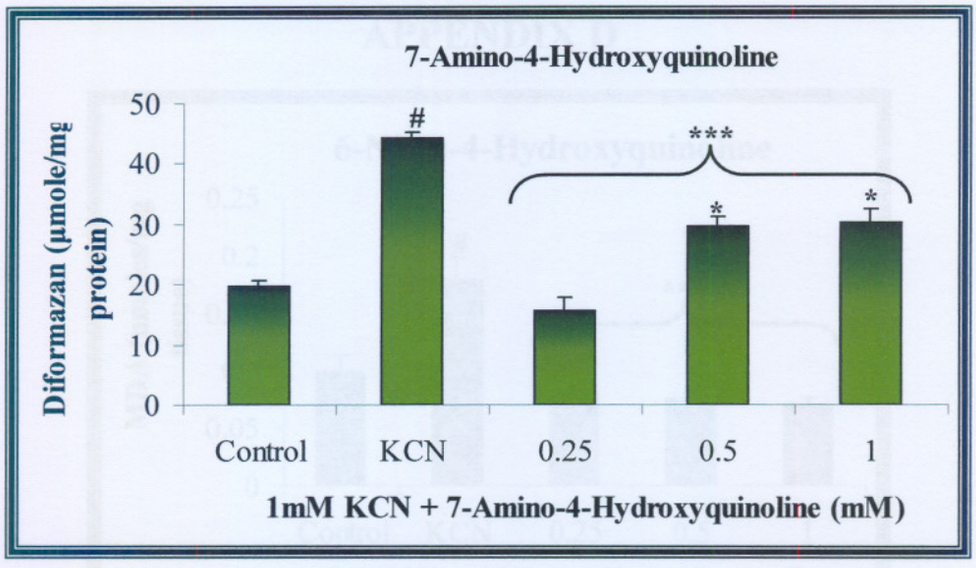


Figure C.5. The superoxide scavenging ability of 7-amino-4-hydroxyquinoline (7). Each bar represents the mean ± S.E.M. ***p<0.001 vs KCN; *p<0.01 vs 0.25mM; #p<0.001 vs. control.

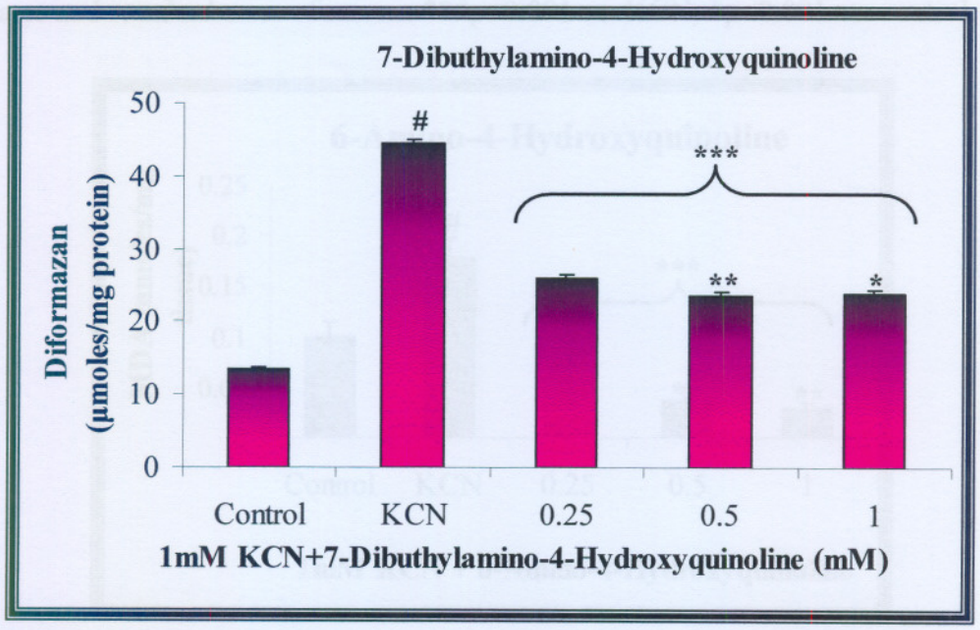


Figure C.6. The superoxide scavenging ability of 7-dibuthylamino-4-hydroxyquinoline (9). Each bar represents the mean ± S.E.M. ***p<0.001 vs KCN; *p<0.01 vs 0.25mM;*p<0.05 vs. 0.25mM; #p<0.001 vs. control.

APPENDIX D

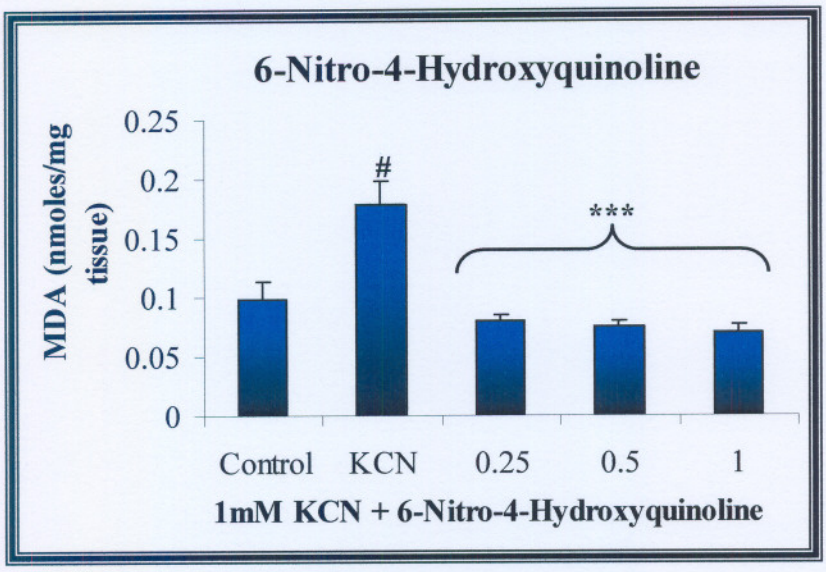


Figure D.1. The effect of increasing concentrations of 6-nitro-4-hydroxyquinoline **(4)** on 1mM KCN induced lipid peroxidation. Data are represented as the mean of 5 replicates and standard error of means. ***p<0.001 vs. KCN; #p<0.001 vs. control.

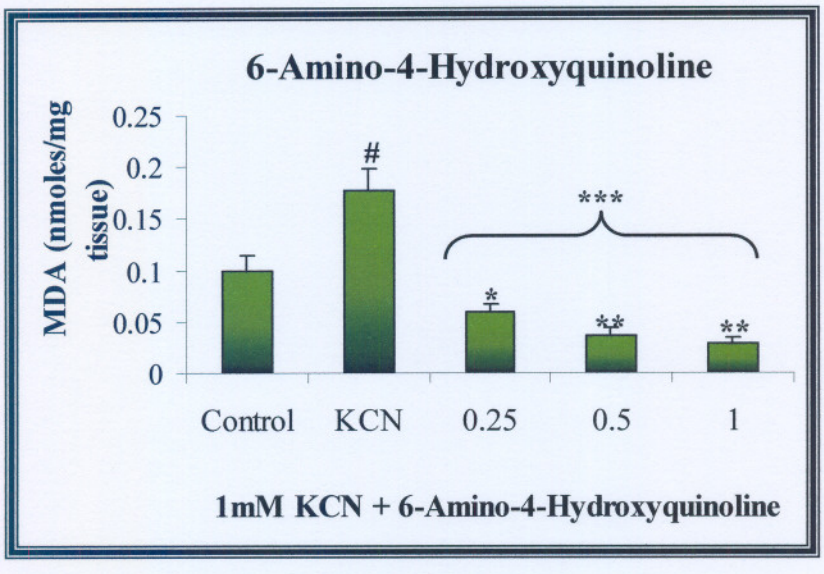


Figure D.2. Inhibition of 1mM KCN induced lipid peroxidation by increasing concentrations of 6-amino-4-hydroxyquinoline **(6).** Data are presented as the mean ± S.E.M; n=5. ***p<0.001 vs. KCN; #p<0.001 vs. control; **p<0.001 vs. control; *p<0.05.

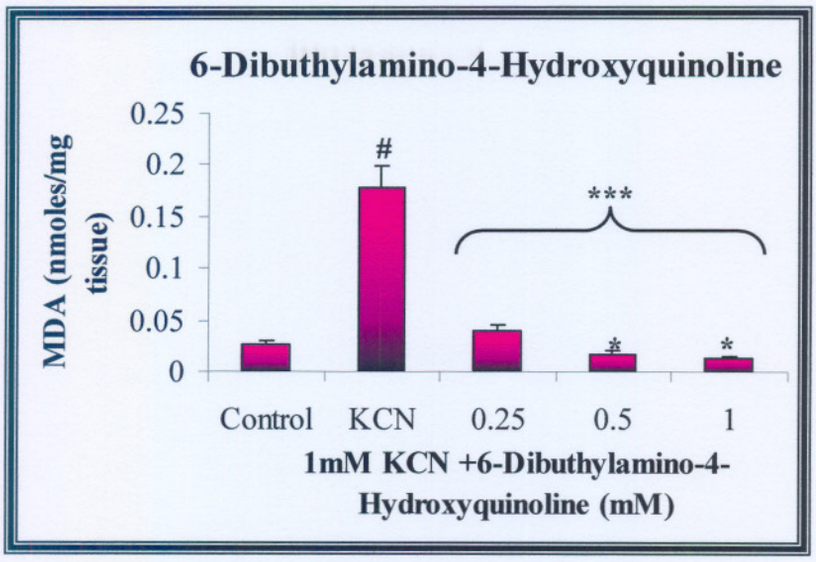


Figure D.3. The dose-dependent inhibition of lipid peroxidation offered by 6-dibuthylamino-4-hydroxyquinolines (8) in the presence of 1mM KCN. Data are presented as the mean ± S.E.M.; n=5. ***p<0.001 vs. KCN; #p<0.001 vs. control; *p,0.05 vs. 0.25mM.

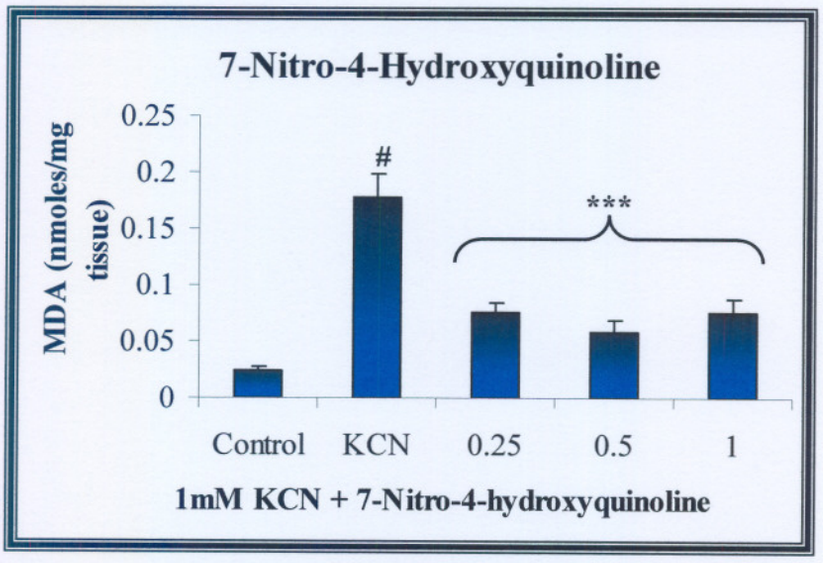


Figure D.4. The effect of 7-nitro-4-hydroxyquinoline **(5)** on lipid peroxidation induced by 1mM KCN. Data are presented as the mean ± S.E.M.; n=5. ***p<0.001 vs. KCN; #p<0.001 vs. control.

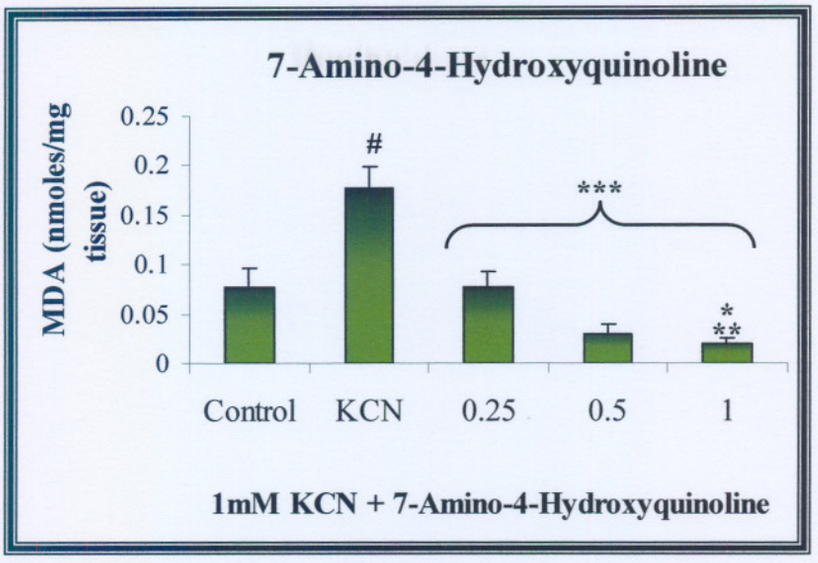


Figure D.5. The dose-dependent effect of 7-amino-4-hydroxyquinolines (7) in the presence of 1mM KCN. Data are presented as the mean ± S.E.M.; n=5. ***p<0.01 vs. KCN; #p<0.001 vs. control; **p<0.05 vs. 0.25mM; *p<0.05 vs. control.

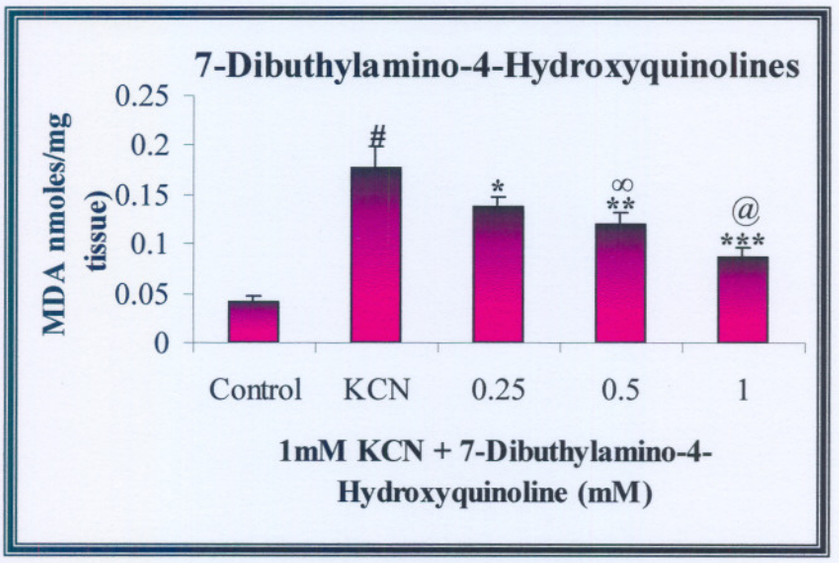


Figure D.6. The dose-dependent effect on lipid peroxidation of 7-dibuthylamino-4-hydroxyquinolines (9) in the presence of 1mM KCN. Data are presented as the mean ± S.E.M.; n=5. ; #p<0.001 vs. control; ***p<0.001 vs. KCN;**p<0.01 vs. KCN; *p<0.05 vs. KCN; @p<0.01 vs. 0.25mM; ∞p<0.05 vs. 1mM.